Statistical thermodynamic basis in drug-receptor interactions: double annihilation and double decoupling alchemical theories, revisited

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Abstract

Alchemical theory is emerging as a promising tool in the context of molecular dynamics simulations for drug discovery projects. In this theoretical contribution, I revisit the statistical mechanics foundation of non covalent interactions in drug-receptor systems, providing a unifying treatment that encompasses the most important variants in the alchemical approaches, from the seminal Double Annihilation Method by Jorgensen and Ravimohan [W.L. Jorgensen and C. Ravimohan, J. Chem. Phys. 83,3050, 1985], to the Gilson's Double Decoupling method [M. K. Gilson and J. A. Given and B. L. Bush and J. A. McCammon, Biophys. J. 72, 1047 1997] and the Deng and Roux alchemical theory [Y. Deng and B. Roux, J. Chem. Theory Comput., 2, 1255 2006]. Connections and differences between the various alchemical approaches are highlighted and discussed, and finally placed into the broader context of nonequilibrium thermodynamics.

INTRODUCTION

The determination of the binding free energy in ligand-receptor systems is the cornerstone of drug discovery. In the last decades, traditional molecular docking techniques in computer assisted drug design have been modified, integrated or superseded using methodologies relying on a more realistic description of the drug-receptor system. It has becoming increasing clear that, in order to reliably rank the affinity of putative ligands for given target, a microscopic description of the solvent is a crucial ingredient. As recently pointed out by Gilson and co-workers,[1] the nature of the entropic term in binding is intimately related to microsolvation phenomena in ligand-receptor association that can bring along very large entropy fluctuations.

In the framework of atomistic molecular dynamics (MD) simulations with explicit solvent, several computational methods have been devised for rigorously determining the absolute binding free energy in drug receptor systems. Most of these methodologies are based on the so-called alchemical route (see Refs. [2, 3] for recent reviews). In this approach, proposed for the first time by Jorgensen and Ravimohan[4], the binding free energy is obtained by setting up a thermodynamic cycle as indicated in Figure 1 and by computing the decoupling free energy of the ligand in the bound state and in bulk water, indicated hereinafter with ΔG_b and ΔG_u, respectively. These decoupling free energies corresponds to the two closing branches of the cycle and are obtained by discretizing the alchemical path connecting the fully interacting and fully decoupled ligand in a number of intermediate nonphysical states, running for each of these states equilibrium, fully atomistic molecular dynamics simulations. Alchemical states are hence defined by a λ coupling parameter entering in the Hamiltonian, varying between 1 and 0 so that at $\lambda = 1$ and at $\lambda = 0$ one has the fully interacting and gas-phase ligand, respectively. ΔG_b and ΔG_u are usually recovered as a sum of the contributions from each of coupling parameter windows by applying the free energy perturbation method (FEP).[5] Alternatively, and equivalently, one can compute the canonical average of the derivative of the Hamiltonian at the discrete λ points, obtaining the decoupling free energy via numerical thermodynamic integration (TI).[6] Finally, the cycle is closed by computing the difference between the two decoupling free energy along the alchemical path, ΔG_b and ΔG_u, obtaining the dissociation free energy in solution.

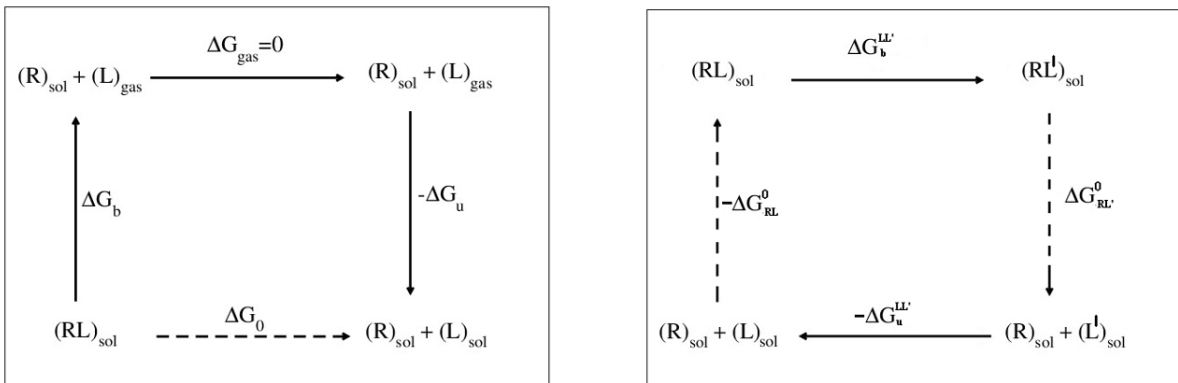Gilson et al. [7] criticized Jorgensen's theory by pointing out that the resulting binding

FIG. 1: The alchemical thermodynamic cycle for computing the absolute and relative dissociation free energy, ΔG_0, in drug-receptor systems. The subscript "sol" and "gas" indicates solvated and gas-phase species, respectively. For the alchemical determination of absolute *standard* free energies (left cycle) the ligand must decoupled in the solvated complex and in bulk solvent obtaining $\Delta G_0 = \Delta G_b - \Delta G_u$. For alchemical determination of *relative* standard free energies (right cycle) the ligand L must transmuted into the ligand L′ in the solvated complex and in bulk solvent obtaining $\Delta\Delta G_0 = \Delta G_b^{\mathrm{LL}'} - \Delta G_u^{\mathrm{LL}'}$.

free energies do not depend upon the choice of standard concentration. In order to define a reference chemical potential for the decoupling ligand when bound to the receptor, Gilson introduced a "restraint" that somehow keeps the ligand in the binding place. This restraint is shown to yield[7] an additive standard state dependent correction to the dissociation free energy of $k_B T \ln(V_r/V_0)$, interpreted as a chemical potential difference of the ligand at concentration $1/V_r$ and $1/V_0$. According to Gilson, the effect of progressively strengthening the restraint, leading to a more negative correction, should be balanced by a larger work integral so that "errors will occur only when the integration region defined by the restraint volume becomes so small that conformations that ought to make important contributions to the work integral are missed." Later Karplus and co-workers[8] noted that in the final ($\lambda \simeq 0$) stages of the decoupling of the complex, the unrestrained ligand in DAM may freely rotate and wander to any point in the simulation system, so that, in order to compute ΔG_b correctly, the ligand would have to sample every possible position in the simulation box, with a standard state correction for the DAM dissociation free energy equal to the additive term $k_B T \ln(V_{\mathrm{box}}/V_0)$ where V_{box} is the volume of simulation box. In the framework of the Gilson's DDM theory, these authors hence proposed to enforce a set of harmonic restraints

(with force constant varying from 5 to 50 kcal mol^{-1}[Å^{-2}/rad^{-2}]) that restrict both the position and the orientation of the ligand. Subsequently, Deng and Roux[9] proposed a DDM variant whereby the restraints for the bound state are not present at the end states $\lambda = 1$ and $\lambda = 0$ of the alchemical process; rather, they are progressively switched on and off during the alchemical transformation with a cancellation effect. In the Deng and Roux variant, in the limit of strong restraints, the standard state correction is no longer dependent on the imposed restraint volume V_r. However, it does requires the estimate of the unknown translational, rotational and conformational binding site "volume" V_{site} in the complex[10, 11] via an independent unrestrained simulation of the bound state.

In a series of recent papers, Fujitani and coworkers,[12–14] successfully applied the unrestricted DAM approach to several drug-receptor systems, in many cases predicting the dissociation free energy via FEP in close agreement with the experimental values with an average error of 2/3 kcal mol^{-1}. Errors were assessed by repeating several times the FEP calculations with runs on the order of few ns on each alchemical states. Most importantly, these authors directly compared their DAM/FEP values, $\Delta G_{\text{DAM}} = \Delta G_b - \Delta G_u$, to the experimental value ΔG_0, openly criticizing the DDM standard state correction: "as far as we know there is no theoretical or experimental proof that [the standard state corrected] ΔG_0 meets the definition of the absolute binding energy.[..] Therefore, we directly compare ΔG_{bind} with ΔG_0."

The standard state correction issue can be bypassed altogether by computing relative binding free energies,[15] due to the transmutation of a ligand into another in the *same binding site* and in the solvent. Relative binding free energy calculations involves as much computations as absolute free energies do (see Figure 1), and completely neglect the possibility of a change of binding site volume due to the transmutation. This approach is hence limited to the assessment of the binding affinities in strictly congeneric series of ligands with the tacit assumption of a constant binding site volume upon transmutation and cannot provide, by any means, a complete tool in MD-based drug design.

In conclusion, the question of the standard state correction, or, equivalently, the issue of the binding site volume in drug-receptor dissociation free energy calculations is either ignored, as in relative free energy calculations, or treated using methodologies relying on the definition of arbitrary set of constraints whose effects on the resulting free energy has never been convincingly assessed. In any case, the standard state issue, that is indeed crucial

for a reliable MD-based in silico tool in drug discovery, is still far from being settled. In this theoretical contribution we revisit the DAM and DDM theory with a spotlight on the binding site volume issue, providing a unifying treatment encompassing Jorgensen, Gilson and Boresch and Roux theories, and finally placing the alchemical methodology into the broader context of nonequilibrium thermodynamics.

ACHEMICAL THEORY OF NON COVALENT BONDING

Molecular recongignition in host-guest or drug-receptor non covalent interactions are based on a highly specific molecular complementary[16], translating in the existence of a single overwhelmingly prevalent binding "pose" defined using an appropriate set of coordinates that are functions of the ligand and receptor Cartesian coordinates x. A natural coordinate in ligand-protein binding is represented by the distance $\mathbf{R}$ of center of mass (COM) of the ligand with respect to a fixed reference system with the origin at COM of the protein and oriented along the inertia axis of the protein. The vector $\mathbf{R}$ (in polar coordinates r, θ, ϕ) defines the precise location of the ligand COM on the protein surface in the bound state. Euler angles can be further introduced to specify the orientation of the ligand frame relative to the protein frame. For non rigid ligands and/or binding pockets, however, a rigorous separation of vibrational and rotational coordinates is not possible as the inertia tensor of the ligand and, to a less extent, that of the protein may change significantly upon binding by coupling to ligand and/or receptor conformational coordinates. The most general definition of a binding pose is hence enforced by supplementing the natural coordinate $\mathbf{R} = r, \theta, \phi$ with an appropriate set of $\boldsymbol{\xi}$ ro-vibrational coordinates defined with respect to a protein frame in terms of the ligand and receptor Cartesian coordinates x. The set $\{\mathbf{R}, \boldsymbol{\xi}\}$ should include all those coordinates whose probability density differ significantly in going from the bound to the unbound states.

Double Decoupling method (DDM)

In DDM, a set of harmonic restraints are introduced on the $\mathbf{Y} = \{\mathbf{R}, \boldsymbol{\xi}\}$ d-dimensional set of coordinates in order to the keep the ligand in the binding pose while the decoupling process proceeds. The easiest way to do so is that of introducing harmonic potentials for

each of these d coordinates, leading to the restraint potential of the kind

$$V_r(x) = \frac{1}{2}K_r(r(x) - r_e)^2 + \frac{1}{2}K_\theta(\theta(x) - \theta_e)^2 + \frac{1}{2}K_\phi(\phi(x) - \phi_e)^2 + \frac{1}{2}\sum_i^{d-3} K_\xi^{(i)}(\xi_i(x) - \xi_i^e)^2 \quad (1)$$

The restraint potential can be compactly written in vector notation as as

$$V_r(\mathbf{Y} - \mathbf{Y}_e) = \frac{1}{2}(\mathbf{Y} - \mathbf{Y}_e)^T \mathbf{K}(\mathbf{Y} - \mathbf{Y}_e) \quad (2)$$

where $\mathbf{K}$ is the diagonal matrix of the harmonic force constants. Note that the function $e^{-\beta V_r(x)}$ may be interpreted as a product of independent univariate Gaussian distributions or equivalently as non normalized multivariate Gaussian distribution in the d dimensional space defined by the coordinates $\mathbf{Y} = \{\mathbf{R}(x), \boldsymbol{\xi}(x)\}$

$$e^{-\beta V_r(x)} = e^{-\frac{1}{2}(\mathbf{Y} - \mathbf{Y}_e)^T \boldsymbol{\Sigma}_r^{-1}(\mathbf{Y} - \mathbf{Y}_e)} \quad (3)$$

where the diagonal covariance matrix $\boldsymbol{\Sigma}_r$ is defined as

$$\boldsymbol{\Sigma}_r = k_B T \mathbf{K}^{-1} \quad (4)$$

As we may not know precisely the geometry of the pose of the ligand in the binding site, the chosen restraint equilibrium parameters, $\mathbf{Y}_e = \{\mathbf{R}_e, \boldsymbol{\xi}_e\}$, can be different from their corresponding true mean values $\mathbf{Y}_c = \{\mathbf{R}_c, \boldsymbol{\xi}_c\}$. In Ref. [17], in the context of single molecule pulling experiments, a simple relation was derived between the free energy of the driven system (i.e. with Hamiltonian including the harmonic potential of an external device coupled to a specific molecular distance R) and the free energy of the system with unperturbed Hamiltonian along the driven coordinate (i.e. the potential of mean force along R). The relation proposed by Marsili (Eq. 7 in Ref. [17]) can be straightforwardly applied to any of the restrained λ alchemical state in DDM as:

$$G_r(\boldsymbol{\Sigma}_r, \mathbf{Y}_e, \lambda) = G(\mathbf{Y}, \lambda) + V_r(\mathbf{Y} - \mathbf{Y}_e) + k_B T \ln\left(\frac{P(\mathbf{Y}|\boldsymbol{\Sigma}_r, \mathbf{Y}_e, \lambda)}{P(\mathbf{Y}_*)}\right) \quad (5)$$

where

$$G_r(\boldsymbol{\Sigma}_r, \mathbf{Y}_e, \lambda) = -k_B T \ln\left[\mathcal{C}\int dx e^{-\beta[H(x,\lambda) + V_r(\mathbf{Y} - \mathbf{Y}_e)]}\right] \quad (6)$$

$$G(\mathbf{Y}, \lambda) = -k_B T \ln\left[\frac{\int dx \delta(\mathbf{Y} - \mathbf{Y}(x))e^{-\beta H(x,\lambda)}}{\int dx \delta(\mathbf{Y}_* - \mathbf{Y}(x))e^{-\beta H(x,\lambda)}}\right] = -k_b T \ln\frac{P(\mathbf{Y})}{P(\mathbf{Y}_*)} \quad (7)$$

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Here, $G_r(\mathbf{\Sigma}_r, \mathbf{Y}_e, \lambda)$ is the free energy of the restrained system ($\mathcal{C}$ is an h dependent constant that makes argument of the logarithm adimensional) and $G(\mathbf{Y}, \lambda)$ is the free energy of the unrestrained system at $\mathbf{Y} = \mathbf{R}, \mathbf{\xi}$ with respect to some immaterial reference state at $\mathbf{Y}_* = \mathbf{R}_*, \mathbf{\xi}_*$ In Eqs 6 and 7, $H(x, \lambda)$ is the Hamiltonian at the alchemical state λ, with x encompassing all solvent, ligand and receptor coordinates. $P(\mathbf{Y}|\mathbf{\Sigma}_r, \mathbf{Y}_e, \lambda) \equiv \langle \delta(\mathbf{R} - \mathbf{R}(x))\delta(\mathbf{\xi} - \mathbf{\xi}(x))\rangle_r$, finally, is the canonical probability density evaluated at $\mathbf{Y} = \{\mathbf{R}, \mathbf{\xi}\}$ for the restrained system with free energy given by Eq. 6.

In the alchemical simulation of the complex, one computes, either via FEP or TI, the free energy difference between the states at $\lambda = 1$ (interacting ligand) and $\lambda = 0$ (gas-phase ligand), subject to the restraint potential V_r, Eq. 1. In force of Eq. 5, we therefore get the $\mathbf{Y}_*$ independent relation

$$
\begin{aligned}
\Delta G_r(\mathbf{\Sigma}_r, \mathbf{R}_e, \mathbf{\xi}_e) &= G_r(\mathbf{\Sigma}_r, \mathbf{R}_e, \mathbf{\xi}_e, 0) - G_r(\mathbf{\Sigma}_r, \mathbf{R}_e, \mathbf{\xi}_e, 1) \\
&= \Delta G(\mathbf{R}, \mathbf{\xi}) + k_{\mathrm{B}} T \ln \frac{P(\mathbf{R}, \mathbf{\xi}|\mathbf{\Sigma}_r, \mathbf{R}_e, \xi_e, 0)}{P(\mathbf{R}, \mathbf{\xi}|\mathbf{\Sigma}_r, \mathbf{R}_e, \mathbf{\xi}_e, 1)}
\end{aligned}
\tag{8}
$$

where I have used the expanded notation for $\mathbf{Y} = \{\mathbf{R}, \mathbf{\xi}\}$ and where

$$
\Delta G(\mathbf{R}, \mathbf{\xi}) = -k_B T \ln \left[\frac{\int dx \delta(\mathbf{R} - \mathbf{R}(x))\delta(\mathbf{\xi} - \mathbf{\xi}(x))e^{-\beta H(x,0)}}{\int dx \delta(\mathbf{R} - \mathbf{R}(x))\delta(\mathbf{\xi} - \mathbf{\xi}(x))e^{-\beta H(x,1)}} \right]
\tag{9}
$$

is the decoupling free energy of the unrestrained system evaluated at $\mathbf{Y} = \{\mathbf{R}, \mathbf{\xi}\}$ and where $\Delta G_r(\mathbf{\Sigma}_r, \mathbf{R}_e, \xi_e)$ corresponds to decoupling free energy of the restrained complex. Note that, since there is no change in the parameters $\mathbf{Y}_e = \{\mathbf{R}_e, \xi_e\}$ in going from the initial (coupled) to the final (decoupled) state, there can't be correspondingly no change in the harmonic potential energy at $\{\mathbf{R}, \mathbf{\xi}\}$ due to the restraint.

The $\mathbf{\xi}$-dependent *decoupling free energy of the unbound state* can be defined as[9]

$$
\Delta G_u(\mathbf{\xi}) = -k_B T \ln \left[\frac{\int dx \delta(\mathbf{R}_\infty - \mathbf{R}(x))\delta(\mathbf{\xi} - \mathbf{\xi}(x))e^{-\beta H(x,0)}}{\int dx \delta(\mathbf{R}_\infty - \mathbf{R}(x))\delta(\mathbf{\xi} - \mathbf{\xi}(x))e^{-\beta H(x,1)}} \right]
\tag{10}
$$

where, $\mathbf{R}_\infty$ represents a ligand-receptor COM distance that is large enough to allow the ligand and the receptor to interact only with the solvent when $\lambda \neq 0$. $\Delta G(\mathbf{\xi})$ represents the reversible work to bring the unbound ligand *and* unbound receptor (set at a relative vector distance $\mathbf{R}_\infty$ and in the ro-vibrational states defined by the vector $\mathbf{\xi}$) from the bulk into the gas-phase. This work may depend on the $\mathbf{\xi}$ coordinates in case of, e.g., competing conformational states of the ligand and/or protein involved in the binding. For a rigid ligand and rigid binding pose, $\mathbf{\xi}$ can be taken to coincide with the three Euler angles, $\mathbf{\Omega}$, defining

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the orientation of the ligand with respect to the protein frame. In this case, all rotational states at $\mathbf{R}_\infty$ (i.e. for the unbound or free ligand) have equal weights so that $\Delta G_u(\mathbf{\Omega}) \equiv \Delta G_u$ is independent of $\mathbf{\Omega}$. In DDM theories, while ligand conformational changes upon binding may[18] or may not[8] accounted for, the fact that the receptor may change as well its conformational state in the binding process is generally overlooked. By subtracting Eq. 10 in Eq. 9, we obtain

$$
\begin{aligned}
\Delta G_r(\mathbf{R}, \xi) - \Delta G_u(\boldsymbol{\xi}) &= -k_B T \ln \left[\frac{\int dx \delta(\mathbf{R} - \mathbf{R}(x)) \delta(\boldsymbol{\xi} - \boldsymbol{\xi}(x)) e^{-\beta H(x,0)}}{\int dx \delta(\mathbf{R} - \mathbf{R}(x)) \delta(\boldsymbol{\xi} - \boldsymbol{\xi}(x)) e^{-\beta H(x,1)}} \right] + \\
&+ k_B T \ln \left[\frac{\int dx \delta(\mathbf{R}_\infty - \mathbf{R}(x)) \delta(\boldsymbol{\xi} - \boldsymbol{\xi}(x)) e^{-\beta H(x,0)}}{\int dx \delta(\mathbf{R}_\infty - \mathbf{R}(x)) \delta(\boldsymbol{\xi} - \boldsymbol{\xi}(x)) e^{-\beta H(x,1)}} \right] \\
&= -k_B T \ln \left[\frac{\int dx \delta(\mathbf{R}_\infty - \mathbf{R}(x)) \delta(\boldsymbol{\xi} - \boldsymbol{\xi}(x)) e^{-\beta H(x,1)}}{\int dx \delta(\mathbf{R} - \mathbf{R}(x)) \delta(\boldsymbol{\xi} - \boldsymbol{\xi}(x)) e^{-\beta H(x,1)}} \right] \\
&= -w(\mathbf{R}, \boldsymbol{\xi}) \quad (11)
\end{aligned}
$$

where we have exploited the fact that the probability densities of the decoupled ligand and receptor ($\lambda = 0$) with respect to $\mathbf{R}$ is uniform. $w(\mathbf{R}, \boldsymbol{\xi})$ on the rhs of Eq. 11 represents the reversible work, or potential of mean force, for bringing a separated ligand and receptor in the $\boldsymbol{\xi}$ ro-vibrational arrangement into the corresponding bound conformation at $\mathbf{R}$.

If in Eq. 8 and Eq. 11 we choose $\boldsymbol{\xi}_e = \boldsymbol{\xi}_c$ and $\boldsymbol{\xi} = \boldsymbol{\xi}_c$ and we use Eq. 11, we obtain

$$
\Delta G_r(\mathbf{\Sigma}_r, \mathbf{R}_c, \boldsymbol{\xi}_c) - \Delta G_u(\boldsymbol{\xi}_c) = -w(\mathbf{R}_c, \boldsymbol{\xi}_c) + k_B T \ln \frac{P(\mathbf{R}, \boldsymbol{\xi} | \mathbf{\Sigma}_r, \mathbf{R}_c, \boldsymbol{\xi}_c, 0)}{P(\mathbf{R}, \boldsymbol{\xi} | \mathbf{\Sigma}_r, \mathbf{R}_c, \boldsymbol{\xi}_c, 1)} \quad (12)
$$

Eq. 12 expresses the fact that the dissociation free energy with a set of harmonic restraints of the kind of Eq.1 computed in DDM simulation via FEP or TI, namely the quantity

$$
\Delta G_d(\mathbf{\Sigma}_r, \mathbf{R}_c, \boldsymbol{\xi}_c) = \Delta G_r(\mathbf{\Sigma}_r, \mathbf{R}_c, \boldsymbol{\xi}_c) - \Delta G_u(\boldsymbol{\xi}_c) \quad (13)
$$

is equal to minus the drug-receptor PMF at $\mathbf{R}_c, \boldsymbol{\xi}_c$ plus a correction related to the logarithm of the ratio of the canonical probability distributions for the *restrained* decoupled and coupled bound states, respectively, evaluated in both cases at the same point $\mathbf{R}_c, \boldsymbol{\xi}_c$. I stress that for Eq. 12 to be valid, the canonical probabilities at the end states, $P(\mathbf{R}, \boldsymbol{\xi} | \mathbf{\Sigma}_c, \mathbf{R}_c, \boldsymbol{\xi}_c, 1)$ and $P(\mathbf{R}, \boldsymbol{\xi} | \mathbf{\Sigma}_c, \mathbf{R}_c, \boldsymbol{\xi}_c, 0)$, must be both evaluated with the restraint in place.

How does then the FEP or TI computed DDM dissociation free energy $\Delta G_d(\mathbf{\Sigma}_r, \mathbf{R}_c, \boldsymbol{\xi}_c)$ relate to the *standard* dissociation free energy ΔG_{d0}? Or, equivalently, how does the potential of mean force $w(\mathbf{R}_c, \boldsymbol{\xi}_c)$ at its minimum value $\{\mathbf{R}, \boldsymbol{\xi}\} = \{\mathbf{R}_c, \boldsymbol{\xi}_c\}$ relate to the dissociation

constant $K_d/C_0 = e^{-\beta \Delta G_{d0}}$? I recall that in the present treatment, the $\{\mathbf{R}, \boldsymbol{\xi}\}$ coordinates are defined with respect to the fixed inertia system of the receptor. It is convenient to further distinguish between rotational coordinates of the ligand relative to the receptor and all other (ligand and receptor) conformational coordinates involved in the definition of the complex, namely $\boldsymbol{\xi} \equiv \boldsymbol{\Omega}, \boldsymbol{\chi}$. While the rotational states $\boldsymbol{\Omega}$ defining the orientation of the ligand frame relative to the fixed protein have all equal probability of $1/8\pi^2$ when the molecules are separated in the bulk (no matter what the conformational states of the partners are), the $\boldsymbol{\chi}$ conformational coordinates of the separated species in standard conditions can be rationalized in terms of *conformational* basins with uneven weights. It can then be shown that the dissociation constant in the *infinite dilution limit* for a fixed conformation $\boldsymbol{\chi}$ is given by[10]

$$\frac{1}{K_d(\boldsymbol{\chi})} = \frac{1}{8\pi^2} \int_{\mathcal{D}_b(\boldsymbol{\chi})} e^{-\beta w(\mathbf{R},\boldsymbol{\Omega},\boldsymbol{\chi})} d\mathbf{R} d\boldsymbol{\Omega} \tag{14}$$

$$= \frac{V_b(\boldsymbol{\chi})}{8\pi^2} e^{-\beta w(\mathbf{R}_c,\boldsymbol{\Omega}_c,\boldsymbol{\chi})} \tag{15}$$

where the integration domain, $\mathcal{D}_b(\boldsymbol{\chi})$, must be restricted to the region of existence of the complex between the receptor and the ligand in the fixed conformational states defined by the $\boldsymbol{\chi}$ coordinates.[7, 10] In the second equality we have written the integral (that has the dimension of a volume and square radiants) in terms of an effective volume $V_b(\boldsymbol{\chi})$ times the potential of mean force at the bottom of the well, $w(\mathbf{R}_c, \boldsymbol{\Omega}_c, \boldsymbol{\chi})$. The physical meaning of such volume is schematically illustrated in Figure 2 for a simple monoatomic ligand. Here, we have assumed a single minimum PMF of the kind $w(\mathbf{R}) = D_e(r)\Omega(r, \theta, \phi)$, where $D_e(r)$ is a Morse potential and $\Omega(r, \theta, \phi)$ is an appropriate square well potential defining the entrance angle of the monoatomic ligand into the binding pocket. Note that (Figure 2**b**) the integral defining the equilibrium constant can be extended beyond the $\mathcal{D}_b$ domain with no appreciable change in K_{eq}.

Returning back to the general Eq. 15, for a polyatomic ligand, $V_b(\boldsymbol{\chi})$ also includes a rotational contribution due to the librations of the ligand in the pocket, [7] when the ligand and the receptor are in the given conformational state $\boldsymbol{\chi}$. We can approximate the integrand in Eq. 15 with respect to the coordinates $\mathbf{X} = \mathbf{R}, \boldsymbol{\Omega}$ with a multivariate Gaussian distribution of appropriate covariance $\boldsymbol{\Sigma}_r$ (see Figure 11 (b)), i.e.

$$\int_{\mathcal{D}_b(\boldsymbol{\chi})} e^{-\beta w(\mathbf{R},\boldsymbol{\Omega},\boldsymbol{\chi})} d\mathbf{R} d\boldsymbol{\Omega} = e^{-\beta w(\mathbf{R}_c,\boldsymbol{\Omega}_c,\boldsymbol{\chi})} \int e^{-\frac{1}{2}(\mathbf{X}-\mathbf{X}_c)^T \boldsymbol{\Sigma}_b^{-1}(\boldsymbol{\chi})(\mathbf{X}-\mathbf{X}_c)} d\mathbf{X} \tag{16}$$
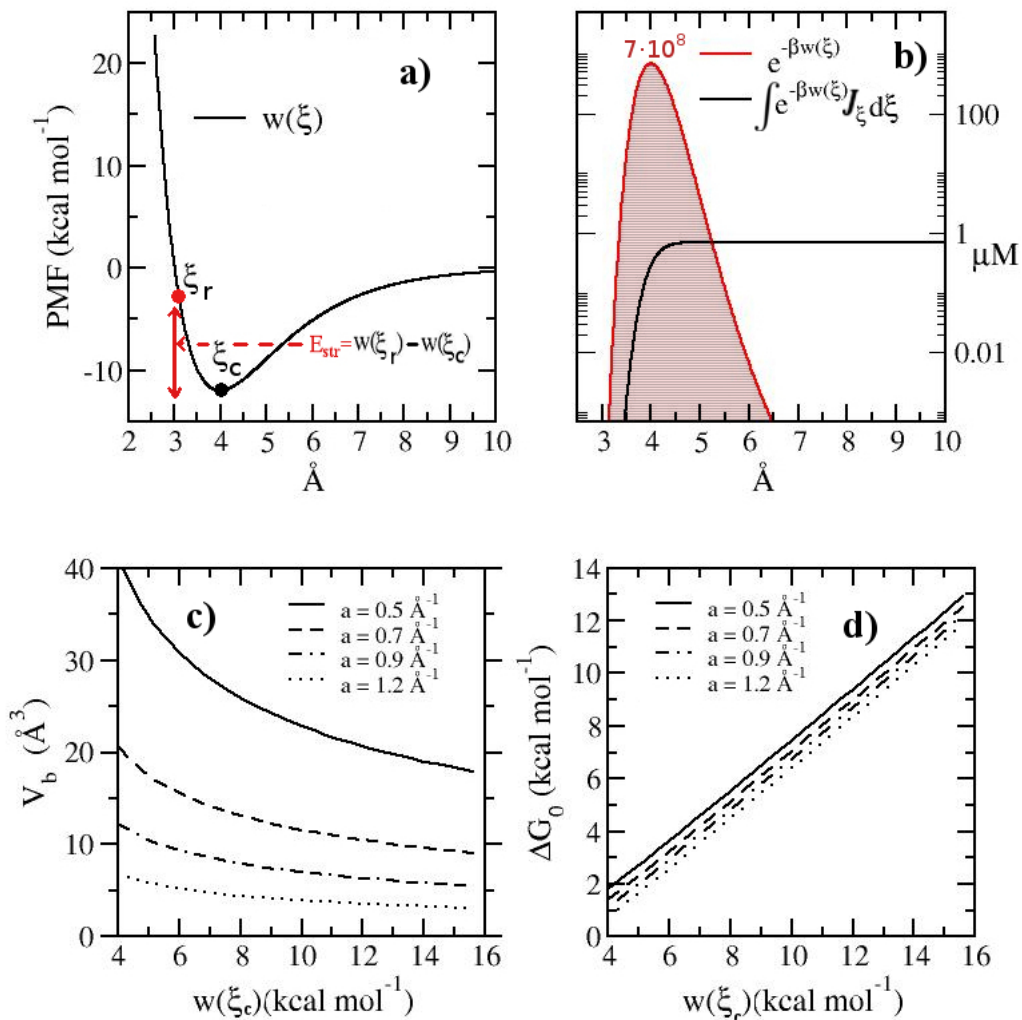
FIG. 2: Relation between V_b and the PMF $w(\xi)$ for a simple monoatomic ligand ($\xi = R, \theta, \phi$) **a**: PMF as a function of the ligand-receptor distance. The PMF is modeled with a distance dependent Morse potential of the form $w(r) = D[1 - e^{-a(r-r_0)}]^2 - D$. The θ, ϕ dependency is such that bonding may occur only in a solid angle of $D_\Omega = \pi/2$ corresponding to one octant of the 4π integrated orientational space. The red segment represents the strain energy due to a wrong choice of the restraints (see text). **b**: adimensional factor $e^{-\beta w(\xi)}$ as a function of the ligand-receptor distance (maximum value at $\xi = \xi_c$). The shaded area defines the volume V_b. The integral of the function $e^{-\beta w(\xi)} J_\xi$ (J_ξ is the Jacobian of the transformation $\xi = \xi(x)$ within the shaded area is the solid black line and yields the equilibrium constant K_{eq} (reported in $1/\mu$ M units). V_b (**c**) and standard dissociation energy (**d**) as a function of $w(\xi_c)$ for various a values (width) of the Morse potential.

so that

$$V_b(\boldsymbol{\chi}) = \sqrt{2\pi^3 |\boldsymbol{\Sigma}_b(\boldsymbol{\chi})|} \tag{17}$$

Going back to Eq. 15, the overall dissociation constant can be calculated as a standard canonical average:

$$\frac{1}{K_d} = \int d\boldsymbol{\chi} P(\boldsymbol{\chi}) \frac{1}{K_d(\boldsymbol{\chi})} = \int d\boldsymbol{\chi} P(\boldsymbol{\chi}) \frac{V_b(\boldsymbol{\chi})}{8\pi^2} e^{-\beta w(\mathbf{R}_c, \boldsymbol{\Omega}_c, \boldsymbol{\chi})} \tag{18}$$

In the infinite dilution limit, the probability density $P(\boldsymbol{\chi}) \equiv \langle \delta(\boldsymbol{\chi} - \boldsymbol{\chi}(x)) \rangle$ is identical to the probability density of the conformational coordinates $\boldsymbol{\chi}$ of the *separated* species. If the binding involves only one conformational state or basin defined by the integration domain $\mathcal{D}_\chi$ of the ligand and the receptor, i.e. if $K_d(\boldsymbol{\chi})$ is overwhelmingly dominated by $\boldsymbol{\chi} \in D_\chi$, we obtain

$$\frac{1}{K_d} = \int_{\mathcal{D}_\chi} d\boldsymbol{\chi} P(\boldsymbol{\chi}) \frac{1}{K_d(\boldsymbol{\chi})} = W(\boldsymbol{\chi}_c) \frac{V_b(\boldsymbol{\chi}_c)}{8\pi^2} e^{-\beta w(\mathbf{R}_c, \boldsymbol{\Omega}_c, \boldsymbol{\chi}_c)} \tag{19}$$

where we have defined the mean (adimensional) conformational weight $W(\boldsymbol{\chi}_c)$. If the conformational states spanned by the $\boldsymbol{\chi}$ coordinates are well separated and characterized by deep minima, then $W(\boldsymbol{\chi}_c)$ can be identified, in first instance, with the canonical weight in dilute solution of the binding ligand/receptor conformation *for the separated species*. If such binding conformation has a low weight for the separated species, then it means that the drug and/or the receptor experiences substantial conformational changes upon binding and that the free energy gain in the association process comes either from the volume (or entropy, *vide infra*) term $V_b(\boldsymbol{\chi})$ or from the enthalpic gain due to the $e^{-\beta w(\mathbf{R}_c, \boldsymbol{\Omega}_c, \boldsymbol{\chi}_c)}$ term.

Taking into account that $\Delta G_{d0} = -k_B T \ln(K_d V_0)$ Eq. 19 can be equivalently written in terms of dissociation free energy as

$$\Delta G_{d0} = -w(\mathbf{R}_c, \boldsymbol{\Omega}_c, \boldsymbol{\chi}_c) + k_B T \ln \left(\frac{V_b(\boldsymbol{\chi})}{8\pi^2 V_0} \right) + k_B T \ln W(\boldsymbol{\chi}_c) \tag{20}$$

Again, note that while the vector distance $\mathbf{R}$ is a collective variable (CV) bearing no coupling with other ligand-receptor CVs, the integration domain of the $\boldsymbol{\Omega}$ CV in the bound state is in principle dependent on the conformational state $\boldsymbol{\chi}$. In DDM theory, it is tacitly assumed that the $\boldsymbol{\chi}$ conformational coordinates pertain the ligand only (i.e. the conformational state of the receptor is invariant upon binding) and that the orientational volume spanned by the ligand relative to the receptor in the binding site is approximately independent of the conformational state of the system. In this rather strong assumption, that can be in essence

identified with the rigid rotor harmonic oscillator (RRHO) approximation, the determinant in Eq. 17 is diagonal and the volume $V_b(\boldsymbol{\chi})$ can be written as product of a $\boldsymbol{\chi}$ independent translational volume V_t and an orientational volume V_Ω (expressed in radiants) leading to the expression

$$\Delta G_{d0}^{[\text{RRHO}]} = -w(\mathbf{R}_c, \boldsymbol{\Omega}_c, \boldsymbol{\chi}_c) + k_B T \ln\left(\frac{V_t}{V_0}\right) + k_B T \ln\left(\frac{V_\Omega}{8\pi^2}\right) + k_B T \ln W(\boldsymbol{\chi}_c) \qquad (21)$$

One can see the three logarithmic terms in Eq. 21 as a translational, rotational and conformational entropy loss of the bound state, producing a penalty in the binding affinity, thus writing Eq. 21 in the familiar form

$$\Delta G_{d0} = \Delta H_d - T\Delta S_{d0} \qquad (22)$$

with the dissociation enthalpy $\Delta H_d = -w(\mathbf{R}_c, \boldsymbol{\Omega}_c, \boldsymbol{\chi}_c)$ given by the PMF at the bottom of the *single* well in the $\mathbf{R}, \boldsymbol{\Omega}, \boldsymbol{\chi}$ space and the standard state dependent and volume related dissociation entropy $\Delta S_{d0} = -k_B\left[\ln\left(\frac{V_t}{V_0}\right) + \ln\left(\frac{V_\Omega}{8\pi^2}\right) + \ln W(\boldsymbol{\chi}_c)\right]$. Hence, the more tightly is bound the ligand in the pocket, the smaller will be the "volumes" V_t, V_Ω and $W(\boldsymbol{\chi})$ and the larger is the entropy loss due to association.

Incidentally, we may hence say that Eq. 21 constitutes the statistical mechanics foundation of the Docking approach, essentially based on the underlying RRHO approximation. If, for example, we assume that N_c represents a set of equally populated conformational states of the free ligand (due to, e.g., rotable bonds[19]), Eq. 21 may be rearranged

$$\Delta G_{d0}^{[\text{RRHO}]} = -w(\mathbf{R}_c, \boldsymbol{\Omega}_c, \boldsymbol{\chi}_c) + k_B T \ln\left(\frac{V_t}{V_0}\right) + k_B T \ln\left(\frac{V_\Omega}{8\pi^2}\right) - k_B T \ln N_c \qquad (23)$$

In molecular Docking, the energetic contribution, $\Delta H = -w(\mathbf{R}_c, \boldsymbol{\Omega}_c, \boldsymbol{\chi}_c)$, is evaluated using molecular mechanics Poisson-Boltzmann surface area (MM/PBSA))[20, 21] or the molecular mechanics generalized Born surface area (MM/GBSA)[21–23] models, while the elusive volume entropic contributions, $k_B T(\ln\left(\frac{V_t}{V_0}\right) + \ln\left(\frac{V_\Omega}{8\pi^2}\right)$, are either evaluated using MD methodologies[24] or by simplified analytical estimates.[25]

Going back to Eq. 12, Eq. 20 provides the searched relationship between the potential of mean force $w(\mathbf{R}_c, \boldsymbol{\xi}_c) = w(\mathbf{R}_c, \boldsymbol{\Omega}_c, \boldsymbol{\chi}_c)$ and the standard dissociation free energy ΔG_{d0} in the context of DDM theory. If we use Eq. 20 in Eq. 12 and using the definition Eq. 13, we finally find

$$\Delta G_{d0} = \Delta G_d(\boldsymbol{\Sigma}_r, \mathbf{R}_c, \boldsymbol{\xi}_c) + k_B T \ln\left(\frac{V_b(\boldsymbol{\xi}_c)}{8\pi^2 V_0}\right) - k_B T \ln \frac{P(\mathbf{R}, \boldsymbol{\xi} | \boldsymbol{\Sigma}_r, \mathbf{R}_c, \boldsymbol{\xi}_c, 0)}{P(\mathbf{R}, \boldsymbol{\xi} | \boldsymbol{\Sigma}_r, \mathbf{R}_c, \boldsymbol{\xi}_c, 1)} \qquad (24)$$

where we have re-defined the overall binding site volume as

$$V_b(\boldsymbol{\xi}_c) = V_t V_\Omega(\boldsymbol{\chi}_c) W(\boldsymbol{\chi}_c) \qquad (25)$$

Equation 24 defines a DDM *general* relation embracing (as we shall see further on) all current binding theories from the DAM approach with no restraints to the Deng and Roux method with strong restraints. Note again that, in the general case, the "rotational volume", $V_\Omega(\boldsymbol{\chi}_c)$, is a function of the conformational states.

Boresch's and Deng's theory: stiff restraint regime

When $K_i \to \infty$, i.e. in the so-called stiff-spring regime[17, 26], the last logarithmic term on the rhs of Eq. 24 is zero since the probability densities for the restrained system in the $\lambda = 1$ and $\lambda = 0$ states becomes identical. According to eq. 12, the alchemically determined dissociation free energy (Eq. 13), $\Delta G_d(\boldsymbol{\Sigma}_r, \mathbf{R}_c, \boldsymbol{\xi}_c)$, can be thus taken to be equal to minus the PMF at $\{\mathbf{R}, \boldsymbol{\xi}\} = \{\mathbf{R_c}, \boldsymbol{\xi}_c\}$, i.e.

$$\Delta G_d(\boldsymbol{\Sigma}_r, \mathbf{R}_c, \boldsymbol{\xi}_c) = -w(\mathbf{R_c}, \boldsymbol{\xi}_c) \qquad (26)$$

Consequently, in order to recover the dissociation standard free energy in alchemical simulations with strong restraints, the strong restraint $\Delta G_d(\boldsymbol{\Sigma}_r, \mathbf{R}_c, \boldsymbol{\xi}_c)$ free energy should be corrected by a volume term $V_b(\boldsymbol{\xi}_c)$ that, in the limit of large force constants K_i, is *independent* on $\boldsymbol{\Sigma}_r$ and is related to the unknown binding site volume V_{site}, i.e.

$$\Delta G_{d0} = \Delta G_d(\boldsymbol{\Sigma}_r, \mathbf{R}_c, \boldsymbol{\xi}_c) + k_B T \ln \left(\frac{V_b(\boldsymbol{\xi}_c)}{8\pi^2 V_0} \right) \qquad (27)$$

$V_b(\boldsymbol{\xi}_c)$ can be taken as a system-dependent volume defined by the domain $\mathbf{R}, \boldsymbol{\Omega} \ \mathcal{D}_b(\boldsymbol{\chi})$ for the bound state when the ligand and the receptor are in the $\boldsymbol{\chi}$ conformational states. It important to stress that the size and the units of the volume $V_b(\boldsymbol{\xi}_c)$ *depends on the choice of the ro-vibrational $\boldsymbol{\xi}$ coordinates used to define the binding site.* Provided that $V_b(\boldsymbol{\xi}_c)$ can be somehow estimated in independent *unrestrained* simulations of the free ligand (needed for measuring $W(\boldsymbol{\chi}_c)$) and of the complex, Eq. 27 allows to compute the absolute dissociation free energy from the difference of the decoupling free energies of the free ligand and of complex obtained by FEP or TI, where the latter is tightly kept around the $\mathbf{R}_c, \boldsymbol{\xi}_c$ ligand-receptor position by a set of strong restraints of the form Eq. 1. Eq. 27 was previously

derived using a different route by Boresch al[8] and by Deng and Roux[11]. In the strong restraint approach, the estimate of the dissociation free energy crucially depends on the estimate of the binding site volume $V_b(\boldsymbol{\xi}_c)$ that can vary by several kcal mol^{-1},[11] hence spanning more than three orders of magnitude in the inhibition constant. Moreover, the parameters $\boldsymbol{\xi}_e$ in the restraint potential, Eq. 1, should be chosen such that they coincides with the corresponding mean values of the unrestrained bound state $\boldsymbol{\xi}_c = \langle \boldsymbol{\xi} \rangle_b$, where the subscript b indicate that the mean must be taken over bound state canonical configurations. If any of the $\{\xi_i^e\}$ differs from the corresponding equilibrium value $\{\xi_i^c\}$, then, as shown in Figure 2, the system is subject to a strain potential that will be reflected in the PMF and hence on V_b. Probably, the major weakness in DDM with strong restraints lies in the choice of the restrained coordinates themselves, that impact on the size and units of $V(\boldsymbol{\xi}_c)$. First of all, the number and the nature of the ligand and receptor conformational coordinates participating to binding is not known from the start. Secondly, whatever their choice, due to the inherent fluxional nature[27] of ligands and receptor, these coordinates will be coupled to other ligand and receptor coordinates so that restraining them may prevent the sampling of configurational states that are relevant for the binding affinity. In some sense, Boresch and Deng theory appears essentially to be based on the traditional picture of "lock and key" model[28] for binding, with a systematic underestimation of the binding site volume $V_b(\boldsymbol{\xi}_c)$ due to the neglect of any effect of receptor and ligand conformational reshaping ("induced fit" model[29]).

Gilson's theory: Intermediate restraint regime

We now assume that we impose only translational and orientational restraints and that these restraint are weak enough to allow the ligand-receptor system, to canonically sample all $\boldsymbol{\chi}$ conformational states that are important for binding. This can be practically achieved, for example, by using only rigid portions of the ligand and the receptor in order to define the relative ligand-receptor orientation $\boldsymbol{\Omega}$ with a possibly negligible impact on the sampling of conformational states. At the same time the translational restraint potential should be strong enough to prevent the ligand to freely drift away from the binding site at any λ alchemical states. In this case, we can identify $\boldsymbol{\xi}_c$ with $\boldsymbol{\Omega}_c$ so that we may write the

probability density of the decoupled restrained bound state as

$$P(\mathbf{Y}|\mathbf{\Sigma}_r, \mathbf{Y}_c, 0) = P(\mathbf{R}, \mathbf{\Omega}|\mathbf{\Sigma}_r, \mathbf{R}_c, \mathbf{\Omega}_c, 0)$$

$$= \frac{1}{\int e^{-\frac{1}{2}(\mathbf{Y}-\mathbf{Y}_c)^T\mathbf{\Sigma}_r^{-1}(\mathbf{Y}-\mathbf{Y}_c)}d\mathbf{Y}} = \frac{1}{V_r} \qquad (28)$$

where we have used Eqs 1 and Eq. 4 and where V_r defines the temperature dependent allowance restraint volume such that $V_r > V_b$. The probability density of the fully coupled restrained system can be written as a product of two multivariate Gaussian distribution with covariance matrix $\mathbf{\Sigma}^{-1} = \mathbf{\Sigma}_b^{-1} + \mathbf{\Sigma}_r^{-1}$ defined in the $\{\mathbf{R}, \mathbf{\Omega}\}$ space, i.e.

$$P(\mathbf{Y}|\mathbf{\Sigma}_r, \mathbf{Y}_c, 1) = \frac{e^{-\beta w(\mathbf{Y}_c)}}{\int e^{-\beta[w(\mathbf{Y})+V(\mathbf{Y}-\mathbf{Y}_c)]}d\mathbf{Y}}$$

$$= \frac{1}{\int e^{-\frac{1}{2}(\mathbf{Y}-\mathbf{Y}_c)^T\mathbf{\Sigma}_b^{-1}(\mathbf{Y}-\mathbf{Y}_c)}e^{-\frac{1}{2}(\mathbf{Y}-\mathbf{Y}_c)^T\mathbf{\Sigma}_r^{-1}(\mathbf{Y}-\mathbf{Y}_c)}d\mathbf{Y}}$$

$$= \frac{\sqrt{2\pi^d \det[\mathbf{\Sigma}_r + \mathbf{\Sigma}_b]}}{V_r V_b} = \frac{\sqrt{\det[\mathbf{1} + \mathbf{\Sigma}_r^{-1}\mathbf{\Sigma}_b]}}{V_b} \qquad (29)$$

where $V_b = \int_{\mathcal{D}_b} e^{-\beta w(\mathbf{Y})}d\mathbf{Y} \simeq \int e^{-\frac{1}{2}(\mathbf{Y}-\mathbf{Y}_c)^T\mathbf{\Sigma}_b^{-1}(\mathbf{Y}-\mathbf{Y}_c)}d\mathbf{Y}$ and where the effective covariance $\mathbf{\Sigma}_b$ no longer depends on the conformational states, whose contribution is supposed to be implicitly integrated away in the PMF $w(\mathbf{R}, \mathbf{\Omega})$. Inserting Eqs. 29 and 28 into Eq. 24, we find

$$\Delta G_{d0} = \Delta G_d(\mathbf{\Sigma}_r, \mathbf{R}_c, \mathbf{\Omega}_c) + k_BT \ln\left(\frac{V_r}{8\pi^2 V_0}\right) + k_BT \ln\sqrt{\det(\mathbf{1} + \mathbf{\Sigma}_r^{-1}\mathbf{\Sigma}_b)} \qquad (30)$$

In the assumption that the last term is small and can be neglected (i.e. $V_r \gg V_b$), and factoring the restraint volume V_r in translational and orientational parts V_I, ξ_I, then Eq. 30 is identical to the Equation proposed by Gilson.[7] I stress that Eq. 30 was derived by introducing ligand-protein rotational coordinates that are supposed to be decoupled from any conformational state, so that $w(\mathbf{R}_c, \mathbf{\Omega}_c)$ represents the reversible work to bring the ligand form the bulk state to the bound state defined by the coordinates $\mathbf{R}_c, \mathbf{\Omega}_c$, irrespective of the conformational states. DDM with weak restraint potentials should be handled with due care by practitioners. In case of highly symmetric ligands like benzene in T-lysozyme,[11] for example, weak orientational restraints may prevent the sampling of the bound conformations that are defined by a mere exchange of the atom labels due to rotational operations of the symmetry group of the ligand (say σ), underestimating the *conformational volume* in the bound state and hence the dissociation free energy. If the weak orientational restraints prevents the sampling of any of the equivalent $\sigma = 12$ states of benzene, then the free

energy should be corrected by an additive term $k_B T \ln 12$ apparently due to "symmetry". If instead the restraints are engineered so that they allow the sampling of the bound states generated by rotations around the six-fold axis of the benzene molecule but not of those that can be generated by rotation around the 2-fold symmetry axis, then the correction factor reduces to $k_B T \ln 2$. Incidentally, I remark that this kind of corrections applies only to DDM with weak restraints and not to the Boresch and Deng variant with strong restraints, provided that in the $V_b(\boldsymbol{\chi})$ measure of the binding site volume for the *unrestrained* system all relevant conformational states have sampled.

As discussed in Ref. [7], for Eq. 30 to hold, it must be that

$$\frac{\partial \Delta G_d(\boldsymbol{\Sigma}_r, \mathbf{R}_c, \boldsymbol{\Omega}_c)}{\partial V_r} = -\frac{k_B T}{V_r} \tag{31}$$

where, in taking the derivative, we have neglected the last term in Eq. 30. Eq. 31 provides in principle a mean to assess whether the chosen restraints obeys the Gilson's regime. In fact, by computing the uncorrected alchemical dissociation free energy for different restraint potentials at constant temperature and pressure and plotting the result as a function of $-1/V_r$ we should find a straight line with slope of $k_B T$.

Jorgensen's theory: Unrestrained (DAM) regime.

What happens when instead we let $\mathbf{K} \to 0$ in Eq. 12? In this case, as first remarked in Ref. [8], the alchemical procedure becomes cumbersome since the standard dissociation free energy should be in principle recovered by the single *equilibrium* simulation at the fully coupled state $\lambda = 1$. The dissociation free energy detected in the unrestrained simulation depends on the nominal concentration of the species imposed by the periodic boundary conditions (PBC), i.e. on the MD box volume V_{box}. The fraction of dissociated species can be expressed as function of the ratio $r = K_d/C_{\text{box}}$, where $C_{\text{box}} = 1/V_{\text{box}}$ is the nominal concentration imposed by the PBC, as

$$f = \frac{r}{2}\left[\left(1 + \frac{4}{r}\right)^{1/2} - 1\right] \tag{32}$$

Note that in the high concentration limit we have that $\lim_{r \to 0} f = 0$ while at infinite dilution $\lim_{r \to 0} f = 1$. In simulations of typical drug-receptor systems, V_{box} may be taken to vary in the range 10^5:10^6 Å^3. Hence, for a micromolar to nanomolar ligand, $1/K_d$ varies in the

range $10^9{:}10^{12}$ Å^3 so that the ratio r is of the order of $10^{-7}{:}10^{-3}$. In this conditions, we have that $f = r^{1/2} + o(r^2)$ and the box dependent free energy evaluated in the equilibrium simulation at $\lambda = 1$ may be computed as

$$
\begin{aligned}
\Delta G_d(V_{\text{box}}) &= -k_B T \ln(K_d V_{\text{box}}) = -k_B T \ln \frac{f^2}{1-f} \\
&= -k_B T \ln r + k_B T \ln(1 - r^{1/2}) \\
&\simeq \Delta G_{d0} - k_B T \ln \frac{V_{\text{box}}}{V_0}
\end{aligned}
\tag{33}
$$

where in the last equation we have neglected the quantity $k_B T \ln(1 - r^{1/2}) \simeq -k_B T r^{1/2}$ and exploited the fact that $\Delta G_{d0} = -k_B T \ln(K_d V_0)$. The standard free energy can hence be determined by a single very long simulation at the fully coupled state using Eq. 33. However, one can also choose to implement the cumbersome alchemical methodology in the unstrained version, by applying the $\mathbf{K} \to \mathbf{0}$ limit of the general Equation 12 and assuming that only a restraint on $\mathbf{R}$ is imposed, i.e.

$$
\lim_{\mathbf{K} \to \mathbf{0}} \Delta G_r(\beta \mathbf{K}^{-1}, \mathbf{R}_c) - \Delta G_u = -w(\mathbf{R}_c) + k_B T \ln \left[\frac{\lim_{\mathbf{K} \to \mathbf{0}} P(\mathbf{R}, |\beta \mathbf{K}^{-1}, \mathbf{R}_c, 0)}{\lim_{\mathbf{K} \to \mathbf{0}} P(\mathbf{R}|\beta \mathbf{K}^{-1}, \mathbf{R}_c, 1)} \right]
\tag{34}
$$

where

$$
\Delta G_{d0} = -w(\mathbf{R}_c) + k_B T \ln \left(\frac{V_T}{V_0} \right)
\tag{35}
$$

$V_T = \int_{\mathcal{D}_b} e^{-\beta w(\mathbf{R})} d\mathbf{R}$ is the allowance oscillation volume of the COM vector distance $\mathbf{R}$ in the complex irrespective of the ligand-receptor orientational and conformational coordinates. In the limit $\mathbf{K} \to \mathbf{0}$, the restraint the probability density of the decoupled system is given by

$$
\lim_{\mathbf{K} \to \mathbf{0}} P(\mathbf{R}, |\beta \mathbf{K}^{-1}, \mathbf{R}_c, 0) = \frac{1}{V_{\text{box}}}.
\tag{36}
$$

The probability density of the coupled system at $\mathbf{R} = \mathbf{R}_c$, $P(\mathbf{R}|\beta \mathbf{K}^{-1}, \mathbf{R}_c, 1)$, is simply given by

$$
\begin{aligned}
\lim_{\mathbf{K} \to \mathbf{0}} P(\mathbf{R}|\beta \mathbf{K}^{-1}, \mathbf{R}_c, 1) &= \frac{e^{-\beta w(\mathbf{R}_c)}}{\int_{V_{\text{box}}} e^{-\beta w(\mathbf{R})} d\mathbf{R}} \\
&= \frac{1}{V_T \left[1 + \frac{(V_{\text{box}} - V_T)}{V_T} e^{\beta w(\mathbf{R}_c)} \right]} = \frac{1}{V_T(1 + c_r)} \simeq \frac{1}{V_T}
\end{aligned}
\tag{37}
$$

where the constant $c_r = \frac{(V_{\text{box}} - V_T)}{V_T} e^{\beta w(\mathbf{R}_c)} \simeq \frac{V_{\text{box}}}{V_0} e^{-\beta \Delta G_0}$ can be neglected as long as $e^{\beta \Delta G_0} \gg V_{\text{box}}/V_0$. Plugging Eqs. 37 and 36 into Eq. 34, using Eq. 35 and defining $\Delta G_d(\text{DAM}) = \lim_{\mathbf{K} \to \mathbf{0}} \Delta G_r(\beta \mathbf{K}^{-1}, \mathbf{R}_c) - \Delta G_u$, we finally obtain for the unstrained (DAM) regime

$$
\Delta G_{d0} \simeq \Delta G_d(\text{DAM}) + k_B T \ln \left(\frac{V_{\text{box}}}{V_0} \right)
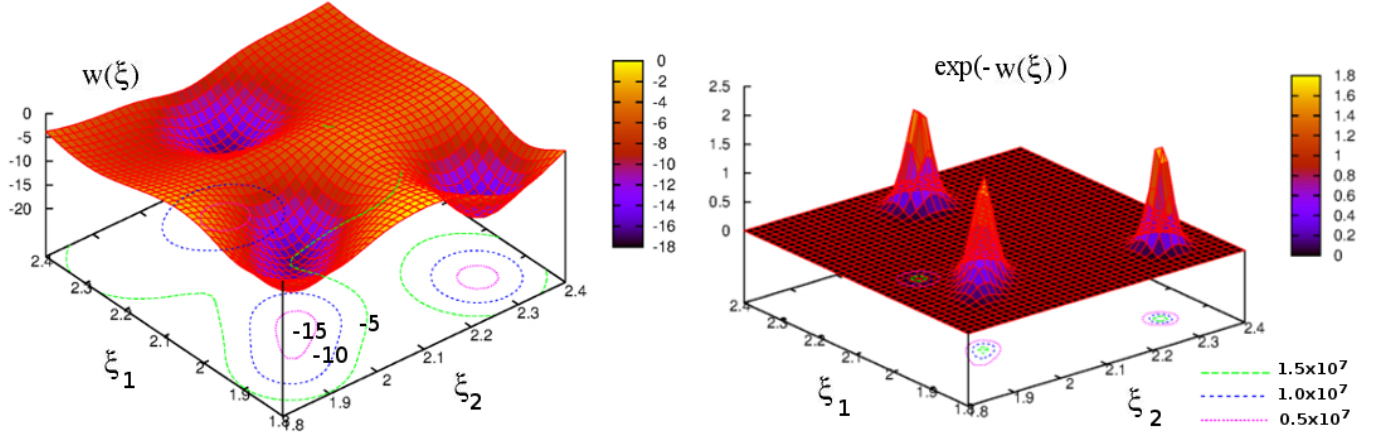\tag{38}
$$

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FIG. 3: Example of a 2D generic PMF with multiple minima (left, energy units in $k_B T$) and corresponding $e^{-\beta w(\xi)}$ factor using a combination of multivariate Gaussian distributions.

thus recovering Eq. 33 with $\Delta G_d(\text{DAM}) = \Delta G_d(V_{\text{box}})$. I stress that Eq. 38 holds only if the MD box volume is such that $e^{\beta G_0} \gg V_{\text{box}}/V_0$. It should also be noticed that, while ΔG_0 in Eq. 35 is a purely *conventional* quantity defined with respect to an arbitrarily selected standard concentration, $\lim_{\mathbf{K}\to 0} \Delta G_r(\beta \mathbf{K}^{-1}, \mathbf{R}_c)$ and ΔG_u in Eq. 34 refer to free energy differences between two *real* thermodynamic states, namely the decoupling of the *unrestrained* ligand in presence of the receptor in the MD box of volume V_{box} and the decoupling of the V_{box}-independent ligand in the bulk phase, respectively. If on the rhs of Eq 37 we let $V_{\text{box}} \to \infty$, we obtain

$$\lim_{V_{\text{box}} \to \infty} \frac{1}{V_T \left[1 + \frac{(V_{\text{box}} - V_T)}{V_T} e^{\beta w(\mathbf{R}_c)} \right]} = \frac{e^{-\beta w(\mathbf{R}_c)}}{V_{\text{box}}} \tag{39}$$

Inserting this result and Eq. 36 into Eq. 34, an using the definition $\Delta G(\text{DAM}) = \lim_{\mathbf{K}\to 0} \Delta G_r(\beta \mathbf{K}^{-1}, \mathbf{R}_c) - \Delta G_u$, we trivially obtain

$$\lim_{V_{\text{box}} \to \infty} \Delta G(\text{DAM}) = -w(\mathbf{R}_c) + w(\mathbf{R}_c) = 0 \tag{40}$$

i.e the V_{box}-dependent dissociation DAM free energy goes to zero for $V_{\text{box}} \to \infty$, or, equivalently the decoupling free energy of the complex coincides with the decoupling free energy of the dissociated state. This happens since in the left branch of the cycle of Figure 1, when the box becomes exceedingly large (so that $e^{\beta G_0} \ll V_{\text{box}}/V_0$) and provided that the *unrestrained* (DAM) transformation of the complex is ideally done at equilibrium, then ligand in

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the fully coupled state at $\lambda = 1$ should be found freely wandering in the bulk with unitary probability, as first remarked in ref. [8].

I conclude this section with some remarks on the nature of V_T appearing in Eqs 35 and 37. This quantity has the unit of a volume and can be identified with the overall (translational) binding site volume of the ligand "pose" on the protein surface. In order to estimate V_T in a unrestrained simulation of the complex, one must define, in each sampled bound configuration, a protein reference frame with respect to which the polar angles θ, ϕ are evaluated. V_T is hence modulated by the ro-vibrational coordinates of *both* ligand and receptor. For fluxional ligands and receptors with conformational configurations widening the COM probability density in the bound state, the pose in the **R** domain can hence be very rugged indeed as schematically shown in Figure 3. This picture of the translational PMF $w(R, \theta, \phi)$ with many crowded competing minima characterizing the "pose" is consistent with the "induced fit" or conformational proofreading model for binding whereby the ligand and/or the receptor kinetically adjust their conformational states due to their mutual interaction.[30]

Dissociation free energy via non equilibrium alchemical transformation

In spite of the previously outlined wandering ligand problem, the DAM theory has been used for many years before the advent of DDM theory, incorporated in popular MD packages[31] and often producing reliable free energy values.[32] Even quite recently,[12] Fujitani and coworkers used the unrestrained DAM and FEP to compute the binding free energy of the FKBP12-FK506 drug-receptor system. In all these early DAM simulations, as well as in the recent examples due to Fujitani and co-workers,[13, 14] the decoupling process in the left branch of the cycle in Figure 1(a), was performed, *starting from a bound state*, in a total simulation time (along the whole alchemical decoupling path) never exceeding, at most, the few tens of nanoseconds. For states with λ approaching to zero, the unrestrained ligand could hence easily leave the binding site and start to freely drift off in the MD box. The time scale of a random encounter in typical MD box of volume V_{box} containing a single drug-receptor pair can be straightforwardly estimated from the mean free path, $\frac{V_{\text{box}}}{\pi d^2}$ (with d being the mean radius of the receptor assumed to be much larger than that of the ligand), and the diffusion coefficient of a ligand in water,[33] typically obtaining collision rates of the order of 0.1:0.01 ns^{-1}, i.e. a *random* collision every 10 to 100 ns. In the light of this

estimate, we can safely say that *all* of the DAM/FEP or DAM/TI simulations appeared on the literature were actually non equilibrium processes hence providing *a non equilibrium estimate* of the decoupling free energy. The same argument applies to DDM simulations as well, where Boltzmann sampling is in principle required for all conformational states of the complex that are not subject to restraints. Conformational transitions in flexible protein side chains occur in a wide range of time scale, from picoseconds to milliseconds and longer.[34] A converged sampling of these CVs, for *all λ states*, that should be highly relevant in induced fit ligand-receptor association, is in many cases out of the reach in DDM/FEP or DDM/TI simulations lasting at most few ns per alchemical state.

In the following, I shall discuss how alchemical non equilibrium decoupling processes can be used to derive reliable estimates of the standard dissociation free energies. The Jarzynski theorem[35] represents one of the few exact results in non equilibrium thermodynamics, relating the work done in a non equilibrium (NE) transformation between two thermodynamic states A, B to the corresponding free energy difference, that is to the work done *reversibly*:

$$e^{\beta \Delta G_{AB}} = \langle e^{-\beta W_{AB}} \rangle_A \tag{41}$$

While the configurations of the *starting* state A are canonically sampled, the *arrival* configurations of B are not distributed canonically. The mean NE work, when averaged over many realizations, all done according to a common prescribed time schedule, is always larger than the free energy, i.e. the minimum, reversible work connecting two states. The difference between the average NE work and the free energy correspond to the mean *dissipation* of the NE process, a function of the speed of the NE realizations. For infinitely slow (quasi-static) realizations, the work is always equal to ΔG_{AB} and the Jarzynski work average is equivalent to TI, while for instantaneous processes, it can be shown that that Jarzynski theorem becomes equivalent to the Zwanzig free energy perturbation formula. The work probability distributions for the forward (A to B) and reverse process (B to A) obey the the Crooks

$$\frac{P_{A \rightarrow B}(W)}{P_{B \leftarrow A}(-W)} = e^{-\beta(W_{AB} - \Delta F)} \tag{42}$$

The sign of the work in the reverse distribution is due to the fact that the reverse process is assumed to be done with identical but inverted time schedule. It has been observed[36–38] that the work distribution obtained from fast annihilation/creation NE processes (lasting no more than few hundreds or even tens of picoseconds) of small to moderate size organic

molecules in polar non polar solvents has a marked Gaussian character and that the corresponding dissipation is surprisingly small, ranging from 0.05 to 0.1 kcal mol^{-1} per atom. In case of Gaussian work distributions for the (forward) annihilation process, the Crooks theorem, Eq. 42, provides an *unbiased* estimate of the free energy in the form of

$$\Delta G = \langle W_{A \to B} \rangle - \frac{\beta \sigma^2}{2} \tag{43}$$

where $\langle W_{A \to B} \rangle$ and σ are the mean work and variance of many NE realizations. This fact has been recently exploited[39, 40] to implement a non equilibrium approach to alchemical simulation. In this methodology the dissociation free energy is again accessed via the thermodynamic cycle, but this time the annihilation processes on the two branches are done irreversibly at fast speed, starting form the fully coupled equilibrated states. The free energy is recovered either from the Jarzynski theorem, Eq. 41 or, in case of Gaussian work distribution, from the unbiased estimate, Eq. 43. As such, the NE alchemical variant is compatible either with the version with strong or weak restraints or with the unrestrained approach. In case the NE alchemical simulations with restraints, the quantities $\Delta G_r(\mathbf{\Sigma}_r, \mathbf{R}_c, \boldsymbol{\xi}_c)$ and in $\Delta G_u(\boldsymbol{\xi}_c)$ in Eq. 13 or $\Delta G_r(\mathbf{\Sigma}_r, \mathbf{R}_c, \boldsymbol{\Omega}_c)$ and in $\Delta G_u(\boldsymbol{\Omega}_c)$ in Eq. 30 are not evaluated using TI or FEP; rather they are computed applying Eq. 43 or Eq. 41 to the work histograms obtained by launching in parallel few hundreds of fast (0.1 to 0.5 ns) decoupling alchemical independent trajectories. For the unrestrained (DAM) NE version, it has been shown[40, 41] that the dissociation free energy can be recovered exploiting the Crooks theorem applied to mixture of Gaussian distributions, landing on Eq.

$$\Delta G_0 = \Delta G_b - \Delta G_{\mathrm{u}} + k_B T \ln \frac{V_{site}}{V_0} \tag{44}$$

where ΔG_b is NE free energy Gaussian estimate for the fast annihilation of the bound state, ΔG_u is NE free energy estimate for the fast annihilation of the ligand in and V_{site} should correspond to the effective cumulative "volume" of the binding site or, using a definition due again to Gilson[42], to the *exclusion zone* of the receptor, defined by a measurable (in principle) probability of re-entrance in an hypothetical reverse process for the complex. As long as the NE process is much faster compared to the time scale of the relative ligand-receptor diffusion, the NE estimate ΔG_b via Eq. 41 or 43 is essentially independent of the box volume and on the duration time of the NE process, so that we can identify V_{site} with V_T in Eq. 38. This is a rather trivial consequence of the insensitivity of the equilibrium

constant integral $K_e = \int_{\mathcal{D}_b} e^{-\beta w(\mathbf{R})}) d\mathbf{R}$ to the integration domain $\mathcal{D}_b$ defining the region of existence of the complex and to the fact that in the fast switching alchemical decoupling of the bound state, the decoupled ligand does not have the time to explore regions that are far away from $\mathcal{D}_b$.

The NE alchemical approach, whether in the restrained or unrestrained version, bypass completely the need for an equilibrium sampling at the intermediate alchemical states, requiring a canonical sampling only at starting fully coupled $\lambda = 1$ thermodynamic state. The latter can be obtained using enhanced sampling techniques such as H-REM or Umbrella Sampling.[43] With this regard, the apparent ability of *equilibrium* FEP or TI based approaches to produce reliable estimates of the binding free energy in conventional simulation lasting few ns per alchemical states (i.e. for a timescale that is well below the characteristic ergodicity timescale in drug-receptor systems) is actually a fortuitous consequence of non equilibrium processes. These techniques are in fact unaware applications of non equilibrium approaches whereby a mean alchemical *work*, rather than a free energy, is determined. Such work, if the alchemical process is done in a cumulative time of the order of the tens of nanoseconds, is Gaussianly distributed over few $k_B T$ or less and, in force of the Crooks theorem, must be close to the true decoupling free energy. The similarity of the dissipation energy on the two branch of the cycles provides a further fortuitous compensation effect when evaluating the dissociation free energy as a difference of two non equilibrium mean work.

CONCLUSIONS

In this paper I have revisited the statistical mechanics of non covalent bonding in drug-receptor systems. I have shown that all existing alchemical theories in binding free energy calculations can be rationalized in term of a unifying treatment encompassing the original unrestrained DAM[4], the Gilson's restrained DDM variant[7] and the sophisticated docking approach proposed by Deng and Roux.[11] The cited alchemical theories differ in the definition (explicit or implicit) of the binding site volume through the enforcement of a set of appropriately selected restrained potentials. Strong restrained approaches[11] relies on a precise knowledge of the binding pose and volume in the context of the traditional picture of the lock and key model. The DDM and DAM theories make weaker assumptions on the

pose topology and nature, hence being progressively shifted towards a more realistic induced fit/ conformational proofreading model in drug-receptor interaction. All alchemical theories are finally placed into the broader context of non equilibrium thermodynamics, discussing the application of the Crooks and Jarzynski non equilibrium theorems to the evaluation of alchemical decoupling free energies.

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