Can Absolute Free Energies of Association Be Estimated from Molecular Mechanical Simulations? The Biotin−Streptavidin System Revisited

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Employing state-of-the-art molecular dynamics protocols, we carried out free energy calculations in the (*N*, *P*, *T*) ensemble on a fully hydrated biotin−streptavidin assembly of 27 702 atoms. The reported absolute binding free energy of -16.6 ± 1.9 kcal/mol is in good agreement with the experimental estimate of −18.3 kcal/mol by Weber et al. [*J. Am. Chem. Soc.* **1992**, *114*, 3197−3200]. These simulations illustrate that the use of massively parallel architectures in conjunction with efficient algorithms allows us to tackle biologically relevant problems involving large molecular systems and to access key properties, like the association of a protein with its ligand, under rigorous thermodynamic conditions.

Introduction

Novel developments on both the hardware and the software fronts have made feasible state-of-the-art statistical simulations that explore a significant range of the phase space in apt thermodynamic ensembles, within reasonable time frames. Biomolecular simulations targeted at the determination of free energies constitute an area that has gained from these developments because of the inherent slow convergence properties of such calculations, requiring long simulation times to yield accurate estimates. Approaches based on the construction of a thermodynamic cycle rely on the assumption that a reaction coordinate connecting these states and characterized by a coupling parameter, λ, can be defined unequivocally.[1−5] This procedure can be further extended to obtain absolute free energies of binding in solution in what is referred to as a "double annihilation/decoupling experiment".[6,7] A basic premise for the successful applicability of this methodology supposes that the free energy change between any two states be calculated in an ensemble of configurations accessible to both states, which is often achieved by varying λ slowly. Furthermore, intermediate states require appropriate equilibration to overcome the so-called Hamiltonian lag that develops when moving along successive λ values, or windows.[8] These prerequisites make "λ"-based free energy calculations highly CPU intensive. In the past decade, significant enhancements in the methodology of molecular dynamics (MD) simulations have been made by implementing accurate algorithms,[9,10] which generate more precise isothermal−isobaric ensembles[11,12]—a thermodynamic requisite for the computation of Gibbs free energies. Moreover, methodological developments in the treatment of boundary conditions and long-range electrostatic forces have improved further both the quality and the reliability of molecular simulations.[13,14]

Here, we present the results of such a state-of-the-art free energy calculation, addressing several issues often approximated in similar large-scale, statistical simulations for the sake of computational effectiveness. The free energy of binding of protein streptavidin to its substrate biotin has been evaluated using over 1 ns of MD trajectory. Streptavidin is a tetrameric protein known to bind biotin very strongly through noncovalent interactions. This feature has been the object of an increased interest in the biotechnology community for labeling and affinity purification methods.[15] X-ray crystallographic studies of biotin−streptavidin complexes (PDB[16,17] identifier 1stp[18,19]) reveal that the binding site accommodates biotin with steric complementarity for optimal van der Waals contacts, accompanied by a network of hydrogen bonds that form a rigid lattice. The streptavidin monomer in the crystal structure contains 118 residues organized in an 8-stranded β-barrel, one end of which forms the active site occupied by biotin.

Elaborate work on this system has been carried out earlier,[19−22] making it an ideal prototypical assembly for probing free energy methodologies. It should be pointed out that the biotin−streptavidin system was used earlier in a free energy perturbation (FEP) study by Miyamoto and Kollman (MK),[20,21] wherein the simulation environment was defined somewhat differently than here to comply with the computational resources and methods at that time. The present study offers a unique opportunity to inspect the approximations commonly adopted in yesteryears to describe similarly large molecular assemblies—for instance, in the calculations of MK—the drawbacks of such representations, and the improvements characteristic of modern, state-of-the-art calculations.

In statistical simulations, limitations in the computational resources generally dictate not only the size of the system but also the level of sophistication at which its boundary conditions are treated. In silico investigations of biological molecules, for which an accurate description of the surroundings is required, have opened the way to alternative approaches for explicit solvation, using a reduced number of solvent molecules. The "solvent cap" representation used in the work of MK is one such procedure, wherein a limited number of water molecules are confined in the portion of a sphere centered about some site of interest, e.g., the binding site of a protein. The cohesion of the liquid is enforced by means of semiharmonic restraining potentials that prevent water molecules from escaping the solvent shell. It has been noted that the surface tension associated with such a definition of the solvent leads to artificially large pressures at the center of the system, reduced atomic fluctua-

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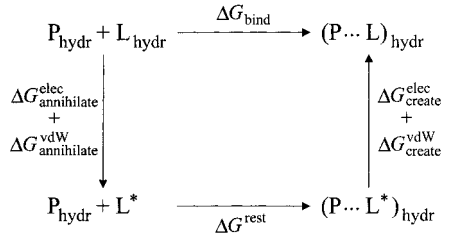

Figure 1. Thermodynamic cycle used for estimating the binding free energy, ΔG_{bind}, of biotin to streptavidin: $\Delta G_{bind} = \Delta G^{elec}(P\cdots L_{hydr}) + \Delta G^{vdW}(P\cdots L_{hydr}) + \Delta G^{elec}(L_{hydr}) + \Delta G^{vdW}(L_{hydr}) + \Delta G^{rest}$, where $\Delta G^{elec}(P\cdots L_{hydr})$ and $\Delta G^{vdW}(P\cdots L_{hydr})$ are the electrostatic and the van der Waals contributions to the free energy for creating or annihilating the ligand within the active site, respectively. $\Delta G^{elec}(L_{hydr})$ and $\Delta G^{vdW}(L_{hydr})$ are the nonbonded contributions for annihilating or creating the free ligand in aqueous solution. ΔG^{rest} is the positive work arising from the loss of rotational and translational entropy when the ligand is locked in the active site.[53] Ideally, creation and annhilation are energetically equivalent processes, opposite in sign, and their difference should provide an estimate of the error.

tions[23,24] and also distortion in the orientation of the side chain of those residues pertaining to the active site.[25] Moreover, artifacts resulting from the use of spherical cutoffs or equivalent truncation schemes, which could inhibit atomic motions or disrupt the structure of the liquid, have been reported recurrently over the past years.[26−28] If truncating $1/\mathbf{r}^3$, dipole−dipole interactions often constitute a legitimate approximation, considering that it only affects moderately the structural characteristics and the thermodynamic ensemble averages of the system, the use of finite spherical cutoffs for $1/\mathbf{r}^n$, $\forall\ n < 3$, is admittedly inappropriate. On account of the net charge borne by the biotin−streptavidin complex, handling of long-range electrostatic forces should be tackled circumspectly. Another key criterion that deserves careful examination is the length of the simulation. Several studies indicate that excessively short molecular dynamics simulation tends to mask the ability to assess the true nature and effect of the simulation protocol, thereby often resulting in fortuitous cancellation of errors, generally left unnoticed.[24] This issue becomes all the more important in the course of free energy calculations, where convergence needs to be monitored cautiously.[3,29] The massive increase of computational power witnessed in recent years has allowed the study of these issues in greater detail and the development of simulation techniques that handle the system in a rational, less approximate fashion. The application of periodic boundary conditions in conjunction with Ewald lattice sums or related methods[14] has reduced significantly the pitfalls resulting from an improper treatment of long-range electrostatic forces. It is inspiring to note that molecular dynamics simulations of complex molecular assemblies employing such state-of-the-art protocols and new-generation force fields have proven to yield stable trajectories over the multinanosecond time scale without the need of unnatural restraints controlling conformational equilibria, thus shedding light on conformational and structural issues that were earlier unavailable.[30−35]

Methods and Computational Details

The binding free energy, ΔG_{bind}, was calculated as a difference of free energy changes during the creation or the annihilation of biotin (i) in the active site of streptavidin and (ii) in water. This is summarized in the thermodynamic cycle of Figure 1. The free energy change associated with each leg of the thermodynamic cycle was evaluated using both FEP and thermodynamic integration (TI) methodologies.[1−5] The integral in the TI scheme was approximated by means of the trapezoidal rule.

Preparation of the system, starting from the crystal structure of the complex, including addition of hydrogen atoms and solvation, was performed with the CHARMm suite of programs.[36] The tetrameric complex was solvated in a cubic cell of ca. $62 \times 62 \times 62$ Å^3, containing 6898 TIP3P water molecules.[37] The net charge of -8 borne by the complex was neutralized using eight Na^+ counterions placed randomly. The simulation of free biotin in aqueous solution was performed by solvating the charged ligand together with a Na^+ counterion in a cubic box of 980 TIP3P water molecules.

The CHARMm param22 force field[38] was employed to describe the protein, in conjunction with the parameters of biotin reported by MK,[20] and free energy calculations were carried out with the NAMD2 code.[39] NAMD2 incorporates the appropriate features to perform state-of-the-art MD simulations on sizable chemical systems, in a massively parallel environment, and has been suitably modified to estimate the free energy associated with alchemical transformations. Here, turning biotin into a ghost molecule that does not interact with the rest of the system was performed in the framework of a "dual topology in single topology" scheme.[40,41] The major difference between this approach and the standard dual topology method[42] lies in the scaling of the nonbonded parameters as a function of λ—as is done in the single topology approach—rather than the Hamiltonians representative of the initial and the final states. It should be mentioned that modifying internal parameters requires a rigorous treatment of the free energy contribution due to shrunk or grown chemical bonds[43] and is normally avoided in the calculation of free energy differences.[40,44]

Long-range electrostatic forces were taken into account by means of the particle mesh Ewald (PME) approach,[45,46] with a direct space sum tolerance of 10^{-6} and a spherical truncation of 11 Å—corresponding to a separation parameter, α, of 0.248 Å^{-1}, a cubic spline interpolation of the charges, and a grid consisting of $72 \times 72 \times 72$ points. Trajectories were integrated with a 1 fs time step. All simulations were carried out in the (N, P, T) ensemble using the Nosé−Andersen scheme,[9−11] which maintains the pressure at a nominal value of 1 atm. The temperature was fixed at 300 K using a stochastic temperature control technique.[47] The system was equilibrated over 50 ps.

To ensure electric neutrality of the system in the course of the alchemical transformation, one Na^+ counterion was also neutralized as a function of λ. This counterion was located at an initial distance of 19.3 Å from the active site and free to move throughout the simulation. An analysis of the complete MD trajectory showed that it remained well solvated and independent of changes in the active site. Separate simulations in the forward and the reverse directions were performed to estimate the hysteresis, albeit transformations in opposite directions may not necessarily share identical convergence properties.[3] The same trajectory was employed for the estimation of the binding free energy via TI and FEP.

The alchemical annihilation of biotin consisted of 10 windows. To avoid numerical instability resulting from the presence of finite charges on atoms bearing very small Lennard-Jones parameters, the electrostatic decoupling approach[48] was utilized, whereby electrostatic and van der Waals contributions were scaled in separate runs. This scheme also allows the analysis of the nonbonded contributions to the net binding free energy. Although the validity of such a decomposition analysis has been questioned on account of the path dependence of the computational scheme, and the fluctuations resulting from cross-

TABLE 1: Estimated Binding Free Energy of Biotin−Streptavidin Complex Using the FEP and TI Methodologies (Free Energies in kcal/mol)

Free Energy Perturbation[a] (FEP)				
biotin ↔ ghost biotin	electrostatic	van der Waals	total	experiment[19]
in complex	-307.8 ± 0.4	-1.8 ± 0.3	-309.6 ± 0.7	
in aqueous solution	298.5 ± 0.5	-6.4 ± 0.2	292.1 ± 0.7	
restraint component[b]			1.8 ± 0.3	
ΔG_{bind}	-9.3 ± 0.9	-8.2 ± 0.5	-16.6 ± 1.9	-18.3
Thermodynamic Integration[a] (TI)				
biotin ↔ ghost biotin	electrostatic	van der Waals	total	experiment[19]
in complex	-326.0 ± 0.1	-17.3 ± 0.3	-343.3 ± 0.4	
in aqueous solution	317.1 ± 0.6	9.3 ± 0.7	326.4 ± 1.4	
restraint component[b]			1.8 ± 0.3	
ΔG_{bind}	-8.9 ± 0.7	-8.0 ± 1.0	-16.0 ± 1.2	-18.3

[a] The reported FEP and TI results are averages of the forward and the reverse simulations. The FEP simulations yielded a net binding free energy of −18.1 and −15.0 kcal/mol, respectively. The TI simulations yielded a net binding free energy of −16.4 and −15.5 kcal/mol, respectively. [b]Restraints were applied only during the reverse simulation and, hence, are included only in the estimate of the net binding free energy for the reverse simulation.

correlation among the energy components,[49,50] its applicability and usefulness in structure-based drug design remains important.[51,52] The present work sheds new light on this debated issue.

In each window, 25 ps of equilibration were followed by 25 ps of data collection, from which a free energy change was estimated; i.e., the overall transformation in each direction corresponded to a total simulation time equal to 1 ns, broken down into 500 ps plus 500 ps for the electrostatic and the van der Waals components—ca. 10 times longer than that of MK. A 1 ns simulation takes approximately 15 days to complete, using 16 R12000 (300 MHz) processors of an SGI Origin 2000. The reverse simulation, wherein the nonbonded interactions of biotin with its environment are created, corresponded to the same simulation length. The Lennard-Jones parameters, however, were grown prior to the charges.

Results and Discussion

The estimated binding free energies are reported in Table 1. Our results match closely the experimental values reported by Weber et al.,[19] as well as the estimate of MK.[21,20] The main difference between the present results and the latter lies in the balance between the nonbonded terms. This discrepancy is likely to stem from differences in the simulation protocols and the force field employed. A critical aspect concerns the accurate treatment of long-range electrostatic forces, as was done here. This example highlights the difficulties associated with component analysis—whereas free energy is a state function, its components are not. Any interpretation based on these quantities should, therefore, be undertaken with this aspect in mind.[49−52] This remark also holds when comparing the results of Table 1 obtained with FEP and TI—two methodologies that do not necessarily share the same convergence properties.[3] Since the choice of the λ dependence of the configurational energy is not unique, the computed free energy components are likewise not unique. Furthermore, in FEP, component analysis is clearly dependent upon the reference ensemble.[3] Put together, this implies that any assessment of the accuracy of FEP versus TI should be performed over the complete thermodynamic cycle schematized in Figure 1, rather than individual legs.[51] Accordingly, a direct comparative analysis of the electrostatic and the van der Waals contributions estimated by means of these two computational methods is not straightforward.

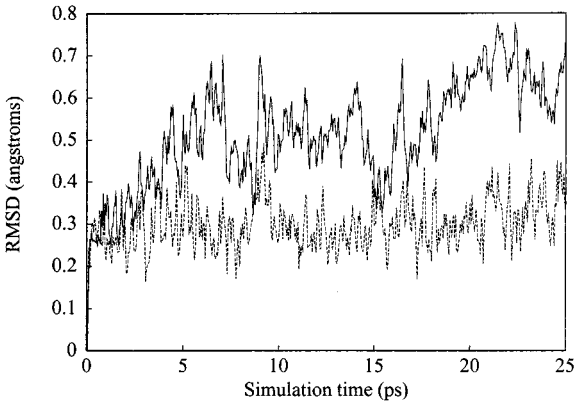

Figure 2. Distance root-mean-square deviation (RMSD) of the atomic positions of biotin over a 25 ps time scale: In aqueous solution (solid line) and in the active site (dashed line). The mobility of biotin is strongly restricted due to packing and hydrogen bonding interactions.

Alchemical creation of biotin in the active site of streptavidin, starting from a ghost ligand, required positional restraints to warrant conservation of the relative orientation and conformation that are appropriate for the formation of the correct hydrogen bonds, viz., between the valeryl carboxyl group and the hydroxyl group of Ser[112] and Ser[88]. Experimental studies indicate that binding of biotin to streptavidin is accompanied by a change in conformation of the surface loop 3−4 (residues 35−46) causing a closure of the active site, thereby locking biotin inside.[18] Analysis of the distance root-mean-square deviation (RMSD) of biotin in the active site confirms that a tight binding through optimal van der Waals contacts and a hydrogen bond network strongly restricts the mobility of the ligand, compared to the available degrees of freedom for the free biotin in water (see Figure 2). The need for a correction term to account for the standard state of the unbound ligand molecule in those simulations where the ligand is restrained in the active site has been discussed previously.[7,53] This term corresponds to the effective volume accessible to the restrained ligand against the volume accessible to the ligand at its standard concentration. The effective volume that biotin explores in the active site was estimated to range between 0.53 and 0.91 Å^3 for MD trajectories generated with 100 kcal/(mol Å^2) restraints and without them. This would correspond to a free energy correction of 4.7 and 4.4 kcal/mol, respectively.

The energetic contribution of the restraints applied to those hydrogen bonds formed between streptavidin and biotin during the MD simulation was also investigated using a perturbation approach.[54] Using a force constant of 100 kcal/(mol Å^2) to coerce the eight hydrogen bonds[18] to their equilibrium lengths in the crystal structure over a 10 ps simulation carried out with the CHARMm suite of programs yields a net average contribution of 1.8 ± 0.3 kcal/mol, where the error bar is based on three simulations, using distinct starting configurations. The analysis of the RMSD of biotin and the effective volume that it explores[7,53] indicates that the binding site harbors the ligand strongly, independent of the restraints enforced during the simulation. Although the perturbation procedure[54] and the method based on evaluating the effective volume are both intended to quantify the effects of the restraints, it is worth noting that they provide quantitatively different answers. That the current approach is still not capable of capturing all facets of the true binding process, despite increased computational resources, constitutes yet another concern. For instance, con-

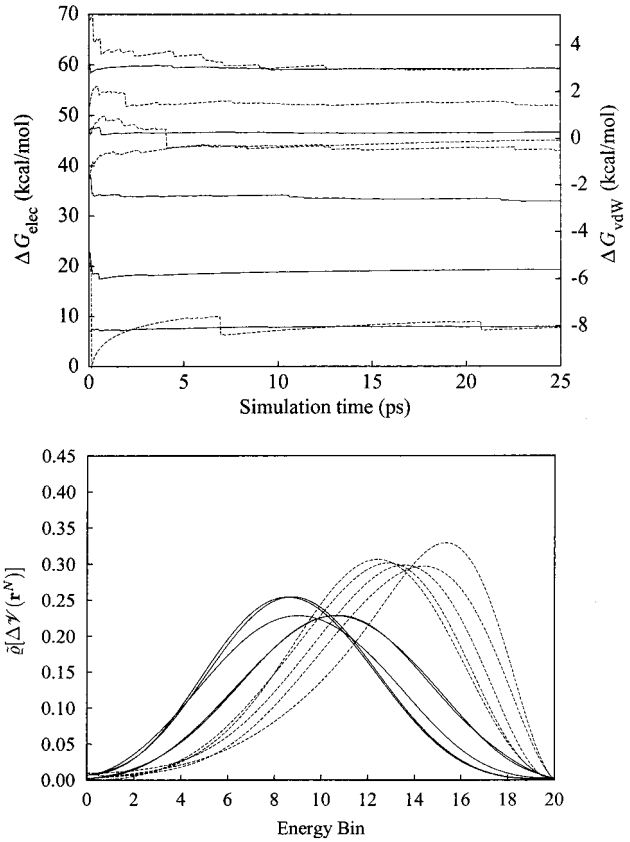

Figure 3. (a) Typical convergence profile of the electrostatic and the van der Waals energies in the annihilation leg of the thermodynamic cycle. Only odd windows are displayed here. All the electrostatic energies are plotted in a solid line scaled to the primary *y* axis and the dashed lines refer to the van der Waals energies scaled to the secondary *y* axis. Whereas the electrostatic energy varies linearly with λ, the dependence of the van der Waals energy appears to be nonlinear, with significant contributions from early windows. (b) Normalized, splined probability distributions of the differences in configurational energies, $\Delta\mathcal{I}(\mathbf{r}^N)$, for selected windows. Although the electrostatic energy distribution is normal, the van der Waals energy distribution is skewed, in particular near the annihilated state of the ligand.

formational changes in the protein occurring over long timescales, e.g., the motion of the loop that closes the active site upon binding, or the occupation of the binding site by water molecules in apo-streptavidin, and their subsequent release in the presence of the ligand, are processes that obviously contribute to the binding free energy and should, hence, be addressed in future studies.

The ensemble averages for the intermediate states and the tendency of the energy distributions to obey a normal law were also examined. Figure 3a shows typical convergence properties of the nonbonded energies throughout the production simulation. The electrostatic and the van der Waals components appear to be well converged within the 25 ps time scale, although the variation with λ of the latter is not as smooth as that of the former. The normalized probability distribution, $\tilde{\rho}[\Delta\mathcal{I}(\mathbf{r}^N)]$, where $\Delta\mathcal{I}(\mathbf{r}^N)$ is the difference in configurational energy of the nonbonded contributions within the windows, are reported in Figure 3b. Here, the electrostatic component can be modeled by a Gaussian distribution, whereas the van der Waals energies exhibit a marked skewness, and hence, fit a Gram−Charlier function better.[55] The probability distribution can be employed to estimate the free energy change:[56]

$$\Delta G = -k_B T \ln \int \tilde{\rho}[\Delta\mathcal{I}(\mathbf{r}^N)] e^{-\Delta\mathcal{I}(\mathbf{r}^N)/k_B T} d[\Delta\mathcal{I}(\mathbf{r}^N)] \quad (1)$$

Fitting $\tilde{\rho}[\Delta\mathcal{I}(\mathbf{r}^N)]$to a Gaussian distribution yields a binding free energy of -13.1 ± 5.5 kcal/mol broken down into $+1.8 \pm 0.3$, -6.8 ± 0.6, and -8.0 ± 4.6 kcal/mol for the restraint, electrostatic, and van der Waals contributions, respectively. The significant error for the van der Waals component clearly demonstrates that it cannot be fit by a simple Gaussian distribution. Employing a Gram−Charlier function leads to an estimated free energy contribution of -6.6 ± 1.8 kcal/mol. This analysis indicates that a linear change in λ might not be the most appropriate way to perform alchemical transformations, in particular, for their van der Waals components. An alternative approach could consist of adopting a modified formulation of the interaction potential energy function to avoid possible end-point singularities when van der Waals sites are created or annihilated.[57,58]

Conclusion

The significant increase of available computational resources, together with milestone developments in the methodology of molecular statistical simulations have paved the way over the past decade for the estimation of free energies with minimal approximations or simplifications. In this contribution, the absolute free energy of association of the prototypical biotin−streptavidin complex is reported. The good agreement with the experimental estimate of Weber et al.[19] suggests that increasingly large macromolecular systems can be described realistically with a high level of confidence, using state-of-the-art simulation protocols. Component analysis at a qualitative level illuminates the effect of methodological differences, e.g., boundary conditions, on the individual contributions of the binding free energy.[20,21] The set of simulations reported herein illustrates the requirement of massively parallel architectures in conjunction with appropriate paradigms for determining free energies in complex molecular assemblies within a reasonable time frame. This is particularly true when use is made of sophisticated algorithms guaranteeing a physically realistic description of the system, and when the nature of the latter calls for significant sampling to attain converged ensemble averages and, thus, reliable free energy estimates.

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