Inclusion of Loss of Translational and Rotational Freedom in Theoretical Estimates of Free Energies of Binding. Application to a Complex of Benzene and Mutant T4 Lysozyme

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Abstract: We present the first complete treatment for calculating theoretical estimates of free energies of formation of macromolecule−ligand complexes with molecular dynamics simulations, as the free energy for transforming the ligand into a non-interacting state by gradually diminishing the forces between macromolecule (plus solvent) and ligand. The calculations become possible due to the introduction of a specially designed potential ("molecular tweezers") which restrains the spatial position and orientation of the ligand molecule and is gradually applied as the transformation proceeds from complexed to non-interacting components. The binding of benzene to a mutant T4 lysozyme (Morton et al. *Biochemistry* **1995**, *34*, 8564−8575) has been used as a test case. The simulations reproduce the value of the free energy of binding (−5.19 kcal/mol if the standard state of benzene is a 1 M aqueous solution) within the sum of experimental and statistical error. Another series of such simulations with rigid protein models provides an estimate of the dependence of the free energy of binding on the protein conformation. The free energy of binding is found to decrease in the series: energy-minimized ligand-free protein (−3.5 kcal/mol), energy-minimized ligand-containing protein (−6.3 kcal/mol), and crystal structure (−8.5 kcal/mol). The free energy of binding to a series of snapshots from a protein−ligand dynamics trajectory varies between −7 and −9 kcal/mol. The "cratic" free energy contribution, which corresponds to the loss of translational and rotational freedom of the ligand molecule, was estimated at 7 kcal/mol. It has proved possible to decompose this into translational and rotational components and, from these free energies, estimate the remaining freedom of the benzene in the binding pocket, at 0.6 Å for positional range and 10−15° for angular range, in excellent agreement with the motion observed in a dynamics trajectory.

I. Introduction

One approach to obtaining a theoretical estimate of the binding constant (or of the standard free energy of binding) for forming a complex of a macromolecule and a ligand, such as an enzyme inhibitor, substrate, or transition state analogue, is to separate the free energy into components. This has as advantages that the individual contributions are often easy to evaluate and that the results of using a well-chosen decomposition scheme provide insight into the balance between effects that stabilize and destabilize the complex. A disadvantage is that the decomposition is an approximation, as the components are not all well separable. Nonbonded interactions between the molecules will contribute a component of the free energy that will usually strongly favor the bound state. Free energy terms for changes of interactions with solvent of both molecules as a result of binding and changes in internal energy and entropy should also be included. More or fewer terms may be considered, depending on the desired accuracy. However, all such estimates should include a term for the loss of freedom of molecular translation and rotation as a result of association of two molecules to form a single complex; this produces an entropic contribution which favors the dissociated state.

The magnitude of this last term, which is called the cratic free energy contribution, has been estimated in a general way in papers by Jencks and subsequently by others including Janin and co-workers and Williams and co-workers, both according to basic theoretical principles and by consideration of a variety of experimental data.[1−4] (We follow Janin[3] in using the expression "cratic free energy" in a general sense, not identified with a particular equation.) However, it is not easy to evaluate this term accurately while taking into account the molecular details of any particular complex. In this paper, we present a general method for computing the cratic free energy term from a knowledge of atomic coordinates and an appropriate interatomic force field.

A second way to calculate the free energy of binding, given atomic coordinates and an appropriate interatomic force field, follows a global approach, which produces a single answer for the free energy of binding and little or no information about component terms. This approach uses free energy perturbation techniques by which the complex of two molecules is gradually converted into two well-separated molecules, in a molecular dynamics or Monte Carlo simulation. It is usually preferable to do this via a molecular transformation calculation, rather than by a potential of mean force calculation along a physically realizable path. In the latter method, the distance between the two molecules is gradually changed. In the former, the forces between the smaller ligand molecule and the macromolecule−solvent system are gradually reduced until the macromolecule is in a free, solvated state and the ligand is, in effect, in an ideal gas state. To estimate the standard free energy of binding for macromolecule and solvated ligand, the free energy of

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transfer of the ligand from solution to vacuum must be added to the free energy change for dissociation. (A value of the transfer free energy may be available from experiment, or else it may be computed in another molecular transformation simulation, in which the ligand molecule is removed from a solvent medium.)

A successful molecular transformation calculation is reversible, i.e., the ligand molecule can be reintroduced into the binding site, for a change in the free energy equal, but of opposite sign, to that calculated for its removal. Furthermore, as the standard free energy change for ligand binding depends on the volume available to the ligand molecule in the ideal gas state, this volume must be clearly defined. These two problems have been solved in past work for the case of *monatomic* or nearly monatomic molecules (Xe, H_2O) by use of a positional restraint which is applied to the ligand molecule in the gas state.[5−7] The effect of this restraint is twofold: it prevents the ligand molecule from moving away from the binding site when the intermolecular forces are absent, and then being caught in the wrong location when the intermolecular forces are increased, and it defines an intermediate standard gas state of the ligand in such a way that the difference in free energy with respect to a common standard state follows from a simple relationship.

It has been recognized that the application of the molecular transformation method to binding of *large* ligand molecules requires an additional device that will hold the isolated ligand in a binding mode similar to that of the bound ligand. Otherwise the complex will form with the ligand roughly in the right place, but with wrong orientation and wrong contacts and higher free energy.[8] We describe here an extension of the positional restraint described above, to a *body restraint* by the addition of a rotational restraint. The angular part of the restraint has been designed so that (1) it does not alter the internal energy or dynamics of the isolated ligand molecule and (2) the free energy for releasing the restraint is easily calculated.

We have applied this new method in order to estimate the loss of translational and rotational freedom and to compute the free energy of forming a complex of benzene with a mutant of T4 lysozyme. This mutant lacks an internal leucine side chain of the wild-type protein and contains a hydrophobic cavity that is large enough to bind a molecule of benzene with a modest affinity ($K_a = 5.7 \times 10^3$ M^{-1}).[9,10] This is an excellent test system for a number of reasons, among which are the following: the structure of the complex has been determined by X-ray crystallography at a resolution of 1.9 Å;[9] the binding site is inaccessible to solvent; the benzene molecule is small and rigid; the free energy of transferring benzene from vacuum to water is accurately known from experiment; being surrounded by a hydrogen-bonded protein framework, the binding site is well defined and (one infers) not subject to the flexing motions typical of many enzyme binding sites.

II. Methods

Benzene−T4 Lysozyme System. The crystal structure of the T4 lysozyme mutant complexed with a benzene molecule in its cavity was retrieved from the Protein Data Bank (PDB18L.ENT). The resolution of the structure is 1.9 Å. Compared with the wild-type T4 lysozyme, the mutant has three point mutations: Leu 99 → Ala (L99A), Cys 54 → Thr (C54T), and Cys 97 → Ala (C97A). The enzyme has 64 residues, but only 162 residues are resolved in the crystal structure. As the two unresolved residues are at the C-terminus and far away from the cavity where the benzene binds and therefore should have little influence on the binding, we have used a structure with 162 residues in our simulations. All hydrogen atoms were added in stereochemically acceptable positions and the energy terms for bonds, angles, and planar groups were minimized. This structure was used as is, or after energy minimization or short dynamics simulation, in simulations of the interaction with benzene with rigid models of the protein.

For the most accurate simulations, a system was used which had been obtained by placing this molecule, which measures roughly 48 × 42 × 42 Å^3, at the center of a box of 64 × 58 × 58 Å^3 and filling the rest of the space with 5668 water molecules. The hydrated system was first subjected to energy minimization and then 20 ps of equilibration by molecular dynamics to generate a reasonable low-energy conformation.

Molecular Dynamics Simulations. All molecular dynamics simulations were carried out with the program SIgMA,[11] and the all-atom CEDAR force field[12,13] was used. SIgMA employs the SHAKE algorithm to constrain bond lengths in the simulations.[14] Unless otherwise stated, the dynamics integration time step was 2 fs and at each step the temperature was globally restrained to 300 K by adjustment of the kinetic energy.[15] The calculations used a cutoff on nonbonded interactions equal to 10 Å, or as stated.

In one series of simulations, the benzene molecule and parts of the protein molecule were allowed to move. The moving atoms of the protein were those contained in a sphere around the center of mass of the benzene molecule: several simulations were done to assess the effect of the choice of the radius of this sphere.

In another series of simulations, only the benzene molecule was free to move. In all simulations the atoms of the benzene molecule were subjected to Brownian dynamics, i.e., had a combination of friction and random forces applied.[16] As this serves to control the mean temperature of the system, the global temperature control method was not applied in calculations where only the benzene molecule moved.

Molecular Transformation Calculations. The process for which the free energy change is to be determined is the conversion of the (solvated) complex of macromolecule and ligand molecule to a state in which the two components are separated into a (solvated) uncomplexed macromolecule and a non-interacting ligand molecule, i.e., a ligand molecule in the ideal gas state. In this case the transformation achieves the following reaction:

$$\underset{[=\mathrm{A}]}{(\text{T4 lysozyme}-\text{benzene})_{\text{water}}} \Rightarrow \underset{[=\mathrm{B}]}{(\text{T4 lysozyme})_{\text{water}}} + (\text{benzene})_{\text{gas,R}} \quad (1a)$$

In order to compare the results of simulation and experiment, one should in addition consider the following two transformations:

$$(\text{benzene})_{\text{gas,R}} \Rightarrow (\text{benzene})_{\text{gas,S}} \quad (1b)$$

$$(\text{benzene})_{\text{gas,S}} \Rightarrow (\text{benzene})_{\text{water,S}} \quad (1c)$$

The subscripts R and S indicate "restrained" and "standard state", respectively.

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The state on the left of eq 1a is represented in the model with full intermolecular forces between protein and solvent water, benzene and water, and protein and benzene. The state on the right-hand side is represented with intermolecular forces between protein and solvent water, only. Intramolecular forces are used always. The body restraint potential is applied to the benzene molecule in the state on the right of eq 1a, only. Equations 1b,c show two additional steps, i.e., conversion of the restrained isolated benzene molecule to a benzene molecule in a standard state and convertion of this to a benzene molecule in solution in a similar standard state; these steps are needed to obtain the conversion studied in the solution measurements of Morton et al.,[10] i.e.

$$(\text{T4 lysozyme}-\text{benzene})_{\text{water}} \Longrightarrow (\text{T4 lysozyme})_{\text{water}} + (\text{benzene})_{\text{water,S}} \quad (2)$$

Writing the net free energy change for this reaction as a sum of three terms, one obtains

$$\Delta A^\circ_{2,\text{w}} = \Delta A^\circ_{2,\text{g}} + \Delta A^\circ_{1\text{c}} = \Delta A^\circ_{1\text{a}} + \Delta A^\circ_{1\text{b}} + \Delta A^\circ_{1\text{c}} \quad (3a)$$

and if the reference state for free benzene is the ideal gas, one gets

$$\Delta A^\circ_{2,\text{g}} = \Delta A^\circ_{1\text{a}} + \Delta A^\circ_{1\text{b}} \quad (3b)$$

These terms are, the free energy change calculated in the simulation, $\Delta A^\circ_{1\text{a}}$, the free energy for releasing the body restraint to attain a standard state of 1 M ideal gas, $\Delta A^\circ_{1\text{b}}$, which is compared with equations given in the final section of this paper, and the standard free energy of transfer of a benzene molecule from vapor to water, $\Delta A^\circ_{1\text{c}}$, which is known from experiment to be −0.3 kcal/mol (based on data reported by Hine and Mookerjee[17]). The result, $\Delta A^\circ_{2,\text{w}}$, is to be compared with the experimental value, which is −5.19 kcal/mol.[10] (The difference between ΔA and ΔG for the equilibrium between protein and *dissolved* benzene is negligible.)

The free energy changes were calculated with slow-growth thermodynamic integration, whereby the integral

$$\Delta A^\circ = \int_0^1 \langle \partial U/\partial\lambda \rangle \, \mathrm{d}\lambda \quad (4a)$$

(where $\langle\ \rangle$ denotes the average over a Boltzmann distribution) is approximated as

$$\Delta A^\circ_{\text{a}} = \sum \partial U/\partial\lambda \, \delta\lambda \quad (4b)$$

Here, the potential energy function, U, depends on the coupling parameter, λ, so that $U(\lambda{=}0)$ corresponds to the starting molecule (=A in eq 1a) and $U(\lambda{=}1)$ corresponds to the product of the transformation (=B in eq 1a). The value of λ is changed by an increment $\delta\lambda$ after every integration time step.

As in previous work, a nonlinear coupling scheme was used for the dependence on λ of the interactions of the benzene molecule with protein and solvent. The body restraint potential was applied to the benzene molecule in the ideal gas state (=B in eq 1a). In most of the reported simulations, the force constants for the component restraints varied linearly with λ. Each cycle produced two estimates of the free energy difference. If these differed significantly, higher precision was attained by performing each cycle several times and averaging the independent free energy values. An estimate of the statistical error was also obtained thereby. One cycle consisted of 10 ps each for equilibrations at $\lambda = 0$ or 1 and 100 ps each for simulations with increasing and decreasing λ, for an aggregate simulation time per cycle of 220 ps.[18]

There is no reason to believe that materially different results would have been obtained had stepwise perturbation[19] or point-by-point thermodynamic integration protocols[20] been used instead.

The body restraint potential (see Body Restraint Algorithm) was applied as follows. For the benzene molecule, the first reference point, $\mathbf{X}_i$, was chosen to be the molecular center of mass, the second reference point, $\mathbf{X}_j$, was one of the carbon atoms (C^1), and the third reference point, $\mathbf{X}_k$, was the center of mass of atoms C^2, C^3, H^2, and H^3. Values for the reference parameters of the restraint potential ($\mathbf{X}_{i,\text{o}}$, $\mathbf{e}_\theta$, and $\mathbf{e}_\chi$) were determined as the mean values observed in a short simulation and were not varied during the simulation. The force constant for the restraints was set at 100 kcal/(mol·Å^2) or 100 kcal/(mol·rad^2).

Table 1. Computed and Experimental Free Energies of Binding of Benzene to L99A Mutant T4 Lysozyme[a]

radius of dynamic sphere (Å)	cutoff on nonbonded forces (Å)	$-\Delta A^\circ_{1\text{a}}$ or $-\Delta A^\circ_{1\text{a}}$* (cf. eqs 1a and 5)	$-\Delta A^\circ_{1\text{r}}$ (cf. eq 5)	$-\Delta A^\circ_{2,\text{w}}$ (cf. eq 2)
12	6	-15.37 ± 0.7		−4.01
12	10	-16.44 ± 0.4		−5.08
12	8	-16.27 ± 0.6		−4.91
15	10	-16.50 ± 0.4		−5.14
12	8	-12.03 ± 0.1[b]	-3.90 ± 0.3[b]	−4.54
experiment[10]				−5.19

[a] The standard state of benzene is 1 M aqueous solution. Units are kcal/mol. [b] Computed in a two-step simulation scheme.

III. Results

Protein is Dynamic. One-Step Simulation. The binding free energy of the benzene molecule in the cavity of the T4 lysozyme mutant was calculated for the hydrated system with periodic boundary conditions, two different sizes of the dynamic sphere (12 and 15 Å), and three different cutoff radii (6, 8, and 10 Å). In these simulations the magnitude of the restraint potential was varied in proportion to that of the coupling parameter, λ. The resulting values of $\Delta A^\circ_{1\text{a}}$ are listed in Table 1. These were used to compute corresponding values of ΔA°_2 according to eq 3, with $\Delta A^\circ_{1\text{c}} = -0.3$ kcal/mol and $\Delta A^\circ_{1\text{b}} = -11.06$; the latter value was computed according to eq B1, with values from Table 5 corresponding to $K_\text{f} = 100$ and using eq B15 for ΔA°_s, with $z_\text{s} = 12$. Use of the symmetry term ΔA°_s implies an assumption that only a single one of the 12 physically indistinguishable binding modes of the benzene molecule is represented in the simulation. (This assumption will be challenged in the next section.) The results are given in the fourth column of Table 1. It is seen that a similar answer is obtained for cutoffs of 8 and 10 Å, and for dynamic spheres with radii 12 and 15 Å. This result is in good agreement with the experimentally measured value. The 6 Å cutoff is obviously too short, as the magnitude of the free energy is too small by approximately 1 kcal/mol.

Two-Step Simulation. In long dynamics simulations with static or dynamic protein, in which no restraint was applied, the benzene molecule retained its general orientation, except that on rare occasions the benzene ring abruptly rotated by 60° or more, jumping to a new binding mode in which the carbon atoms (and of course also the hydrogen atoms) had changed places. This raised the possibility that such conformation changes occurred during the transformations; in particular, this might happen when the value of λ is small, or when it is zero (during the equilibrations at $\lambda = 0$). If this were so, the simulations would report the thermodynamics of a mixture of several symmetry-related, and physically indistinguishable, bound states, rather than of a single state, as assumed.

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The following alternative procedure for calculating ΔA°_2 has been designed to eliminate alternate binding modes. The transformation is performed with a constant value of the body restraint potential, i.e., the benzene molecule's position and orientation are restrained both in the starting and in the final state of the transformation. With this simulation one computes a modified free energy difference

$$\Delta A^\circ_{1,a}{}^* = \Delta A^\circ_{1,a} - \Delta A^\circ_{1,r} \qquad (5)$$

which contains a term $\Delta A^\circ_{1,r}$ to account for the difference between the restrained and the unrestrained system. The value of $\Delta A^\circ_{1,r}$ can be calculated in a simulation in which no transformation occurs, but in which the magnitude of the restraint potential is systematically varied by multiplication with a coupling parameter, λ_r. The free energy difference is again calculated according to eqs 4a,b.

To prevent the benzene molecule from jumping to a different binding mode during the equilibration at $\lambda_r = 0$ (when the restraint is absent), the angular distribution of the benzene molecule was limited by application of a modified body restraint, which is non-zero only when the angle θ in eq B7 exceeds a certain minimum, here set to 30°. (This modified restraint was absent in the subsequent simulation in which λ_r was varied.) In addition, the orientation of the benzene molecule was monitored to ensure that in these simulations only a single binding mode was represented. A series of simulations, in which the radius of the dynamic sphere was 12 Å and the cutoff on nonbonded forces was 8 Å, gave $\Delta A^\circ_{1,a}{}^* = 12.0 \pm 0.1$ and $\Delta A^\circ_{1,r} = 3.90 \pm 0.3$, yielding a value for $\Delta A^\circ_{1,a}$ of 15.9 kcal/mol.

The result from the two-step calculation differs by 0.4 kcal/mol from the values obtained with the one-step simulation scheme. Apparently, the mobility of the benzene molecule is sufficiently large that use of the former scheme is to be preferred. However, as benzene is a very special case of a small molecule with high symmetry number, the two-step process will not be needed in a majority of studies of protein−ligand interactions. The agreement between this theoretical and the experimental value of the free energy of binding is very good, just inside the limits set by experimental and statistical error.

Protein is Static. Dependence of the Free Energy of Binding on the Protein Conformation. These calculations were done also with a static protein model (without solvent or periodic boundary conditions), for several different conformations. In this case, quite short calculations were found to produce adequately converged answers, i.e., the magnitude of ΔA for the forward and backward parts of the cycle was the same within a few tenths of a kilocalorie/mole, and longer simulation cycles produced the same answer as did shorter ones.

The simulations were done with the protein atoms having the coordinates of the crystal structure, and with each of two structures of which the energy had been lowered by 50 cycles of conjugate gradient minimization, in one case with the benzene in place and in the other case with the binding cavity empty. The calculation was done also with each of five snapshots from a molecular dynamics calculation collected at intervals of 2 ps. The results are given in Table 2; the individual answers are seen to differ considerably amongst themselves.

Magnitude and Components of the Cratic Free Energy. The cratic component of the free energy of binding reflects the loss of molecular translational and rotational freedom when two separate molecules join to form a complex. The free energy of binding includes the cratic term and the binding energy and also terms due to changes in the structure and internal dynamics of macromolecule, solvent, and ligand. We note again that this is an artificial distinction and that there is no unique decomposition into terms.

Table 2. Free Energies of Binding of Benzene (Dynamic) to Different, Static Conformations of Mutant T4 Lysozyme[a]

protein conformation	$-\Delta A^\circ_{2,s}$
experiment (crystal) coordinates	−8.5
energy minimum with benzene	−3.5
energy minimum with benzene	−6.3
dynamics snapshot at time t_0	−6.8
dynamics snapshot at time $t_0 + 2$ ps	−8.2
dynamics snapshot at time $t_0 + 4$ ps	−8.9
dynamics snapshot at time $t_0 + 6$ ps	−8.3
dynamics snapshot at time $t_0 + 8$ ps	−7.5
experiment[10]	−5.5

[a] The standard state of benzene is 1 M ideal gas.

Magnitude. In the free state, a ligand molecule can assume any orientation in space and its position can be anywhere within the molecular volume, *V/N*, which is the reciprocal of the concentration; in a first approximation, the cratic term corresponds to the loss of this freedom when the ligand molecule binds to the macromolecule, and this is an entirely entropic term. A more advanced treatment recognizes that the ligand still has some freedom to move relative to the macromolecule and takes into account the six new vibrational modes of the complex as a correction. The magnitude and the distribution into energetic and entropic parts depend on the details of the energy surface, but in first approximation, the energetic part can be equated with the binding energy and the entropic part with the cratic free energy. In a short simulation the mean binding energy was found to be −15.2 kcal/mol; with the free energy change of −8.5 kcal/mol from Table 2 this gives the cratic free energy component for binding of a molecule of benzene into the cavity (from a standard state of 1 M gas) as the entropic part, i.e., 6.7 kcal/mol.

Components. It proved possible to obtain separate estimates of the positional and the angular parts of the cratic free energy by following a three-step insertion process. Step i: in the first step, the translational and rotational degrees of freedom of the benzene molecule in the ideal gas standard state are very nearly frozen by application of a body restraint potential with extremely large force constants. Step ii: in the second step, this restrained molecule is introduced into the binding cavity in the position and orientation of minimum energy. Step iii: in the third step, the restraints are released with the benzene molecule in the binding cavity. The cratic component is calculated as the difference between the free energies for freezing the benzene molecule in the ideal gas standard state (step i) and in the cavity (reverse of step iii).

The free energy change for step i is found with equations given in Body Restraint Algorithm. The free energy change for step iii was found by a three-step dynamics simulation in which the force constants of the components of the body restraint were *successively* decreased to zero from a large value.

The free energy change for step ii is simply equal to the energy change for placing the benzene molecule in the binding cavity into the position of minimum energy. Of course, this requires the benzene molecule's position to be adequately restricted so that the protein−benzene energy and forces do not fluctuate as a result of the remaining motion of the benzene molecule. It was found that a body restraint potential with force constants of 2000 kcal·mol^{-1}·Å^{-2} and 2000 kcal·mol^{-1}·rad^{-2} was adequately tight. (The simulation time step was reduced to 1 fs.)

As mentioned, the simulation in step iii was done in three stages, first the dihedral restraint, then the angle restraint, and

Table 3. Free Energies to Successively Apply Component Restraints with $K_f = 2000$

restraint component	ΔA for step iii	ΔA for step i	difference	effective range
1. translation (x)	5.0	10.1	5.1	(0.4 Å)^3
2. angle (θ)	2.1	4.9	2.8	(0.3 rad)^2
3. dihedral (χ)	1.0	3.0	2.0	0.2 rad

Table 4. Free Energies to Successively Apply Component Restraints with $K_f = 2000$, with Different Order of Imposition

restraint component, in order of application	ΔA for step iii
1. angle (θ)	1.9
2. dihedral (χ)	1.0
3. translation (x)	4.0

finally the positional restraint was released, and a free energy difference was obtained for each stage (see Table 3). This then allowed a decomposition into three terms, which are given in the fourth column of Table 3; a corresponding effective translation or angular range is given for each in the fifth column. As the decomposition is arbitrary, the results may depend on the choice of the order in which the restraints have been released. However, in this case, a change of order was found to have very little effect on the magnitude of the component terms (Table 4). The sum of the three components in the fourth column of Table 3 equals 9.9 kcal/mol; by subtracting the symmetry term $kT \ln z_s = 1.5$ kcal/mol, one obtains from this a value for the cratic free energy of 8.4 kcal/mol, in reasonable agreement with the value of 6.7 kcal/mol obtained from the entropic part of the binding free energy.

Freedom of Motion of the Bound Ligand. The result of a short simulation of the motion of benzene in the cavity was analyzed in terms of extent and frequency of the displacement the benzene molecule. The root-mean-square (rms) displacement of the molecular center of mass normal to the plane of the ring was found to be 0.13 Å, with a period of 0.3 ps. The rms displacement in the plane of the benzene ring was 0.3 Å in two orthogonal directions, with a period of 1 ps. The rms angular displacement of a vector normal to the ring was 3.5° in one direction and 6° in the other, both with a period of 0.3 ps, while the rms angular displacement for rotation about the normal vector was 7.5°, with a period of 0.5 ps. In a longer run, sudden rotations about the normal vector by 60° or more, that correspond to transitions in which the carbon atoms exchange positions, were observed at a rate of 0.1 ps^{-1}. By equating rms deviation with one-half of a range, one sees that these rms deviations agree very well with the estimates of range of positional and angular displacement that had been determined quite independently on the basis of decomposition of the cratic free energy component (cf. the preceding paragraph and Table 3).

IV. Discussion

Free Energies of Binding from Simulations. Free energy differences between physically realizable states can be computed along physically nonrealizable paths, by dynamics or Monte Carlo simulations in which molecular interactions are gradually changed, and a molecular system is transformed into one with different composition.[21,22] With the advent of faster computers and development of reasonably accurate empirical force fields, the molecular transformation method has found numerous applications; application to the calculation of free energies of transfer from vacuum into solvent for a series of small molecules has been an early success,[23,24] and this has encouraged more complex studies, such as calculations of differences in free energy of binding of related small molecules to a common enzyme active site.[25,26]

However, use of molecular transformation for calculation of the absolute binding free energy of a small molecule−macromolecule complex has been found to run into unique problems, whose complete solution has not been obvious, but has been presented in this paper. As mentioned in the Introduction, these unique problems are due to two requirements. The first requirement is that the process must be reversible. At the end of the first half of a cycle of transformation, when the interactions of the ligand molecule with the rest of the system have been reduced to zero, the ligand will leave its binding position. Generally, it will not return there when the interactions are reintroduced, finding instead one of many alternative positions of (locally) minimum energy. This problem has been solved by the use of position restraints,[5] and this solution has since been adopted in a number of studies.[6,7,27−29] In two other studies, no restraints were used; in one of these, the transformation was not permitted to go to completion, so that the ligand would remain in the binding site,[8] and in the other, the atomic masses of the ligand were made very high, so that the ligand's motion was sluggish, and the ligand did not have time to escape from the binding site when it did not interact with the protein.[30] However, these last two methods are not recommended because they do not permit one to satisfy the second requirement.

The second requirement is that the computed free energy of binding must reflect the choice of standard state. Free energy changes computed by molecular transformation methods correspond, in the real world, to differences in *standard* free energy. (The latter are formally identified with the superscript °, which has been used throughout in this paper and in other papers from our laboratory in order to emphasize this fact.) For example, transformation of one molecule of P into one molecule of Q, in solution or bound to a macromolecule, gives the difference between the free energies of one molecule of P and Q in a standard state, the details of which (temperature, pressure, molar volume) are defined by the simulation conditions. In this instance, the molar volumes of the standard states of P and Q are identical; although the free energy of each standard state depends on its molar volume, a change of the simulation volume will have no effect on the difference $A°_Q - A°_P$ (except for boundary effects in small systems).

However, this is not the case when the number of independent molecular species changes in the process.[1,3] The standard free energy change for the process P + Q → PQ, $A°_{PQ} - A°_P - A°_Q$, changes when the standard state volumes of all components (P, Q, and PQ) are changed in the same way. It is convenient to consider that any changes in standard state volume of macromolecule (call it P) and macromolecule−ligand complex, PQ, cancel and focus attention solely on the standard state of the ligand. The choice of standard state in experimental studies

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in arbitrary (in practice, conventions limit the choice to 1 M ideal solution, 1 M ideal gas, ideal gas at a pressure of 1 bar, or pure liquid); therefore, a computed binding free energy cannot be validly compared with the corresponding experimental value, unless the method used to compute it has explicitly taken into account the choice of standard state of the ligand in the experimental system. This second condition has been satisfied in only three of the cited studies; in these, a harmonic position restraint was applied to a small ligand molecule (Xe or H_2O) in studies of binding in cavities inside protein molecules.[5−7]

The condition was not satisfied in the two studies in which no restraints were applied;[8,30] Miyamoto and Kollman did point out in their paper that no method yet existed for presenting such a large ligand (biotin) to a binding site in a protein (avidin). In a study by Sun and Kollman,[29] restraints were applied in order to introduce a bound K^+ ion into a macrocycle binding site; while the study applied a correction for the restraint free energy, it did not provide a theoretical estimate of binding free energy in terms of the standard state of the ligand used in the corresponding experimental study. Because distance restraints between ion and the oxygen atoms of the macrocycle, rather than a single position restraint, were used to position the ion in the binding site, it is not at present clear how to analyze the results of this study so as to provide the sought connection with the experimental binding free energies. (As outlined below, in Body Restraint Algorithm, the ideal choice is here a restraint relating the position of the ion to that of the center of mass of the oxygen atoms of the macrocycle.) Mordasini-Denti et al.[28] applied 80 simultaneous distance restraints when introducing a pyrene molecule to form an inclusion complex with a molecule of cyclophane but did not attempt to evaluate a corresponding restraint free energy.

Differences between binding free energies, to two different chelators in the first study,[29] and in two different solvents in the second study,[28] agreed well with experimental results, but the reported absolute binding free energies were of by larger amounts. For the pyrene−cyclophane complex, the model greatly exaggerated the affinity, which is what one expects as a result of neglect of the restraint free energy. The simulated macrocycle−K^+ ion complex was less stable than found experimentally; this is most probably due to the inherent inaccuracies of representing interactions with metal ions in an empirical, nonpolarizable force field. In contrast, the calculated absolute binding free energies of Xe to myoglobin[5] and benzene to mutant T4 lysozyme (this paper) are in very good agreement with the experimental values.

In a study of protein hydration, Helms and Wade[27] used position restraints to place the water molecule in the sites for which they wished to estimate the hydration but did not convert their hydration free energies to a common standard state. Interestingly, this can easily be done using the published data and then results in the elimination of a systematic dependence on the restraint parameter; however, the authors' conclusion that one site is hydrated, and the other not, is not changed by this. (The form of the restraint potential and therefore also the expression for V_{eff} used in that study differ from those in eqs B2,4, but this can be easily dealt with. We do not show the details of this calculation here.)

The restraints developed and applied here serve as virtual molecular tweezers with which to present a molecule to a binding site in an arbitrarily precise position and orientation relative to the binding site and, when necessary, also in a precisely controlled conformation. This not only guarantees that the transformation form interacting to noninteracting system and back will be reversible but also provides a straightforward means of calculating the absolute binding free energy for any choice of standard state of the ligand molecule. The methodological inaccuracies of some of the previous studies can henceforth be avoided. Of course, other causes of inaccuracies, such as imperfect force fields and inadequate sampling of conformation space, which are common to all molecular simulation studies, remain.

Cratic Free Energy and Flexibility. The cratic free energy is a component identified with the loss of translational and rotational freedom when two molecules (in a solution or in a vapor) associate to form a complex. The concept was first developed some 40 years ago and has since been elaborated in terms of its effects on the stability of molecular complexes and on catalysis and in efforts to determine its magnitude from first principles or from experimental data.[1,2,31−34] We have here presented a new method for estimating this component for an individual ligand−macromolecule complex in terms of a dynamic model and have obtained a value of 7 kcal/mol for the cratic component of the free energy for binding a molecule of benzene in a cavity in a mutant form of T4 lysozyme. This value is in the range of general estimates of this free energy component. The method is general and can be extended to other complexes of known structure.

In this study, we have selected a particular definition of cratic component, as the entropic part of the free energy for a process in which one transfers the ligand from a standard state (here, 1 M gas) into the cavity, while preserving the internal dynamics of the ligand molecule and the relative dynamics of ligand and macromolecule, but keeping the macromolecule rigid. This definition can also include ligand molecules more flexible than benzene.

One sees that the definition is asymmetrical. One might instead choose a definition with both ligand and macromolecule flexible and dynamic. Either way is in principle valid: any decomposition of a binding free energy is obtained by dividing the binding process into successive steps, and the choice of boundaries between steps is arbitrary; it is a matter of convention what to call the terms obtained by a particular division.[35,36] The three-step approach for calculating the cratic component described above can be easily adapted to work with two flexible molecules, instead of one flexible and one rigid molecule; in that case, each reference point−position pair of the body restraint algorithm must consist of a pair of atomic positions (or center of mass of several), one on the ligand and the other on the macromolecule. Of course, simulations with a rigid protein molecule are very fast and much preferable in an "engineering" setting.

Using an elegant alternative approach, Tidor and Karplus have computed the entropy change and estimated a value of 23 kcal/mol for the cratic free energy component upon dimerization of insulin from the changes in the translational, rotational, and vibrational motion with a molecular mechanics model like the one used here.[37] An advantage of that approach is that a quantum-mechanical formalism can be used for computing the free energy change, while the method used by us inherently

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operates in the classical-mechanical limit. Also, it is very difficult to apply the method developed here to a complex of two macromolecules. Disadvantages are that inclusion of explicit solvent may not be feasible and that the vibrational motions are limited to the normal modes, while dynamics simulation can easily work with explicit solvent and will explore conformation space more fully.

Our results with a series of different rigid protein models indicate that the cavity in this T4 lysozyme mutant is quite flexible. When the cavity is empty, energy minimization causes it to change its size and shape, to where it fits poorly around the benzene molecule, and the affinity is low. Energy minimization with the benzene molecule in place leads to a better fit, and during a molecular dynamics simulation, the fit (as measured by affinity) varies widely, above and below the experimental value. The range of affinities computed for the different conformations of the protein is too large for predictive purposes. Jedrzejas et al. proposed a recipe for rapidly computing approximate affinities of drug−macromolecule complexes in terms of free energy terms for packing and solvation.[38] Their recipe is incomplete, as it omits a cratic free energy component;[3] while this can now be estimated with use of the method developed in this paper, the question is if this estimate will be sufficiently accurate. The computed value depends significantly on the selection of a conformation of the protein. The (rigid) conformation obtained by energy minimization with benzene present in the cavity gives affinity in best agreement with the experimental value (or the value from simulations with a dynamic protein model). This is potentially important for predictive work, as energy minimization is a simple first step that can be applied to any complex if the structure of the macromolecule without ligand, or with another ligand, is known. Of course, the generality of this observation will need to be established, and it is premature to actually recommend any such recipe.

Body Restraint Algorithm

The body restraint potential whose description follows serves to limit the translational and rotational degrees of freedom of a molecule to within narrow limits. It is designed in such a way that it does not perturb the internal dynamics of the molecule and hence application of this restraint to a molecule in vacuo does not cause a change in the intramolecular energy or entropy. In addition, this restraint restricts but does not abolish motion along the molecular translational and rotational degrees of freedom, and this makes it possible to compute the free energy for relaxing the restraint to standard conditions. Independently of this study, Gilson et al. have provided an extensive theoretical analysis of the problem of calculating binding free energies from simulation studies and also have given formal derivations of the necessary equations.[39]

The body restraint potential consists of three parts, a position restraint potential, an angle restraint potential, and a dihedral angle restraint potential. The position restraint limits the three translational degrees of freedom, the angle restraint limits two of the three rotational degrees of freedom, and the dihedral angle restraint limits the remaining one. Imposition of these restraints changes the free energy of the restrained molecule by $\Delta A^\circ_{r,x}$, $\Delta A^\circ_{r,\theta}$, and $\Delta A^\circ_{r,\chi}$, respectively. A further free energy term, ΔA°_s, must be added for symmetrical molecules. Finally, it is often necessary to extend the body restraint proper by inclusion of one or more potential energy terms that restrain internal degrees of freedom, i.e., internal rotation about single bonds, which gives a term ΔA°_i.

Overall, the imposition of the body restraint and internal restraints alters the free energy by

$$-\Delta A^\circ_{1b} = \Delta A^\circ_{r,x} + \Delta A^\circ_{r,\theta} + \Delta A^\circ_{r,\chi} + \Delta A^\circ_s + \Delta A^\circ_i \quad \text{(B1)}$$

Position Restraint. The position of the non-interacting ligand molecule can be restrained by application of a potential that confines one atom, or the center of mass of several or all atoms of the ligand molecule, to the neighborhood of a fixed point in space. Such a position restraint has been used earlier in calculations of the binding free energy of proteins with xenon and water molecules.[5−7] A quadratic restraint potential is convenient, but it is possible to use a potential having another form.

In this study, the restraint is applied to a reference atom i or to a reference point, such as the center of mass, that is defined in terms of more than one atomic position:

$$U_{r,x} = (K_x/2)(\mathbf{X}_i - \mathbf{X}_{i,o})2 \quad \text{(B2)}$$

This confines the ligand molecule to a small effective volume:

$$V_{eff} = \int_V \exp[-(K_x/2)(\mathbf{X}_i - \mathbf{X}_{i,o})^2/kT]\, d\mathbf{X}_i \quad \text{(B3)}$$

$$V_{eff} = \left(\frac{2\pi kT}{K_x}\right)^{3/2} \quad \text{(B4)}$$

The corresponding restraint free energy is

$$\Delta A^\circ_{r,x} = -kT \ln(V_{eff}/V_o) \quad \text{(B5)}$$

where V_o is the molecular volume in whatever ideal gas standard state has been selected as appropriate for the problem. (Suitable standard states include: ideal gas of concentration 1 mol/L, ideal gas at a pressure of 1 bar, (vapor in equilibrium with) a pure liquid phase, and vapor in equilibrium with a solution of concentration 1 mol/L.) If we use gas of concentration 1 mol/L as the reference, then $V_o = 1660$ Å^3 at 300 K. (See Table 5 for dependence of $\Delta A^\circ_{r,x}$ on K_x.)

The reference position, $\mathbf{X}_{i,o}$, is determined by the coordinates of the macromolecule. In the most general case, the reference position, and also the reference vectors $\mathbf{e}_\theta$ and $\mathbf{e}_\chi$ (see below), will be functions of atomic positions of the macromolecule and will vary as the latter's conformation changes during the simulation. However, in the present study, the values of these parameters were held fixed during the dynamics simulations.

Angle Restraint. The angle restraint is defined in terms of two vectors. The first of these connects the reference point for the position restraint, $\mathbf{X}_i$, to another reference point (atom or center of mass of several atoms) in the molecule, $\mathbf{X}_j$:

$$\mathbf{r} = (\mathbf{X}_j - \mathbf{X}_i) \quad \text{(B6)}$$

The second vector has a fixed direction which is defined by a unit vector, $\mathbf{e}_\theta$. It is convenient to write the restraint energy as

$$U_{r,\theta} = (K_\theta/2)(1 - \cos\theta) \quad \text{(B7)}$$

where θ is the angle the vectors $\mathbf{r}$ and $\mathbf{e}_\theta$ make with each other, i.e.

$$\cos\theta = \frac{\mathbf{e}_\theta \cdot \mathbf{r}}{r} \quad \text{(B8)}$$

Application of this restraint confines the selected molecular vector to lie within an effective solid angle with value below the value of 4π for an unrestrained molecule. The partition function is given by

$$z_\theta = \int_0^\pi \exp[-(K_\theta/2)(1 - \cos\theta)/kT]\, 2\pi \sin\theta\, d\theta = (4\pi kT/K_\theta)[1 - \exp(-K_\theta/kT)] \quad \text{(B9)}$$

The free energy for applying this restraint to the free ligand molecule is given by

$$\Delta A^\circ_{r,\theta} = -kT \ln(z_\theta/4\pi) = -kT \ln\{(kT/K_\theta)[1 - \exp(-K_\theta/kT)]\} \quad \text{(B10)}$$

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Table 5. Free Energy Cost of Imposing the Body Restraint Potential on a Freely Moving Polyatomic Molecule[a]

K	V_{eff} (Å^3)	$\Delta A°_{\text{r,x}}$ (kcal/mol)	$z_\theta/4\pi$	$\Delta A°_{\text{r},\theta}$ (kcal/mol)	$z_\chi/2\pi$	$\Delta A°_{\text{r},\chi}$ (kcal/mol)
2000	.000082	10.10	.0003	4.87	.007	2.98
1000	.00023	9.47	.0006	4.45	.010	2.77
500	.00065	8.85	.0012	4.04	.014	2.57
200	.00259	8.03	.0030	3.49	.022	2.29
100	.00732	7.40	.0060	3.07	.031	2.08
30	.0445	6.32	.020	2.35	.056	1.72
10	.231	5.33	.060	1.69	.098	1.39
0	∞	undefined	1.000	0.	1.	0.00

[a] K: K_x, K_θ, or K_χ. K_x in kcal/(mol·Å^2), K_θ and K_χ in kcal/(mol·rad^2). The 1 M reference state for the position restraint has $V_o = 1660$ Å^3.

Dihedral Angle Restraint. The dihedral angle, χ to which the restraint is applied is defined by two planes: (1) the plane containing the reference points $\mathbf{X}_j$, $\mathbf{X}_i$, and a third reference point (atom or center of mass of several atoms) $\mathbf{X}_k$, and (2) the plane containing the points $\mathbf{X}_j$ and $\mathbf{X}_i$ and a reference unit vector $\mathbf{e}_\chi$. As a restraint potential we use the following expression:

$$U_{\text{r},\chi} = (K_\chi/2)\chi^2 \tag{B11}$$

This restricts the selected dihedral angle to an effective range which is less than the value of 2π for the free molecule. The partition function is given by

$$z_\chi = \int_{-\pi}^{\pi} \exp[-(K_\chi/2)\chi^2/kT]\, d\chi \tag{B12}$$

$$\Delta A°_{\text{r},\chi} = -kT \ln(z_\chi/2\pi) \tag{B13}$$

For large values of K_χ

$$z_\chi \cong [2\pi kT/K_\chi]^{1/2} \qquad (K_\chi \gg kT) \tag{B14}$$

Molecular Symmetry. A rotationally symmetric ligand molecule can bind to the macromolecule in a number, z_s, of physically indistinguishable orientations. Application of the body restraint potential effectively breaks the symmetry of the molecule and artificially restricts the molecule to a single one of these. Equations B9 and B12 together overestimate the effect of the restraint by a factor z_s. This gives rise to an additional term in the restraint free energy equal to

$$\Delta A°_s = -kT \ln z_s \tag{B15}$$

For benzene, $z_s = 12$.

Restraints on Internal Rotation. Changes in conformation by rotation about single bonds that would alter the geometry of the binding site or the shape of the ligand molecule tend to have an adverse effect on the reproducibility of molecular transformation calculations. If these are a problem, then it is advisable to restrict dihedral angles, ρ, for internal rotation of macromolecule and protein that are involved in these changes of conformation with potentials of the form

$$U_{\text{r},\rho} = (K_\rho/2)[1 - \cos(\rho - \rho_o)] \tag{B16}$$

Since these internal rotation restraints, in contrast to the terms of the body restraint potential, affect the internal energy of the restrained molecules, the corresponding restraint free energies must be evaluated by additional simulations.[18]

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