Efficient Evaluation of Binding Free Energy Using Continuum Electrostatics Solvation

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The linear interaction energy (LIE) method is combined with energy minimization and finite-difference Poisson calculation of electrostatic solvation for the estimation of the absolute free energy of binding. A predictive accuracy of about 1.0 kcal/mol is obtained for 13 and 29 inhibitors of β-secretase (BACE) and HIV-1 protease (HIV-1 PR), respectively. The multiplicative coefficients for the van der Waals and electrostatic terms are not transferable between BACE and HIV-1 PR although they are both aspartic proteases. The present approach is about 2 orders of magnitude faster than previous LIE methods and can be used for ranking large libraries of structurally diverse compounds docked by automatic computational tools.

1. Introduction

Computer-aided approaches for docking libraries of small molecules into proteins of known structure require fast and accurate methods for the evaluation of binding free energies.[1−6] Rigorous approaches to evaluate relative binding affinities such as free energy perturbation and thermodynamic integration have sampling and convergence problems that prevent them from being used routinely.[7] Moreover, the convergence problem is more severe the larger the structural differences are between ligands. Several semiempirical methods based on linear approximations to the free energy have been introduced and used with success.[5] A decade ago Åqvist and co-workers proposed the LIE (linear interaction energy) method to calculate free energies of binding by averaging interaction energies from molecular dynamics (MD) simulations of the ligand and the ligand/protein complex.[8,9] They approximated the free energy of binding by

$$\Delta G_{bind} = \frac{1}{2}(\langle E^{elec} \rangle_{bound} - \langle E^{elec} \rangle_{free}) + \alpha(\langle E^{vdW} \rangle_{bound} - \langle E^{vdW} \rangle_{free}) \quad (1)$$
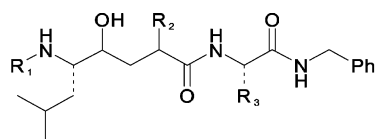
where E^{elec} and E^{vdW} are the electrostatic and van der Waals interaction energies between the ligand and its surroundings. The surroundings are either the solvent (free) or the solvated ligand/protein complex (bound). The $\langle \rangle$ denotes an ensemble average sampled over an MD[8] or Monte Carlo[10] trajectory. The coefficient α is determined empirically.[8] The multiplicative factor $1/2$ for the electrostatic term has a physical justification that can be explained by the fact that the electrostatic contribution to the hydration energy of a single ion is equal to half the corresponding ion−water interaction energy.[11,12] In practice, the electrostatic multiplicative factor is also considered a free parameter in the fitting except for a few studies characterized by either a small number of ligands[8,9] or large deviations in some of the predicted binding energies.[13,14] The main advantage of the LIE method is that it can be used for ligands with

significant differences in their chemical structures where rigorous free energy calculations usually fail to converge. The method was first applied to five endothiapepsin inhibitors leading to an α value of 0.161 and a maximum error of 0.53 kcal/mol for the absolute binding free energies of the training set.[8] Carlson and Jorgensen extended the original LIE method to estimate hydration free energies and added a cavitation term proportional to the solute's solvent accessible surface area.[15] Kollman and co-workers have used LIE with MD sampling for studying the binding of 14 biotin analogues to avidin and yielded results that correlate well with experimental data for 10 of the ligands.[14] For the other four ligands an error of more than 7 kcal/mol was reported to originate mainly from conformational changes in the protein due to bulky substituents. Wall et al. have successfully applied the LIE method to a set of 15 neuraminidase inhibitors.[16] By statistical analysis, they concluded that energy terms accounting for intramolecular strain and cavitation effects do not contribute significantly to relative binding free energies.[16]

Recently, an LIE method based on the generalized Born approximation[17] of electrostatic solvation has been validated on 20, 7, and 8 inhibitors of HIV-1 reverse transcriptase, human thrombin, and factor Xa, respectively.[18] The authors reported similar cross-validated results of about 1.0 kcal/mol accuracy using MD and hybrid Monte Carlo sampling techniques. The same LIE approach was recently applied to a set of 12 BACE inhibitors, and a cross-validated root-mean-square (rms) error of 1.36 kcal/mol was observed using a three-parameter model.[19]

Here, we replace the MD sampling with a simple energy minimization and combine the LIE method with a rigorous treatment of continuum electrostatics, i.e., numerical solution of the Poisson equation by the finite-difference technique.[20] The modified LIE approach is termed LIECE where the last two letters stand for continuum electrostatics. The present work was motivated by two questions: Is it possible to improve the efficiency of the LIE method by replacing explicit water MD or Monte Carlo simulations with energy minimiza-

Inhibitors	R$_1$	R$_2$	R$_3$	K$_i$ (nM)	ΔG (kcal/mol)
1		Me	Me	22423.0	-6.38
2		Me	CHMe$_2$	3134.0	-7.55
3		Me	CHMe$_2$	1129.0	-8.16
4		Me	Me	61.4	-9.90
5		Me	CHMe$_2$	5.9	-11.30
6		Me	CHMe$_2$	50.1	-10.02
7		Me	CHMe$_2$	9.4	-11.02
8		Me	CHMe$_2$	5808.0	-7.19
9		Me	CHMe$_2$	2.5	-11.81
10		Me	CHMe$_2$	8.0	-11.11
11		CH$_2$CHMe$_2$	CHMe$_2$	10491.0	-6.80
12	OM99-2: Glu-Val-Asn-Leu-Ψ-Ala-Ala-Glu-Phe			1.6	-12.06
13	OM00-3: Glu-Leu-Asp-Leu-Ψ-Ala-Val-Glu-Phe			0.3	-13.05

Figure 1. BACE inhibitors tested by Ghosh and co-workers.[31]

tion and a continuum model of the solvent? Are the parameters of the LIECE approach transferable between two enzymes of the same class? β-Secretase (BACE) and HIV-1 protease (HIV-1 PR) are both aspartic proteases, and they represent pharmaceutically important targets in the fight against Alzheimer's disease[21−23] and AIDS,[24−26] respectively. The LIECE results obtained on BACE and HIV-1 PR indicate that the first question can be answered affirmatively, whereas parameter transferability is not possible.
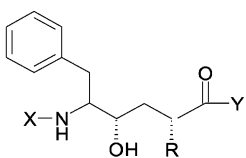
2. Methodology

Preparation of BACE. Tang and co-workers have solved the crystal structure of BACE in the complex with two peptidic inhibitors, OM99-2[27] and OM00-3.[28] Coordinates of BACE in complex with the inhibitor OM00-3 (Glu-Leu-Asp-Leu-ψ-{CHOH-CH$_2$}-Ala-Val-Glu-Phe where ψ{CHOH-CH$_2$} is a hydroxyethylene isostere of the peptide bond) were downloaded from the PDB database[29] (PDB entry 1M4H[28]). The B chain, the inhibitor, and all water molecules were removed. Particu-

lar attention was addressed to the ionization state of the cleavage site, which contains the aspartyl dyad (Asp32/Asp228). At optimal pH for enzymatic activity ($\sim$3.5−4.5[30]), the aspartyl dyad is most probably monoprotonated in the uncomplexed enzyme as well as in the complex with peptidomimetic inhibitors with a hydroxyethylene isostere of the peptide bond. The choice of which of the two catalytic aspartates to protonate should have little effect on the relative binding affinity because all of the inhibitors have the same binding motif at the catalytic site.[19] Asp228 was protonated in this study.

The 13 peptidic inhibitors of BACE used in this study (Figure 1) include OM00-3 (K$_i$ = 0.32 nM), OM99-2 (K$_i$ = 1.6 nM), and a series of 11 related inhibitors (K$_i$ values ranging from 2.5 nM to 22.4 μM) synthesized in the same laboratory.[31] The initial binding conformations were modeled manually according to the binding mode of OM00-3[28] because all inhibitors have similar backbone structure.

Preparation of HIV-1 PR. Coordinates of HIV-1 PR in complex with the inhibitor Ala-Ala-Phe-ψ{CHOH-CH$_2$}-Ala-Val-Val-OMe were downloaded from the PDB database[29] (PDB

Inhibitors	*X*	*Y*	*R*	K_i (nM)	ΔG (kcal/mol)
1	*Boc*	Val-NH$_2$	H	6500	-7.08
2	*Boc*	Val-NH$_2$	Me	260	-8.99
3	*Boc*	Val-NH$_2$	n-Pr	50	-9.97
4	*Boc*	Val-NH$_2$	i-Bu	23	-10.43
5	*Boc*	Val-NH$_2$	Bn	1.4	-12.09
6	*Cbz-Ala*	Val-NH$_2$	H	2500	-7.65
7	*Cbz-Ala*	Val-NH$_2$	Me	50	-9.97
8	*Cbz-Ala*	Val-NH$_2$	n-Pr	20	-10.51
9	*Cbz-Ala*	Val-NH$_2$	i-Bu	3.9	-11.48
10	*Cbz-Ala*	Val-NH$_2$	Bn	0.6	-12.59
11	*Cbz-Ala*	Val-Val-O-Me	H	118	-9.46
12	*Cbz-Ala*	Val-Val-O-Me	Me	1.6	-12.01
13	*Cbz-Ala*	Val-Val-O-Me	n-Pr	0.6	-12.59
14	*Cbz-Ala*	Val-Val-O-Me	i-Bu	0.9	-12.35
15	*Cbz-Ala*	Val-Val-O-Me	Bn	0.6	-12.59
16	*Cbz-Ala-Ala*	Val-Val-O-Me	H	44	-10.05
17	*Cbz-Ala-Ala*	Val-Val-O-Me	Me	0.6	-12.59
18	*Cbz-Ala-Ala*	Val-Val-O-Me	n-Pr	0.4	-12.83
19	*Cbz-Ala-Ala*	Val-Val-O-Me	i-Bu	0.8	-12.42
20	*Cbz-Ala-Ala*	Val-Val-O-Me	Bn	0.4	-12.83
21	*Ala-Ala*	Val-Val-O-Me	H	4.0	-11.47
22	*Ala-Ala*	Val-Val-O-Me	Me	3.0	-11.64
23	*Ala-Ala*	Val-Val-O-Me	n-Pr	1.2	-12.18
24	*Ala-Ala*	Val-Val-O-Me	Bn	0.6	-12.59
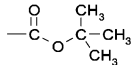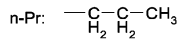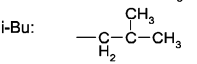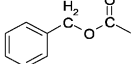
Figure 2. HIV-1 PR inhibitors tested by Dreyer and co-workers.[32]

entry 1AAQ[32]). The ligand and all water molecules but one were removed. The water bridging the two flaps was retained because it mediates the binding of the inhibitors considered in this study. A monoprotonated state at the catalytic aspartates was considered for HIV-1 PR as in the case of BACE.

The crystal structure of the 1AAQ complex contains the largest compound from a set of 24 HIV-1 PR inhibitors (Figure 2) with K_i values ranging from 0.4 nM to 6.5 μM.[32] The remaining 23 inhibitors were modeled manually by deleting parts of the inhibitor in 1AAQ.

Minimization. Hydrogen atoms were added to all structures and minimized with the program CHARMM[33] and the CHARMm22 force field (Accelrys Inc.). Partial charges were assigned using the MPEOE method.[34,35] All protein/inhibitor complexes were minimized by the conjugate gradient algorithm to an rms of the gradient of 0.001 kcal mol^{-1} Å^{-1}. During minimization the electrostatic energy term was screened by a distance-dependent dielectric of $4r$ to prevent artificial deviations due to vacuum effects and the default nonbonding cutoff of 14 Å was used. Protein atoms and the water molecule in HIV-1 PR were kept fixed during minimization. The minimized structures were used for evaluating the van der Waals energy and finite-difference Poisson calculations.

Energy Calculations. The van der Waals and electrostatic interaction energies were calculated by subtracting the values of the isolated components from the energy of the complex. The van der Waals energy was calculated with CHARMM[33] and the CHARMm22 force field (Accelrys Inc.) using the default cutoff of 14 Å.

The electrostatic energy is the sum of the Coulombic energy in vacuo and the solvation energy. The former was calculated with CHARMM[33] using infinite cutoff and neglecting interactions between pairs of atoms separated by one or two covalent bonds. The electrostatic solvation energy was calculated by the finite-difference Poisson approach[20] using the PBEQ module[36] in CHARMM and a focusing procedure with a final grid spacing of 0.3 Å. The size of the initial grid was determined by considering a layer of at least 20 Å around the solute. The dielectric discontinuity surface was delimited by the molecular surface spanned by the surface of a rolling probe of 1.4 Å. The ionic strength was set to zero, and the temperature was set to 300 K. Two finite-difference Poisson calculations were performed for each of the three systems (inhibitor, protein, and inhibitor/protein complex). The exterior dielectric constant was set to 78.5 and 1.0 for the first and second calculation, respectively, while the solute dielectric constant was set to 1.0, which is consistent with the value used for the parametrization

Table 1. Coefficients and Root-Mean-Square Errors

	α	β	$\Delta G_{tr,rot}$ (kcal/mol)	rms (kcal/mol)	cv rms LOO[a] (kcal/mol)	cv rms test set[b] (kcal/mol)	cv q^2 LOO
			BACE				
$\alpha\Delta E_{vdW} + \beta\Delta G_{elec}$	0.2737	0.1795		1.00	1.16		0.71
standard deviation[c]	±0.0395	±0.0461					
$\alpha\Delta E_{vdW} + \beta\Delta G_{elec} + \Delta G_{tr,rot}$	0.2943	0.1458	4.0594	0.95	1.28		0.65
standard deviation[c]	±0.0431	±0.0540	±3.3946				
			HIV-1 PR				
$\alpha\Delta E_{vdW} + \beta\Delta G_{elec}$	0.1690	0.0168		0.89	0.97	1.68	0.64
standard deviation[c]	±0.0196	±0.0199					
$\alpha\Delta E_{vdW} + \beta\Delta G_{elec} + \Delta G_{tr,rot}$	0.3205	0.0636	8.1665	0.73	0.77	1.21	0.77
standard deviation[c]	±0.0575	±0.0257	±2.8346				

[a] Leave-one-out cross-validated rms error. [b] The test set was not used to derive the model. It contains five inhibitors with K_i values of 0.05, 0.38, 3.2, 437, and 1100 nM. [c] Standard deviation from the leave-one-out procedure.

of the charges. The solvation energy is the difference between the two calculations.

Binding Free Energy. The equations used for the fitting are a two-parameter model,[8]

$$\Delta G_{bind} = \alpha\Delta E_{vdW} + \beta\Delta G_{elec} \qquad (2)$$

and a three-parameter model[13]

$$\Delta G_{bind} = \alpha\Delta E_{vdW} + \beta\Delta G_{elec} + \Delta G_{tr,rot} \qquad (3)$$

where, as detailed above, ΔE_{vdW} is the ligand/protein van der Waals interaction energy, ΔG_{elec} is the sum of the ligand/protein Coulombic energy in vacuo and the change in solvation energy of ligand and protein upon binding, and $\Delta G_{tr,rot}$ accounts for the loss of translational and rotational degrees of freedom upon binding.

3. Results and Discussion

The parameters obtained by least-squares fitting and energy values are given in Table 1 and Table 2, respectively, while the correlation between LIECE binding energies and experimental values is shown in Figure 3.

BACE. For the 13 BACE inhibitors, the two-parameter model yields an rms of the error of 1.0 kcal/mol with a leave-one-out cross-validated error rms of 1.16 kcal/mol and a cross-validated q^2 of 0.71. These results are better than those reported in a study that used an LIE method based on the generalized Born approximation.[19] In the latter, although one of the 13 inhibitors was explicitly left out, a three-parameter model yielded an rms of the error of 1.1 kcal/mol and a cross-validated error rms of 1.355 kcal/mol.[19] The better performance of LIECE is probably due to the evaluation of electrostatic solvation by the finite-difference Poisson approach, which is more accurate than the generalized Born approximation. It is interesting to note that the LIECE electrostatic solvation energy of compound **9** (K_i = 2.5 nM) is 6.7 kcal/mol more favorable than the one of compound **11** (K_i = 10491 nM) (Table 2), whereas in the previous LIE study the electrostatic energy of compound **11** was more favorable than compound **9** by about 3 kcal/mol (Table 5 in ref 19). Other differences between the present study and the one published previously[19] include the force field (CHARMm vs OPLS-AA[37]), the use in the latter of a cutoff for the electrostatic interaction, and the sampling which consisted of minimization in the rigid protein in LIECE and a hybrid Monte Carlo search in the previous study. The different force fields are not expected to have a strong influence because interaction energy differences are considered,

Table 2. Energy Components for BACE and HIV-1 PR (in kcal/mol)

	ΔE_{vdW}	$\Delta E_{elec,coul}$	$\Delta E_{elec,solv}$
	BACE Inhibitors		
1	−68.4	−38.19	106.44
2	−75	−39.25	109.26
3	−75.38	−42.85	115.07
4	−74.97	−53.47	114.98
5	−83.3	−51.08	113.86
6	−80.61	−44.94	111.02
7	−83.99	−58.7	124.79
8	−76.53	−39.41	107.08
9	−82.42	−47.58	116.38
10	−86.69	−62.15	129.51
11	−80.47	−47.82	123.12
12	−92.29	45.03	39.69
13	−99.54	43.75	35.64
	HIV-1 PR Inhibitors		
1	−58.39	−33.73	78.54
2	−61.16	−34.33	79.33
3	−66.63	−29.63	79.16
4	−64.9	−30.01	80.14
5	−70.95	−32.22	87.14
6	−64.61	−41.18	105.78
7	−67.42	−41.26	106.4
8	−72.44	−42.12	106.34
9	−72.07	−39.05	106.35
10	−77.51	−40.48	113.68
11	−73.16	−47.75	127.27
12	−76.24	−48.02	127.48
13	−80.66	−48.73	126.16
14	−80.38	−46.76	125.6
15	−81.17	−47.14	125.86
16	−75.36	−50.83	144.16
17	−78.49	−51.13	145.36
18	−82.6	−51.49	144.02
19	−82.37	−49.81	144.69
20	−83.19	−50.53	144.66
21	−69.11	−60.07	127.08
22	−71.73	−57.34	123.94
23	−76.02	−55.42	122.94
24	−76.23	−62.73	126.67

and these have partial cancellation of systematic errors.[13] On the other hand, the electrostatic cutoff introduces an error that is larger for charged inhibitors.

Fitting the BACE data with an additional coefficient (three-parameter model in Table 1) results in a slightly worse predictive power, i.e., a cross-validated error rms of 1.28 kcal/mol and a cross-validated q^2 of 0.65. The deterioration of the predictive accuracy is probably due to the fact that 13 data points are overfitted by three parameters. This observation is corroborated by the standard deviation of the third parameter ($\Delta G_{tr,rot}$), which is almost as large (3.39 kcal/mol) as the value of the parameter itself (4.06 kcal/mol).
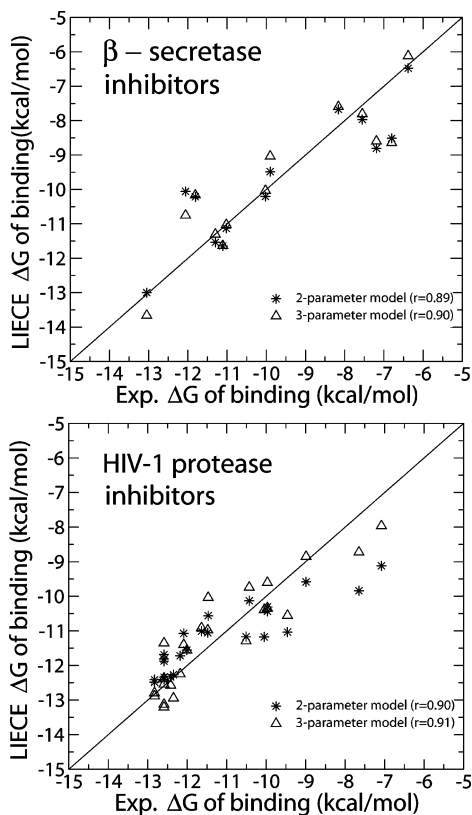
Figure 3. Comparison of the calculated versus experimental binding free energies for 13 BACE inhibitors (top) and 24 HIV-1 PR inhibitors (bottom). The diagonal is drawn for visual help, while the *r* value in parentheses is the correlation coefficient.

HIV-1 PR. For the 24 HIV-1 PR inhibitors, the three-parameter model has a better predictive accuracy than the two-parameter model (cross-validated rms error of 0.77 kcal/mol and cross-validated $q^2 = 0.77$ versus 0.97 kcal/mol and $q^2 = 0.64$, Table 1). This is consistent with the small standard deviations of the parameters in the former, whereas the value of β in the two-parameter model is even smaller than its standard deviation. It is interesting to note that the value of the third parameter $\Delta G_{tr,rot} = 8.2$ kcal/mol is within the range of $7-11$ kcal/mol observed experimentally.[38,39]

The predictive ability of the LIECE approach was further tested on a set of five inhibitors available from a previous work.[40] Their PDB identifiers and K_i values are the following: 1hvr, $K_i = 0.38$ nM; 1hbv, $K_i = 437$ nM; 1htg, $K_i = 3.2$ nM; 1hvs, $K_i = 0.05$ nM; 5hvp, $K_i = 1.1$ μM.[41−45] The five inhibitors were minimized in the HIV-1 PR conformation from the 1hvr complex, which does not contain the water molecule bridging the flaps. The error rms for the five inhibitors of the test set is 1.68 and 1.21 kcal/mol for the two-parameter and three-parameter model, respectively. A similar predictive accuracy was obtained upon minimization in the protein structures of 1hbv, 1htg, and 1hvs, whereas minimization into 5hvp yielded an error rms of 2.47 and 2.45 kcal/mol for the two-parameter and three-parameter model, respectively. The good predictive power is surprising if one considers that the five inhibitors are rather different chemical entities that are characterized by a wide range of torsional degrees of freedom (between 10 and 22 rotatable bonds).

Parameter Transferability. The value of the coefficient α ranges from 0.169 to 0.321 for the four models discussed above (Table 1). Moreover, it is close to the values of α obtained in a previous LIE application to the same set of inhibitors that used a different force field and solvation model.[19] The similar values of the multiplicative parameter for the van der Waals interaction indicate that it is rather robust with respect to the physicochemical characteristics of the binding site. On the other hand, the electrostatic coefficient β varies between 0.0168 (two-parameter model for HIV-1 PR) and 0.1795 (two-parameter model for BACE). This is consistent with the strong hydrophilic character of the BACE S_4 and S_2 subsites,[27] which is not observed in HIV-1 PR.

Using the two-parameter model of BACE ($\alpha = 0.27$, $\beta = 0.18$) to predict the binding free energy of the 24 [32] and 5 (test set) HIV-1 PR inhibitors yields an error rms of 4.1 and 5.9 kcal/mol, respectively. Application of the two-parameter model of HIV-1 PR ($\alpha = 0.17$, $\beta = 0.02$) to the 13 BACE inhibitors yields an error rms of 3.0 kcal/mol. An error rms of about 4 kcal/mol was also obtained for the three-parameter BACE model employed for the HIV-1 PR inhibitors and vice versa. These results indicate that even within two enzymes of the same class (though viral and mammalian aspartic proteases are related very distantly[23]), parameters are not transferable.

Comparison with a Recent MD-Based Model. It is useful to compare the LIECE approach with a recent simplified method for the estimation of absolute binding free energies[46] inspired by, though different from, the LIE approach. Zoete and co-workers[46] performed conformational sampling by MD in vacuo (distance-dependent dielectric function) using harmonic restraints for the protein to prevent excessive conformational deviations from the X-ray structure. For a training set of 16 HIV-1 protease-inhibitor complexes of known three-dimensional structure, they proposed a four-parameter model based on the electrostatic interaction energy between the ligand and the protein, the difference of the electrostatic solvation free energies upon binding, the buried surface, and a constant term. The first three energy terms were averaged over 50 snapshots saved along 100 ps of MD simulation. By use of the energy values printed in Table 2 of ref 46, the LIECE two-parameter and three-parameter models yield a predictive accuracy of 1.85 and 2.01 kcal/mol, respectively. On the other hand, by use of the 24 HIV-1 PR inhibitors of the present study (Figure 2), the simplest four-parameter model of Zoete et al. (based on minimization from the X-ray structure) has a predictive accuracy of 2.32 kcal/mol. Hence, for these sets of HIV-1 PR inhibitors LIECE yields slightly better results than the approach of Zoete et al. Interestingly, for the four-parameter model Zoete et al. found that using a single average structure from the 100 ps MD run provided a slightly better predictive accuracy than the average over 50 MD snapshots and a much better accuracy than using just minimization from the X-ray structure.[46] Unfortunately, the parameters for the model based on the average structure are not given in ref 46, so it is not possible to test the model on the 24 HIV-1 PR inhibitors listed in Figure 2. The authors suggested that it is not the conformational sampling per

se but rather the average structure that is responsible for the improvement with respect to minimization from the X-ray structure. In any case, minimization is easier and more efficient than obtaining an average structure by MD sampling even if one runs MD sampling in vacuo.

Computational Requirements. The LIECE approach requires about 5 or 10 min (mainly for the finite-difference Poisson calculations) of CPU of a single Athlon 2.1 GHz for each HIV-1 PR or BACE inhibitor, respectively. It is about 2 orders of magnitude faster than the most efficient LIE method reported (0.55 days for each inhibitor; see Table 7 of ref 18). The memory requirement for the finite-difference Poisson calculations is about 0.5 GB, which is available on low-cost PCs.

4. Conclusions

Three main results emerge from the present study. First, the LIECE approach, i.e., ligand minimization (in the rigid protein) and finite-difference Poisson calculations, is an efficient procedure that yields accurate predictions of binding free energy values for aspartic proteinase inhibitors. It is important to note that this might not be the case for proteins that bind different inhibitors with some plasticity in the binding site. The original LIE method based on MD (or Monte Carlo) sampling might be more appropriate than LIECE for flexible binding sites. On the other hand, binding site flexibility requires longer MD simulations to reach convergence, which might not be computationally feasible for a large library of compounds in virtual screening.

Second, although the enzymes considered in this work belong to the same class and have similar substrate binding sites, the parameters for LIECE derived using BACE are not predictive for HIV-1 PR and vice versa. This finding provides additional evidence that LIE parameters are not transferable, and therefore, a training set of known inhibitors is a necessary condition.

Third, the simplicity of the approach (no need to add a Born correction term for ionized systems as required in explicit solvent LIE) and the required computational effort (about 5 min per compound) allow LIECE to be used for postprocessing of large libraries of automatically docked compounds. We are currently investigating this issue in our research group.

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