Free Energy Perturbation Calculations on Glucosidase-Inhibitor Complexes

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Abstract: Free energy perturbation studies have been performed on Glucoamylase II (471) from *Aspergillus awamori* var. X100 complexed with three different inhibitors: (+)lentiginosine, (+)(1S,2S,7R,8aS) 1,2,7-trihydroxyindolizidine, (+)(1S,2S,7S,8aS) 1,2,7-trihydroxyindolizidine and the inactive compound (+)(1S,7R,8aS)-1,7-dihydroxyindolizidine. Molecular dynamic simulations were carried out using a recently developed procedure for fast Free Energy Perturbation calculations. In this procedure only a sphere of 1.8 nm around the central atom of the inhibitor is considered in the calculations. Crystallographic restraints are applied over this reduced system using a generated electron density map. The obtained values for the free energy differences agree with experimental data showing the importance of fast calculations in drug design even when the crystallographic structure of the complex is not available. As the method uses only the crystallographic structure of the receptor, it is possible to test the possible efficiency of even still not synthesised ligands, making the pre-selection of compounds much easy and faster.

Key Words: Glycosidase inhibitors, free energy calculation, glycosidase complexes.

INTRODUCTION

Glucoamylase (α-1,4-D-glucan glucohydrolase, EC 3.2.1.3) is an inverting exo-acting hydrolase, that catalyzes the α-D-glucose release from the end of starch and related oligo- and polysaccharides of glucose by hydrolysis of the R-1,4-glucosidic linkages. Glucomaylase occurs naturally in two forms, Glucomaylase I and II. Glucomaylase I consist of a catalytic domain (residues 1 to 470, structure solved by X-ray crystallography [1]), a starch-binding domain (residues 509 to 616 [2]) and a highly O-glycosylated linker region. Glucoamylase II lacks the starch-binding domain but its primary structure is identical to that of the catalytic domain of Glucoamylase I. Glucoamylase II hydrolyses maltooligosaccharides but does not degrade starch granules [3].

Glucoamylase has an inverting glycolisis mechanism. Residues Glu179 and Glu400 have been identified as the active carboxylic residues [4]. No catalytic activity was detectable when two residues in the bottom of the active site, Arg54 and Asp55, were mutated (Arg to Lys or Thr and Asp to Asn or Tyr). In the Glucomaylase native structure Asp55 adopts two conformations, reflecting a high flexibility. A single conformation, involved in hydrogen bonds with the ligand, was found for this residue in the structure of complexes. Asp55 interacts with the two key substrate polar groups 6V-OH and 4V-OH [3]. The Asp55Val mutant has a very low rate of catalysis, binding ascarbose with an association constant reduced nine orders of magnitude compared with the wild enzyme [5]. A Molecular Dynamics study shows the strong inhibitor lentiginosine forming two hydrogen bonds with Arg54 and Asp55 [6].

A big number of glucosidase inhibitors were studied in the recent years in order to understand the enzymatic mechanism but also to generate new potential antibacterial, antiviral [7] (e.g. α–Glucosidase inhibitors interfere with HIV infectivity [8,9]), antitumoral agents [10] or drugs for the treatment of non-insulin dependent diabetes [11]. The most studied inhibitors are the monocyclic azasugars, such as nojirimycin and deoxynojirimycin; see Fig. **1**. The binding geometry of these inhibitors mimics the protonated transition state [11]. A thermodynamic study shows that binding is driven by a negative enthalpy change; the bind is favourable in terms of bond energies [12]. The pH dependence of inhibition of Glucosidase of *Thermotoga maritima* indicates that a charged deoxynojirimycin optimally interacts with a double ionized protein at pH 6.85. However this value is shifted from the maximal activity of the enzyme (pH 5.85), at pH 6.85 the enzymatic catalysis is reduced. The entropy term for the deoxynojirimycin binding was found to be positive, which could be associated with the water molecules becoming ordered in the complex surface. The N atom of this inhibitor makes a hydrogen bond with a bulk water molecule; such interaction has not been observed with the closely related isofagomine inhibitor complexed to Glucoamylase [13].

Despite the efforts putted into the design, synthesis and testing of glycosidase inhibitors only few Glucomaylase - monocyclic azasugar inhibitor complex structures are available. Two different binding sites are observed in the

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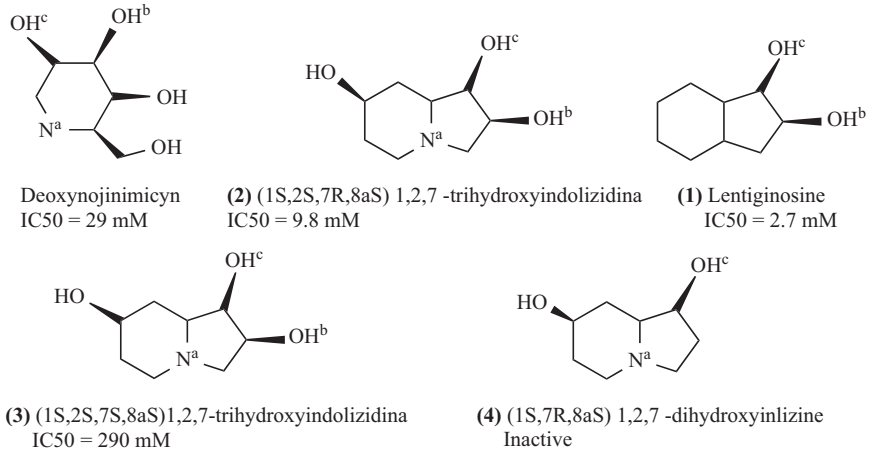


Fig. (1). Deoxynojirimycin -the substrate for which the complex with crystallographic structure is available- and the four molecules studied. The atoms superimposed over the corresponding atoms of crystallographic Deoxymojimrimycin are labeled.

Glucoamylase-deoxynojirimycin complex structure. The first one, with thermal parameters for the inhibitor atoms from 0.7 to 1.0 nm^2, shows a strong electron density (over 10σ) but does not allow the assignment of the protonation state for the central nitrogen atom. The second binding site presents a weak electron density. With thermal parameters between 2.6 and 2.9 nm^2 may not reflect a true binding [3]. A molecule placed in this second site moves to the solvent -loosing all the interactions with neighbour protein residues- during a Molecular Dynamics (MD) run [6].

Another class of Glucosidase inhibitors is formed by nitrogen fused bicyclical alkaloids having pyrrolizidine, indolizidine or quinolizidine ring systems. In this group are: lentiginosine **(1)** [14] and its analogues (1S, 2S, 7R, 8aS)-1,2,7-trihydroxyindolizidine **(2)**, (1S, 2S, 7S, 8aS)-1,2,7-trihydroxyindolizidine **(3)** and the inactive (1S,7R,8aS)-1,7-dihydroxyindolizidine **(4)** [15]; see Fig. **1**. The μM range for the IC50 values of **(1)**, **(2)** and **(3)** evidences how powerful the inhibitors are; even when the analogy with natural substrates is not clear.

The advancement in high throughput screening and combinatorial chemistry techniques has changed drug design. Computational methods that rapidly identify possible ligands have been specially designed. Free Energy Perturbation (FEP) calculations [16-18] have been demonstrated their utility in the study of conformational changes; solvation, relative bind energies [19-24], binding affinities of ions [25] and even absolute bind energies [26]. Despite the simplicity of FEP calculations this technique is frequently ignored for drug design purposes due to its high computational time cost. The main time consuming task is the performing of several MD calculations (multi-steps) for the non-physical intermediates (windows) between the two real systems under study. With the aim of reduce these times different approaches have been studied. Some of them are: considering only a fraction of the whole system in the MD calculations, using short-length simulations (sampling errors are cancelled in the double difference [27]) or using the single-step FEP formalism. This last formalism can be used when a related group of ligands is present, only a trajectory with a non-physical reference state is considered for several mutations [19,24].

In case of no crystallographic structure available manually docked ligands have successfully been used in FEP studies (e.g. [20]).

In this work we report a free energy perturbation study on the complexes formed by Glucoamylase II and manually docked inhibitors **(1)**, **(2)**, **(3)** and **(4)**. The goal is to test a novel procedure for fast FEP calculations in complexes without available crystallographic structure. This procedure

Table 1. Comparison Between Experimental Data and the ΔΔG (in KJ/mol) Values Obtained with the Two FEP Formalism Used, Multi-Steps ($\Delta\Delta G_m$) or Single-Step ($\Delta\Delta G_s$)

	IC50 (μM)	$\Delta\Delta G_e$	$\Delta\Delta G_m$	$\Delta\Delta G_s$
Lentiginosine (1)	2.7	--	--	--
(1S, 2S, 7R, 8aS)-1,2,7-trihydroxyindolizidine (2)	9.8	0.76	0.19	23.35
(1S, 2S, 7S, 8aS)-1,2,7-trihydroxyindolizidine (3)	290	2.78	0.63	47.06
(1S, 7S, 8aS)-1,7-di trihydroxyindolizidine (4)	inactive	--	30.4	--

is based on the use of crystallographic restraints over a reduced system[§]. The results agree qualitatively with experimental data allowing identifying the worst inhibitor of the group. Using the single-step FEP formalism the energetic differences differ considerably, even when long trajectories are considered.

RESULTS AND DISCUSSION

The ΔΔG obtained values –using multi or single-step FEP formalisms- are summarized in table **1** with the experimental IC50 values [6]. The experimental ΔΔG values ($\Delta\Delta G_e$) are calculated from the IC50 ratios using the following expression: $\Delta\Delta G_e(2,1) = RT\ln\{IC50(2)/IC50(1)\}$. The energetic differences in single-step FEP calculations between **(2)** and **(1)** and between **(3)** and **(1)** are calculated as: $\Delta\Delta G_s(2, 1) = (\Delta\Delta G(2,$ reference sate) - $\Delta\Delta G(1,$ reference sate) and $\Delta\Delta G(3, 1) = (\Delta\Delta G(3,$ reference sate) - $\Delta\Delta G(1,$ reference sate), respectively.

The ΔΔG values, obtained with the procedure that uses crystallographic restraints on the reduced system, agree with experimental data. The higher difference is obtained when the inactive (1S,7R,8aS)-1,7-dihydroxyindolizidine **(4)** is analysed. The differences between the other three inhibitors are small but even in this case FEP calculations can distinguish that (+)(1S,2S,7S,8aS) 1,2,7-trihydroxyindolizidine **(3)** is the worst of the group. These results confirm the importance of fast FEP calculations, even when it is necessary to make a manually docked complex because a crystallographic structure is not available. We wish to remark this point since it gives the possibility of evaluate free energy differences between complexes before the inhibitor synthesis.

Even using 360 ps trajectories the free energetic difference values obtained with the single-step FEP formalism do not agree with experimental data. Not definitely reasons for this were concluded. This could be explained by a poor sampling of the phase space or by the use of two soft Van der Waals atoms united to the same carbon, a situation that has not been previously considered [19].

Fig. (**2a**), Fig. (**2b**), Fig. (**2c**) and Fig. (**2d**) –made using the program PyMOL [28]- show the residues involved in the active site of the four enzyme-inhibitor complex structures. All found interactions are labelled in these geometries obtained averaging 400 ps of MD.

Such geometries show appreciable differences in the bindings of compounds **(2)** and **(3)**. The interaction with Arg 54 and Asp 55 is observed in each of the three complexes but **(2)** curves over itself. The OH group proper of this molecule could establish then a new interaction with Arg 54 with a water molecule as intermediate. This geometry is not observed in the Glucoamylase-**(3)** complex were the interaction with Tyr311 and Tyr48 pulls of the OH group from the opposite position of the active site. This could be a possible explanation for the small difference between the compounds observed experimentally and by means of these fast FEP calculations. In the Glucoamylase-**(4)** complex the h-bond interaction with Arg54 and Asp55 is lost. The inhibitor only interacts by means of a water molecule. Such situation could explain the lack of inhibitor activity.

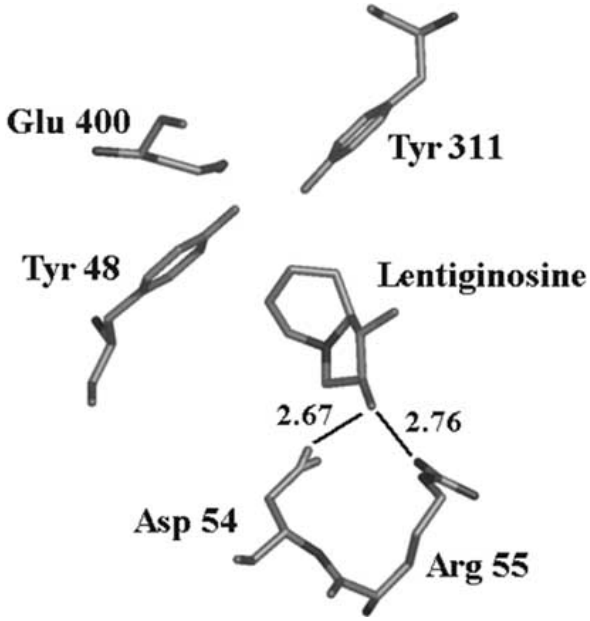


Fig. (2a). Active site structure obtained for Glucoamylase-lentiginosine **(1)** complex by averaging over 400 ps of molecular dynamics trajectory.

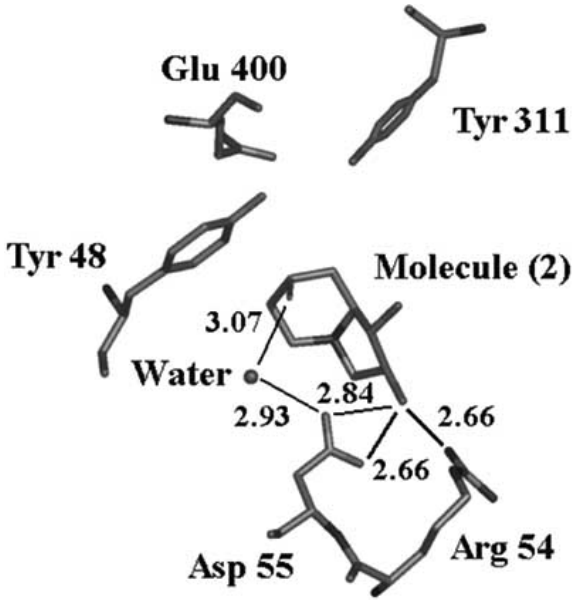


Fig. (2b). Active site structure obtained for Glucoamylase-(1S, 2S, 7R, 8aS)-1,2,7-trihydroxyindolizidine **(2)** complex by averaging over 400 ps of molecular dynamics trajectory.

CONCLUSION

Using a reduced system with crystallographic restrains for the calculation of binding Free Energy differences we have been able to obtain the correct order between the

[§] Ruiz F., Podjarny A., Burt S., Cachau R, in preparation.

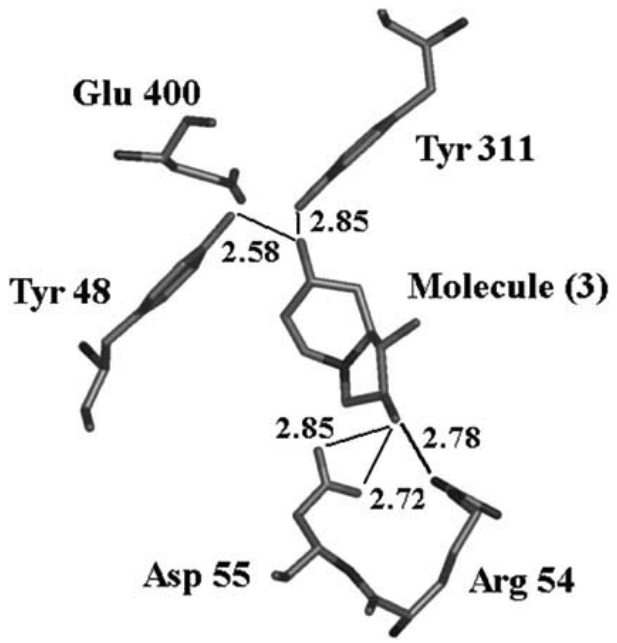


Fig. (2c). Active site structure obtained for Glucoamylase-(1S, 2S, 7S, 8aS)-1,2,7-trihydroxyindolizidine **(3)** complex by averaging over 400 ps of molecular dynamics trajectory.

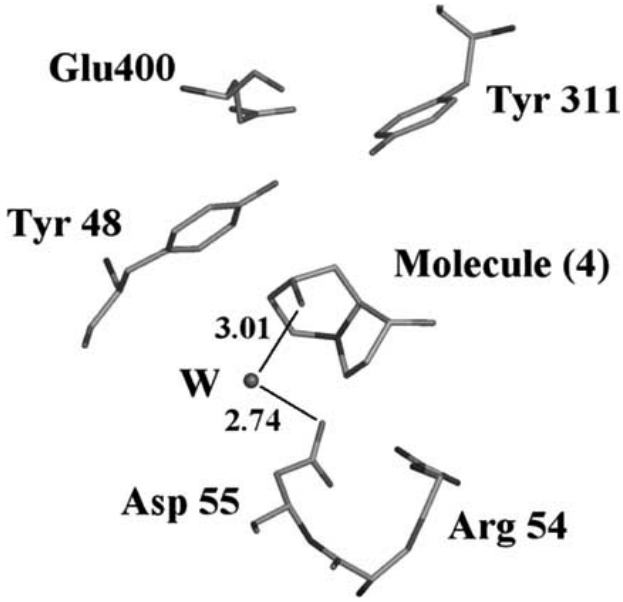


Fig. (2d). Active site structure obtained for Glucoamylase-(1S, 7S, 8aS)-1,7-dihydroxyindolizidine **(4)** complex by averaging over 400 ps of molecular dynamics trajectory.

differences in the binding free energies of a family of compounds. Although the agreement is only qualitative it is remarkable that we have not used the crystallographic structure of any of the considered complexes. Since the time demanded by the calculation using such recently developed fast FEP procedure is not excessive, this methodology opens the possibility of exploring the possible efficiency of different ligands; even for still not synthesised ones, making the pre-selection of compounds much easy and faster.

EXPERIMENTAL

We have obtained the atomic structure of Glucoamylase II (**4 7 1**) from *Aspergillus Awamori* complexed with deoxynojirimycin from the Protein Data Bank (entry 1DOG, [3]). Both molecules of deoxynojirimycin present in the crystallographic structure were deleted and the inhibitor under study was manually docked in the first binding site. Both, crystallographic [3] and simulation studies [6] consider a low –if null- occupation of the second site, therefore we kept it empty. The central nitrogen atom and the two OH groups of the 5 members ring were manually placed over the corresponding deoxynojirimycin atoms, following the a fitting previously used [6] (see Fig. (**1**)). Based in previous studies indicating that molecules of this inhibitor class should be positively charged at physiological pH [6,29] we have protonated the nitrogen atom of the inhibitors. We adopted the criteria of consider explicitly all the hydrogen atoms for the inhibitor molecules but only the polar ones for the protein. We have taken atomic hydrogen mass of 2 in order to use a 2 fs integration step during the data acquisition runs. The hydrogen atoms were added and all the parameters obtained using Quanta 2000 (Molecular Simulations 2000). We used parameters PARAM19 for the protein and PARAM22 for the inhibitors. The X-Plor 3.851 program [30] was used in all the calculations.

Complexes enzyme-inhibitor (**2**) and enzyme-inhibitor (**3**) -systems of maximal inhibitor volume- were solvated using a 6.0x6.0x6.0 nm box of SPC/E water [31]. Water molecules at distance from the complex lower than 0.26 nm were deleted. Crystallographic waters were conserved. The total number of considered atoms is close to 17000 in both cases.

The geometries of the solvated complexes were subsequently minimized using the conjugate gradient method. We made a total of 3000 steps, taking five groups of 600 steps applying positional harmonic restraint with decreasing constant values k (the first: k=300 kcal mol^{-1} Å^2 for the protein, k=70 kcal mol^{-1} Å^2 for the inhibitor, and k=50 kcal mol^{-1} Å^2 for water; the second: k=50 kcal mol^{-1} Å^2 for the protein, k=30 kcal mol^{-1} Å^2 for the inhibitor, and k=25 kcal mol^{-1} Å^2 for water; the third: k=25 kcal mol^{-1} Å^2 for the protein, k=10 kcal mol^{-1} Å^2 for the inhibitor and water; the fourth: k=15 kcal mol^{-1} Å^2 for the protein and finally the fifth: k growing from 0.1 to 5 kcal mol^{-1} Å^2 for the protein atoms inside the region between 1.5 and 1.8 nm from the nitrogen of the inhibitor and k=5 kcal mol^{-1} Å^2 for larger radius). Using the last mentioned harmonic restraints conditions MD was performed with 1 fs integration step in order to relax the whole system. Temperature was controlled using the Berendsen's thermostat [32]. The temperature of the coupled bath was 150 K for the first 1000 steps and then it was increased during 6 ps to 400 K. After that, the temperature was decreased to 300 K in 5 ps. Finally, the system was equilibrated at 300 K over 5 ps.

The size of the system described is extremely large to allow computations in a reasonable time, even with very large computers; therefore we have proceeded to the

computation with a small fraction of the complete system. Several methods have been used in the past, all considering only the part of the system containing the active site; we have used the principles pointed by Cachau *et al*. [33].

From the structure of the interest region (a sphere around the inhibitor) a low-resolution (6Å) electron density map (F_{calc},t) is computed. The initial structure is then annealed inside this envelope. In this way, the overall shape of the parent structure is transferred onto the model structure. Compared to the high-resolution maps, normally used to solve the experimental structure, a map at 6Å resolution does not contain detailed information on the position of the side chain atoms, although the main secondary motifs can be easily recognized. A restrain energy function using the crystallographic $F_{calc,t}$ is defined as:

$$E_{Xray} = W_A/N_A \Sigma_{h,k,l}[\ |F_{calc,t}(h,k,l)| - |F_{calc,m}(h,k,l)|\ ]^2,$$

where h,k,l are the Miller indices of the selected reflections, $F_{calc,t}$ are the observed structure factors (in our case calculated from the initial geometry), $F_{calc,m}$ the computed factors for the structure being modelled, N_A is a normalization factor, and W_A is an overall weighting factor, following the usual protocol for the refinement of crystallographic structures. This restrain implies an extra term in the Hamiltonian function to be used. A total equilibration time of 50 ps was used, the others parameters were the same than in the previous case.

From the equilibrated structures we selected a sphere of 1.8 nm radius centred in the central nitrogen atom of the inhibitor. We deleted the external atoms of the protein. Also we deleted water molecules with oxygen atoms located beyond the sphere. Around 1750 atoms form the remaining reduced systems, ten times smaller than the complete systems.

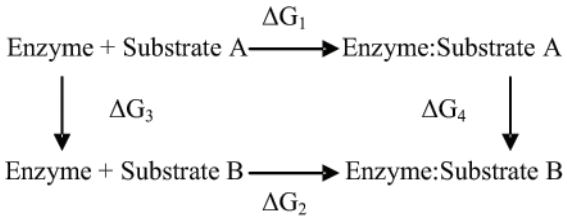
We computed the structure factor (F) for the reduced system using X-Plor 3.851 [30] in a box of 50x5.0x5.0 nm, with a lower resolution limit equal to 2 Å.

Furthermore, in the last part of the preparation procedure and during the calculations, harmonic restraints were applied on the protein atoms located into the 0.3 nm external shell –mimicking the protein main chain continuity near the border of the system. With these two restraints the general details of the whole system geometry are kept in the reduced system.

The temperature has grown from 150 K to 300 K in 4 ps starting with atomic velocities assigned with a Maxwell distribution. Last 5000 steps of MD were used to rise up the integration step from 1fs to 2 fs, each 1000 steps 0.25 fs were added. The last step was a 10 ps equilibration at 300 K. This last equilibration run (integration step of 2 fs) was 25 times faster than the equivalent MD run for the whole system (5 ps with an integration step of 1 ps). From the last trajectory the final atomic positions and velocities were taken as initial points to the trajectory captures.

Over the system inhibitor-solvation water a similar equilibration protocol was applied without harmonic restraint.

We used a thermodynamic cycle –Fig. (**3**) – to evaluate the energetic double difference in the binding of inhibitors. Three different mutations were considered: **(2)** to **(1)**, **(2)** to **(4);** adding both of them it is possible to analyse the **(4)** to **(1)** mutation) and **(3)** to **(1)**. In all the cases the Van der Waals atomic parameters and atomic charges were changed linearly for the OH group mutating to H. The atomic charge of the carbon where the OH is bound was also changed, conserving the total charge of this mutating C-O-H group. The complete charge of the mutating inhibitor kept unchanged (+1). The bonds carbon-mutating oxygen and mutating oxygen-mutating hydrogen mutated both, equilibrium distance and bond force. We used the intermediate reference states method [34] considering 15 windows. In each 100 ps window the first 30 ps were used to equilibrate the system after the change of parameter. 70 ps trajectories were taken to energetic calculations. Final position and velocities were taken as initial points to the next window.



$$\Delta\Delta G\ (A, B) = \Delta G_2 - \Delta G_1 = \Delta G_4 - \Delta G_3$$

Fig. (3). Thermodynamic cycle employed in FEP calculations.

Furthermore single-step FEP calculations were carried out for the mutations **(2)** to **(1)** and **(3)** to **(1)**. Soft van der Waals interactions were considered for the studied two mutating sites. The soft-core parameters used for the mutating atoms in the reference state correspond to an α-parameter of 1.51 [35]. Although 400 ps trajectories were considered for the complex and inhibitor solvated reference states, only the last 360 ps were used in energetic calculations, allowing to the system equilibrates during 40 ps. The values obtained correspond to energetic differences between the reference state and molecules **(1)**, **(2)** and **(3)**.

Four different trajectories of 400 ps were taken with the original parameters of inhibitors **(1)**, **(2)**, **(3)** and **(4)** in order to analyze the inhibitor binding modes.

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