Convergence Properties of Free Energy Calculations: α-Cyclodextrin Complexes as a Case Study

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Abstract: By considering all possible mutations among four para-substituted phenols, *p*-chlorophenol, *p*-methylphenol, *p*-cyanophenol, and *p*-methoxyphenol, which bind as inclusion compounds in α-cyclodextrin, the convergence properties of thermodynamic integration free energy calculations using slow growth as compared to numerical quadrature are investigated and interpreted in terms of structural and dynamical properties of the molecular system. It is shown that a systematic increase in the calculated hysteresis can be expected with increasing simulation time in slow-growth calculations if the system is perturbed faster than the rate at which the various states that make up the equilibrium ensemble are sampled. Using numerical quadrature the effects of nonequilibrium can be largely separated from the effects of insufficient sampling. It is shown, however, that the apparent degree of convergence when using numerical quadrature does not necessarily reflect the accuracy of the calculation. The utility of formulating closed cycles in both the bound and unbound states as a means of determining the minimum error in a given calculation is demonstrated. The effects of the choice of pathway and of the choice of integration scheme on convergence within closed cycles are also discussed. Finally, the quality of the force field used and the relative importance of the force field as opposed to sampling considerations are assessed by comparing the estimated free energy differences to experimental data. It is shown that a meaningful appraisal of a specific force field cannot be made independent of sampling considerations. A modification to the GROMOS force field that improved the agreement between the calculated and experimental free energies for the mutation of *p*-chlorophenol to *p*-methylphenol is also proposed.

Introduction

As a global property the absolute free energy of a system is dependent on the total phase space that is available to that system. Complete sampling of phase space is not possible except for the simplest of model systems. Free energy calculations on systems of biological interest have, thus, concentrated on the determination of the relative free energy between two closely related states. In this way only differences between the two states need be considered. Irrelevant regions of phase space can be ignored. Although this greatly simplifies the problem, it does not eliminate the need to effectively sample relevant regions of phase space.[1] This is especially true if entropic contributions to the total free energy are to be correctly estimated. As the number of free energy calculations in the literature grows, it is becoming clear that simulation times in excess of many hundreds of picoseconds (ps) are normally required to obtain reliable free energy estimates even on simple systems.[2,3] It is also clear that a single simulation protocol is not appropriate for all systems and that the reliability of a given calculation, excluding force field quality, is primarily determined by the effectiveness of sampling of configurational space.[4] Thus, convergence properties in free energy calculations have become a major question.

The general problem of convergence in free energy calculations has been addressed in a number of recent papers which attempt to analyze the origin of various sources of error.[1-7] In this paper we consider in detail the convergence behavior of a specific system. By considering only closed thermodynamic cycles, our aim is to isolate and analyze effects due to insufficient sampling and/or lack of equilibrium from those due to force field and other system parameters.

The phenomenon chosen as a test case is the binding of guest compounds to α-cyclodextrin. Cyclodextrins (CD) are a family of cyclically closed, torus-shaped oligosaccharides consisting of six (α), seven (β), or eight (γ) glucose units linked by α(1–4) bonds. Their most interesting property is the inclusion of guest molecules in their annular cone-shaped cavities. This inclusion of compounds by CDs has been studied in great detail using a variety of techniques including NMR and crystallography. The system is of interest both for specific applications such as the transport of small molecules and as a simple model system for understanding basic principles of recognition and binding. The nature of the guest molecule can vary from hydrophilic to hydrophobic in character. The primary condition for inclusion is that they fit spatially into the central cavity which ranges in size from 0.5 to 0.7 nm in diameter. The cavity itself is hydrophobic. The rims of the cavity are, however, lined with hydrophilic OH groups. It has been previously shown that molecular dynamics simulations of crystalline α-CD using the GROMOS force field[8] yield very good agreement with X-ray

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and neutron diffraction data.[9] The experimental positions of α-CD atoms are reproduced to within 0.025 nm. In addition, features such as hydrogen bond flip-flops and three-centered hydrogen bonding are also well reproduced in the simulations.[10,11] Structural and dynamical differences between crystalline and aqueous α-CD's have been studied[12] as has the conformational behavior of complexes of α-CD with *p*-chlorophenol and *p*-hydroxybenzoic acid in water.[13] We may be confident that the force field is capable of describing the structural and dynamical properties of the system in detail and that these properties are well understood from previous work.

In the current study we investigate the binding to α-CD of four para-substituted phenols, *p*-chlorophenol, *p*-methylphenol, *p*-cyanophenol, and *p*-methoxyphenol, for which experimental binding data is available. By determining the change in free energy for all possible cyclic mutations among the four guest molecules, we consider, in addition to testing the ability of the force field to reproduce the experimental results, the convergence properties of the calculations.

Theory

The difference in free energy between two states, A and B of a system whose atomic interaction functions are denoted by V_A and V_B, can be determined using simulation techniques if the effective potential energy function V is made a function of a coupling parameter λ such that $V(\lambda_A) = V_A$ and $V(\lambda_B) = V_B$. Since the force field $V(\lambda)$ is dependent on λ, the free energy of the system is also dependent on λ. Using statistical mechanics it can be readily shown that the change in free energy ΔG_{AB} may be expressed as the following integral (e.g., see refs 14 and 15):

$$\Delta G_{AB} = \int_{\lambda_A}^{\lambda_B} \left\langle \frac{\partial V}{\partial \lambda} \right\rangle_\lambda d\lambda \qquad (1)$$

where the brackets $\langle ... \rangle_\lambda$ denote averaging over an equilibrium ensemble generated with the potential energy function $V(\lambda)$. If molecular dynamics simulations are used to generate the ensemble of molecular configurations, the integral (eq 1) can be evaluated in different ways. The coupling parameter λ can be made a function of time such that it slowly changes from λ_A to λ_B during the course of the simulation:

$$\Delta G_{AB} = \sum_{t_i=t_A}^{t_B-\Delta t} \left.\frac{\partial V}{\partial \lambda}\right|_{\lambda(t_i)} [\lambda(t_i+\Delta t) - \lambda(t_i)] \qquad (2)$$

The time points in the simulation separated by Δt are indicated by t_i, where $\lambda = \lambda_A$ at $t = t_A$ and $\lambda = \lambda_B$ at $t = t_B$. This procedure is commonly referred to as continuous change or slow growth. Alternatively, the ensemble average in (1) may be evaluated at a number of discrete λ-values by performing a separate simulation at each chosen λ-value λ_i. These ensemble averages can then be used to determine the integral by numerical quadrature:[16]

$$\Delta G_{AB} = \sum_{i=1}^{M} \left\langle \frac{\partial V}{\partial \lambda} \right\rangle_{\lambda_i} W_i \qquad (3)$$

The points λ_i (i = 1, 2, ..., M) and weights W_i can be chosen according to any appropriate numerical quadrature method. This procedure will be referred to as thermodynamic integration by numerical quadrature. In the current work both formulas 2 and 3 were used in order to compare the convergence properties of the two methods. In the case of formula 3, the λ-values and weights W_i were chosen in accordance with Gauss–Legendre quadrature, Simpson's rule, or a trapezoidal approximation as noted in the text.

Material and Methods

(i) **Simulation Setup.** All simulations were performed using the GROMOS package of programs in conjunction with the standard GROMOS force field.[8] In this force field, nonpolar hydrogen atoms are treated as united atoms together with the carbon atoms to which they are attached. Polar hydrogens are treated explicitly and experience electrostatic but no Lennard–Jones interactions. The α-CD molecule (Figure 1 (top)) was modeled by 36 carbon atoms, 30 oxygen atoms, and 18 hydroxyl hydrogen atoms. The phenol group (Figure 1 (bottom)) was modeled by six aromatic carbons, one hydroxyl oxygen, and one hydroxyl hydrogen. Where available standard GROMOS parameters were used to describe the interactions between atoms. Additional parameters for α-CD were taken from Koehler et al.[9] The parameters for the chloro group were taken from a personal communication with W. L. Jorgensen. Cyano parameters were taken from Picken et al.[17] The charges of the cyano group were modified such that there was no net charge on the substituent. The GROMOS parameters for the methoxy group were modified such that the methyl group lies in the plane of the ring as in anisole.[18] This was achieved by reducing the GROMOS 1–4 interaction from the methyl group (C7) to carbons C3 and C5 of the ring by a factor of 2. A summary of the interaction parameters is given in Tables 1 and 2.

The starting structure for the simulations of the α-CD complexes was taken from the crystal structure of α-CD complexed with *p*-hydroxybenzoic acid.[19] The *p*-hydroxybenzoic acid was modified to the appropriate guest molecule and the system energy minimized. The complex and the isolated guest molecule were then placed at the centers of two truncated octahedrons, both with a box length of 3.12 nm. For the complex this corresponded to a closest distance of a solute atom to the box wall of 0.8 nm or approximately three water solvation shells. Each box was filled with SPC (simple point charge)[20] water molecules, and all water molecules that lie outside the box or whose oxygen atom lies within 0.23 nm of a non-hydrogen solute atom were removed. The final systems contained 508 and 544 water molecules for the complex and the isolated guest molecule, respectively. Each system was first energy minimized to relax the water–water contacts while keeping the solute atoms fixed, then the entire system was minimized to obtain the starting structure for the dynamics calculations. All calculation were performed using periodic boundary conditions at a temperature of 293 K and a pressure of 1 atm. The temperature and pressure were maintained by weak coupling to an external bath.[21] The relaxation times were 0.1 and 0.5 ps, respectively. Bond lengths were held fixed using the SHAKE algorithm[22] with a geometric tolerance of 10^{-4}. The integration time step was 2 fs. The cutoff radius for all nonbonded interactions was 1.0 nm. Interactions were calculated using a pairlist which was updated every 10 steps. Each complex was equilibrated for 20 ps before starting the free energy calculations. The isolated guest molecules were equilibrated for 10 ps before starting the free energy calculations.

(ii) **Perturbations.** Two sets of perturbations were performed. To test the convergence properties of slow-growth calculations using (2), the

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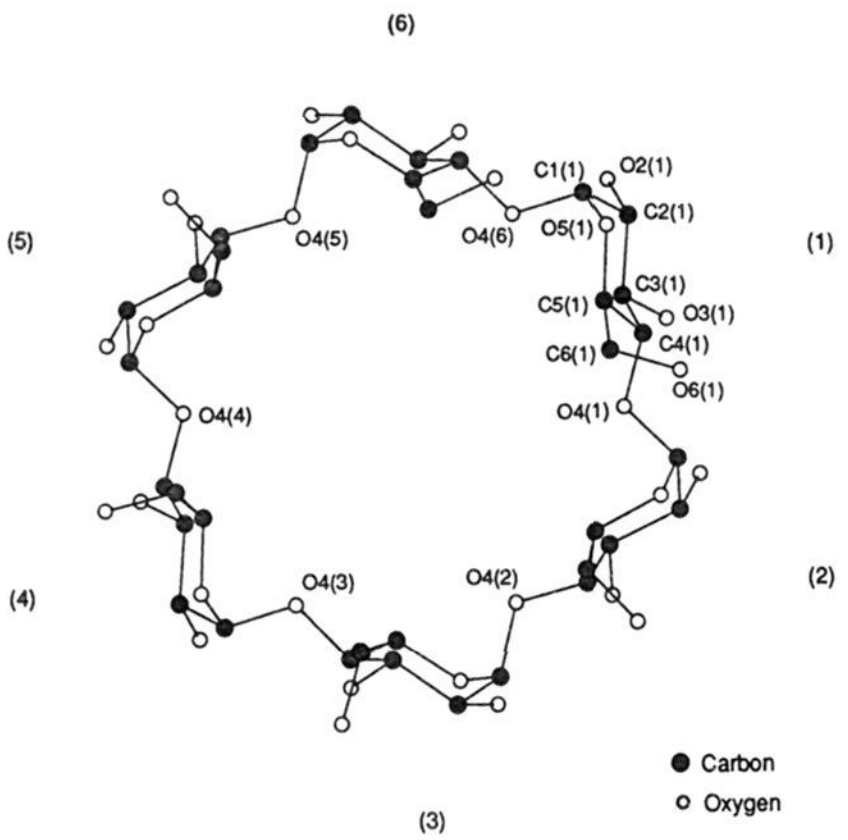

Figure 1. Structures of (top) α-cyclodextrin, showing the numbering scheme of the cyclodextrin, and (bottom) the guest molecule *p*-methoxyphenol together with the cyclodextrin, showing the orientation of the guest when it is inserted into the cyclodextrin and the numbering scheme of the guest molecule.

mutation from *p*-chlorophenol to *p*-methylphenol was investigated. These simulations were performed using *p*-chlorophenol as a starting topology. To test the convergence of closed cycles involving *p*-cyanophenol and *p*-methoxyphenol, dummy atoms were included in the topology files of *p*-chlorophenol and *p*-methylphenol. The mass, bond, angle, and dihedral parameters of the dummy atom were set identical to those of the N atom in *p*-cyanophenol. The dummy atom, however, experienced no nonbonded interactions. The inclusion of the dummy atoms even in the mutation from *p*-chlorophenol to *p*-methylphenol was important to ensure that the initial and final states of the system in each cycle were identical. Contributions to the free energy from changes in constrained bond lengths were not determined.[15,23]

The partial derivatives $\partial V/\partial\lambda$ used to calculate the free energy change were determined analytically assuming a linear dependence of the effective potential on the coupling parameter λ

$$V(\lambda) = (1 - \lambda)V_A + \lambda V_B \qquad (4)$$

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Table 1. Summary of the Interaction Parameters Used in the Simulations

compd	atom	partial charge (e)	$[C6(i,i)]^{1/2}$ [a] [(kcal mol^{-1} Å^6)$^{1/2}$]	$[C12(i,i)]^{1/2}$ [a,b] [(kcal mol^{-1} Å^{12})$^{1/2}$]	mass (amu)
phenol	H1	0.398	0.0	0	1.0080
	O1	–0.548	23.25	421/600	15.9994
	C1	0.15	23.65	898	12.0110
	C2, C3, C5, C6	0.0	36.30	1901	13.0190
	C4 substituent	[c]	23.65	898	12.0110
p-Cl	Cl	–0.14	45.77	1912	35.4530
p-CH_3	CH_3	0.0	46.06	2500	15.0350
p-CN	N	–0.368	24.13	636/900	14.0067
	C	0.184	23.65	898	12.0110
p-OCH_3	CH_3	0.18	46.06	2500	15.0350
	O	–0.36	23.25	421/600	15.9994

[a] Lennard–Jones parameters $C6(i,j)$ and $C12(i,j)$ were obtained using the following combination rules: $C_6(i,j) = [C_6(i,i)]^{1/2}[C_6(j,j)]^{1/2}$ and $C_{12}(i,j) = [C_{12}(i,i)]^{1/2}[C_{12}(j,j)]^{1/2}$. [b] To mimic the effects of hydrogen bonds, the C12 parameters of polar atoms were increased for hydrogen bond donor and acceptor pairs. This is the second value in this column. [c] The charge on the C4 atom was opposite that of the parasubstituent so that a neutral group was obtained.

Table 2. Force Field Parameters between Bonded Atoms of the Guest Molecule

compd	bond	K_b (kcal mol^{-1} Å^{-2})	b_o (Å)
phenol	C—C	1000	1.39
	C1—O1	900	1.36
	O1—H1	750	1.00
p-Cl	C4—Cl	600	1.74
p-CH_3	C4—CH_3	800	1.53
p-CN	C4—C	900	1.42
	C≡N	1200	1.125
p-OCH_3	C4—O	900	1.36
	O—CH_3	600	1.43

compd	angle	K_θ (kcal mol^{-1} rad^{-2})	θ_0 (deg)
phenol	C—C—C	100	120.0
	C—C—O1	100	120.0
	C—O1—H1	95	109.5
	C—C4—X	100	120.0
p-CN	C4—C≡N	120	180.0
p-OCH_3	C4—O—CH_3	80	120.0/109.5[a]

compd	improper dihedral	K_ξ (kcal mol^{-1} rad^{-2})	ξ_0 (deg)
phenol	C—C—C—C	40.0	0.0
	C—O1—C—C	40.0	0.0
	C—X—C—C	40.0	0.0

compd	dihedral	K_ϕ (kcal mol^{-1})	δ (deg)	n
phenol	C—C—O1—H1	1.7	180	2
p-OCH_3	C3—C4—O—CH_3	1.7	180	2

[a] Value used by van Helden.[29]

for the dihedral angle and nonbonded interaction terms and a nonlinear dependence for the bond angle and improper dihedral angle terms.[14] A linear dependence is normally appropriate for bonded and electrostatic interactions. Problems in integrating the Lennard–Jones contribution to the free energy can, however, arise when atoms are created or annihilated.[24] In such cases a nonlinear dependence may be more appropriate.

Results and Discussion

(1) Slow Growth. Since the (Gibbs) free energy G is a state function, the total change in free energy, ΔG, for any cyclic mutation is zero. This is the basis of the concept of a thermodynamic cycle and is the primary means by which the internal consistency of free energy calculations can be checked independent of system and force field considerations. A common method to assess a given free energy simulation is to perform the

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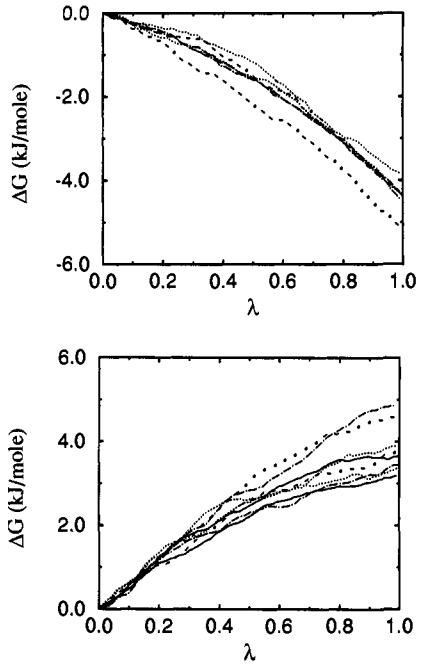

Figure 2. Change in free energy (kJ mol^{-1}) as a function of the coupling parameter λ determined by slow-growth thermodynamic integration for the mutation of *p*-chlorophenol (λ = 0) to *p*-methylphenol (λ = 1) (top) in water and (bottom) complexed with α-cyclodextrin. The different lines styles represent simulations of different lengths: 25 (···), 50 (- - -), 100 (—·—), and 300 ps (—). The curves have been shifted such that ΔG = 0 at λ = 0 for ease of comparison.

Table 3. Calculated Free Energies for the Mutation from *p*-Chlorophenol to *p*-Methylphenol Using Slow Growth[a]

	water		α-CD		α-CD – water
t (ps)	ΔG	hysteresis	ΔG	hysteresis	ΔΔG
25	−4.33	0.25	3.68	0.51	8.0
50	−4.90	1.22	4.16	0.77	9.1
100	−4.50	0.21	4.18	1.48	8.7
300			3.42	0.51	7.9[b]

[a] Values are in kJ mol^{-1}. [b] Value calculated using the ΔG for water calculated over 100 ps.

mutation in the forward and reverse directions. If the process is performed in a reversible manner, the overall free energy change will be zero, no net work having been done on the system. If the process is not performed in a reversible manner, excess work will be done during the mutation which will manifest itself as a hysteresis or difference between the free energy calculated for the forward and reverse directions. In such cases it is often assumed that the average value of the forward and reverse processes represents the true value of ΔG and that the magnitude of the hysteresis provides an estimate of the error in the calculation.

Figure 2 shows the change in free energy as a function of the coupling parameter λ for the mutation of *p*-chlorophenol to *p*-methylphenol in water (a) and in α-CD (b). Curves for the respective reverse mutations of *p*-methylphenol to *p*-chlorophenol are shown on the same plots. For ease of comparison the curves for the reverse mutation have been shifted such that ΔG = 0 when λ = 0. The free energy was calculated using (2) (slow growth). A series of simulations were performed in each direction. The simulations differ only in the rate of change of λ as a function of time and are distinguished by different line styles. The average changes in free energy for the forward and reverse mutations and the hysteresis are given in Table 3. It should be noted that Figure 2 was generated by summing values of $\partial V/\partial\lambda$ written only every 20 steps during the course simulation, and because of this, there are apparently slight differences between Figure 2 and Table 3. It can be seen on inspection of Figure 2 (top) and Table 3 that the hysteresis when the mutation from *p*-chlorophenol to *p*-methylphenol was performed over 25 ps (···) was 0.25 kJ mol^{-1}. When the same perturbation was performed more slowly, over 50 ps (- - -), the hysteresis increased to 1.22 kJ mol^{-1}. Further increasing the length of the simulation to 100 ps (—·—) resulted in a decrease in the magnitude of the hysteresis to. 0.21 kJ mol^{-1}. The effect of decreasing the rate of change of λ on the magnitude of the hysteresis follows a similar pattern in the case of the α-CD complex shown in Figure 2 (bottom). In this case the hysteresis increases systematically as the length of the simulation is increased from 25 to 50 to 100 ps. A simulation of 300 ps (—) was required to reduce the hysteresis to the same value as obtained for the 25-ps simulation.

It is clear from Figure 2 that the form of the curve and the hysteresis do not directly indicate the adequacy of sampling in slow-growth free energy calculations nor the magnitude of the intrinsic error. Such plots simply indicate the degree to which the process is reversible. Reversibility is dependent on two factors: (1) the relaxation rate of the system in response to the specific mutation and (2) the rate that states which make up the equilibrium ensemble of the system are sampled. If the rate of perturbation is much slower than both the relaxation rate of the system and the rate at which different equilibrium states are sampled, then the process will be reversible and the system will be adequately sampled (ΔG = 0). If the rate of perturbation is much faster than the rate of relaxation, the system will not respond during the perturbation but remain trapped in a local state which is quickly sampled. In this case the mutation will again be reversible. The nonlocal states of the system, however, will not be adequately sampled, and the calculated free energy will be unreliable. Most cases do not correspond to either extreme. In such cases, the process is not reversible and some degree of hysteresis is observed.[5]

It is important to distinguish between the effects of performing a perturbation faster than the relaxation rate of the system as opposed to insufficient sampling of the equilibrium ensemble. By definition the equilibrium state of a system corresponds to the lowest free energy state. If the perturbation is not performed significantly slower then the relaxation rate of the system, the system will in effect lag behind the changing Hamiltonian.[25] The system will not correspond to the lowest free energy state, and the calculated change in free energy will be systematically overestimated. If the system is equilibrated before performing the reverse mutation, the free energy in this direction will also be overestimated. In this case the true value for ΔG will lie between the two extremes. If the process of relaxation is similar, the amount by which the free energy is overestimated in both directions may also be similar and the true value of ΔG may lie close to the average of the forward and reverse values. However, if both the forward and reverse simulations do not start from equilibrium configurations or the relaxation processes are not similar, the average value of ΔG has little meaning.

Superimposed on the effect of the relaxation time of the system are effects due to insufficient sampling of the equilibrium ensemble. An equilibrium ensemble is not in general characterized by a single state but is instead a collection of related states populated according to their Boltzmann weights. Since in a molecular dynamics simulation it is only possible to deal with a single molecule, or at most a small number of molecules, at one time, it is necessary to approximate the ensemble averages in (1) and (3) by time averages. The values of $\partial V/\partial\lambda$ are thus correlated in time. Unless the perturbation is performed much slower than the rate of sampling of different states, the calculated free energy will depend on the precise starting configuration and the trajectory followed during the simulation. This has the effect of introducing

(25) Wood, R. H. *J. Phys. Chem.* **1991**, *95*, 4838–4842.

statistical errors into the calculation.[26] If the perturbation is performed very rapidly, only a small number of states within the ensemble will be sampled and the overall fluctuations will be small. As the perturbation is performed more slowly, progressively more states will be sampled during the simulation and the magnitude of the fluctuations in $\partial V/\partial\lambda$ will increase. Only as sampling of a representative ensemble is approached will the effects of the fluctuations in $\partial V/\partial\lambda$ be suppressed and a reliable average obtained.

The mutation from *p*-chlorophenol to *p*-methylphenol represents a minor perturbation to the system, primarily resulting from the removal of the dipole along the C–Cl bond. The Lennard–Jones, bond, angle, and dihedral angle force field parameters remain largely unchanged. Assuming both compounds bind in a similar fashion inside the cavity, very little structural rearrangement is expected. The relaxation time of the system with respect to this mutation is expected to be short and to be dominated by the time required for the surrounding water molecules to reorientate. The dipolar relaxation time of SPC[20] water is in the order of 6–8 ps.[27] Even when the mutation is performed over the shortest time (25 ps) it is to be expected that the system will remain close to equilibrium. This expectation is borne out in Figure 2, where it can be seen that by starting from the same initial structure there is no systematic decrease in the free energy for the forward mutation as the rate of change in λ is decreased for either the guest free in solution or for the complex. The variation between the runs is primarily due to insufficient sampling of the equilibrium ensemble. Although the system may respond to the change in dipole when the mutation is performed over 25 ps, this is insufficient to sample alternative states that make up the equilibrium ensemble. For example, due to the imposition of boundary conditions, the calculated free energy will depend on the orientation of the molecule inside the box. When the mutation is performed over 25 ps only a small number of the possible alternative states are sampled. The end state strongly reflects the initial state and a small hysteresis is observed. However, when the mutation is performed over 50 ps, there is a partial sampling of alternative states leading to an increase in the hysteresis. To understand why for the complex at least 300 ps, considerably longer than many published free energy studies, is required to obtain a reliable estimate of the free energy change for such a simple mutation, it is instructive to consider the variety of substates that constitute the equilibrium ensemble in this case. The structural behavior of α-CD complexes has previously been described in detail by van Helden et al.[13] This work showed that there were three primary modes of motion of the guest molecule relative to the α-CD: (a) rotation of the guest inside the α-CD about an axis perpendicular to the plane of the α-CD ring, (b) out-of-plane motion of the guest between the center of the cavity and the outer ring of the α-CD, and (c) tilting of the plane of the guest molecule with respect to the plane of the α-CD. An illustration of the range of possible configurations of the complex is shown graphically in Figure 3, which shows the superposition of five snapshots from the trajectory of the guest molecule superimposed on a representative α-CD structure. The time dependence of the motions are shown in Figure 4. Insertion of the guest molecule into the α-CD results in an elliptical distortion of the cavity. Figure 4 (top) shows the diagonal distances between glycosidic oxygen atoms [O4(1)–O4(4) (—), O4(2)–O4(5)(- - -), and O4(3)–O4(6)(- - -)] as a function of simulation time, indicating rotation of the guest inside the cavity.[13] The distances vary between 0.70 and 0.95 nm. Complete rotation of the guest molecule requires 50–100 ps. The symmetry of the α-CD means, however, that the effects of rotation average on the order of 20–30 ps. The periods of the out-of-plane and tilting motions are considerably longer. Figure 4 (center) shows the distance between the center of mass of the guest molecule and the center of mass of the α-CD as a function of simulation time. A distance of 0.2 nm or less (dashed line) indicates that the guest molecule is included inside the cavity. A larger distance indicates that the guest molecule has moved out toward the rim. Figure 4 (bottom) shows the time dependence of the angle between the plane of the α-CD ring and that of the guest molecule. From Figure 4 (center and bottom) it can be seen that periods in excess of 100 ps would be required to obtain effective averaging of the out-of-plane and tilting motions.

In slow-growth calculations the system is continuously changed throughout the simulation. Therefore, the sum given in (2) must approximate an equilibrium ensemble over a small range of

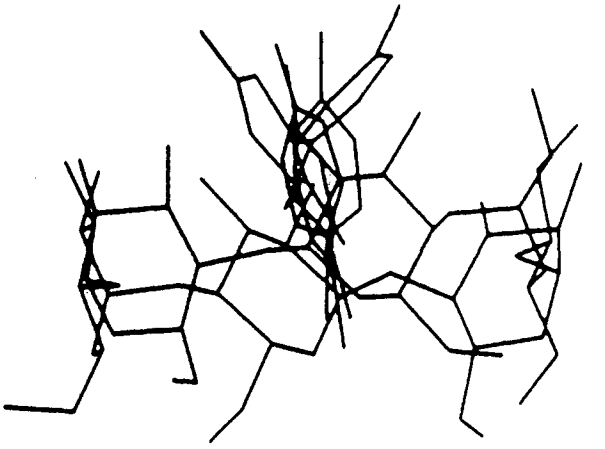

Figure 3. Superposition of five snapshots from a trajectory of *p*-chlorophenol complexed to α-cyclodextrin showing the range of motion of the guest within the host molecule.

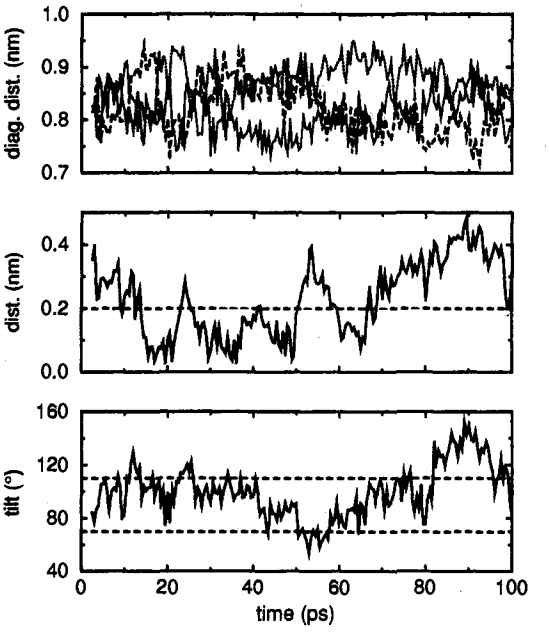

Figure 4. Illustration of the motions of the guest molecule with respect to the α-cyclodextrin host: (top) diagonal distances between glycosidic oxygen atoms [O4(1)–O4(4)(—), O4(2)–O4(5)(- - -), and O4(3)–O4(6)(···)] during a simulation of *p*-chlorophenol complexed to α-cyclodextrin, indicating the rotational motion of the guest within the host cavity; (center) distance between the center of the benzene ring of the guest and the center of the plane of the α-cyclodextrin as a function of simulation time (A distance of less than 2 Å indicates that the guest is fully included within the cavity. Larger distances correspond to the guest having moved toward the edges of the rim of the α-CD molecule.); (bottom) angle between the plane of the benzene ring of the guest molecule and the plane of the α-cyclodextrin as a function of simulation time, indicating tilting motion of the guest.

(26) Straatsma, T. P.; Berendsen, H. J. C.; Stam, A. J. *Mol. Phys.* **1986**, *57*, 89–95.
(27) Smith, P. E.; van Gunsteren, W. F. *J. Chem. Phys.* **1994**, *100*, 3169–3174.

Table 4. Gauss–Legendre Quadrature[a]

three-point		five-point		five-point atom annihilation	
λ_i	W_i	λ_i	W_i	λ_i	W_i
0.1127	0.2778	0.0469	0.1185	0.6005	0.2528
0.5000	0.4444	0.2308	0.2393	0.7832	0.1354
0.8873	0.2778	0.5000	0.2844	0.8909	0.0845
		0.7692	0.2393	0.9572	0.0496
		0.9531	0.1185	0.9920	0.0206

[a] λ_i values and weight factors W_i (eq 3) for three- and five-point integration using a linear dependence on λ as well as transformed values for five-point integration using a dependence of $\lambda^{1/6}$.

λ-values. The rate of change in λ must be significantly slower than the sampling of configurational space. With this in mind we would conclude from Figure 2 that the increase in hysteresis in going from 25 to 100 ps, and the decrease as the total simulation time is increased to 300 ps, for the α-CD complex relates primarily to the sampling of rotational conformers and that much longer simulations would be required to sufficiently sample the out-of-plane and tilting modes. In 25 ps, little or no rotational motion of the guest in the complex is sampled. The complex remains effectively frozen and small hysteresis is observed. When the mutation is performed over 300 ps, the rotational motion can be fully sampled and again the observed hysteresis is small. It is possible, however, that if the length of the simulation was increased to 600 or 1000 ps the observed hysteresis would again increase. In this case it might be argued that it would be beneficial to "freeze" the low-frequency motions by restraining the molecule to a single well-characterized conformational state. This would clearly reduce the hysteresis and the time to convergence. The low-frequency modes, however, form part of the overall entropy of the system. Thus, removing such modes may significantly perturb the calculated free energy, introducing errors that do not cancel within the context of a thermodynamic cycle. The resulting net free energy change would, therefore, be unreliable, and restraining the system in this manner is not recommended.

(2) Numerical Quadrature. As an alternative to slow-growth calculations, one may perform independent simulations at a number of λ-values and determine the integral in (1) by numerical quadrature. This has the advantage that an ensemble average can be obtained at each point rather then being approximated by a continuous sum. Thus, the effects of incomplete equilibria can be largely separated from the effects of incomplete sampling of the ensemble.[28] Nevertheless, the method has two disadvantages. First, it must be assumed that the free energy is a smooth function of λ, and second, a sufficient number of points must be taken to ensure that the integral is evaluated to the required precision. In the case of the mutation from *p*-chlorophenol to *p*-methylphenol, it can be seen from the slopes of the curves in Figure 2 that the sign of $\partial V/\partial\lambda$ is constant for $\lambda = 0$ to 1 and that there is little change in the magnitude. A small number of points should, therefore, be sufficient to accurately determine the integral numerically. To confirm this expectation the results of taking three and five Gaussian quadrature points were compared. The appropriate λ-values and weights are given in Table 4. The starting configurations for the simulations at fixed λ-values were taken from the 300-ps slow-growth trajectory of the α-cyclodextrin complex and the 100-ps slow-growth trajectory of the isolated guest molecule. Each system was equilibrated for a further 20 ps in the case of the complex and 10 ps in the case of the isolated guest. A plot of $\partial V/\partial\lambda$ for $\lambda = 0.5$ for the complex as a function of time is shown in Figure 5. The system had been simulated over 150 ps of the slow-growth mutation from $\lambda = 0$ to 0.5 and by a further 20 ps with λ fixed at 0.5. The dashed line shows the instantaneous derivative while the solid line in Figure 5 shows the accumulative average. Despite the extensive equilibration, large

(28) Straatsma, T. P.; McCammon, J. A. *J. Chem. Phys.* **1991**, *95*, 1175–1188.

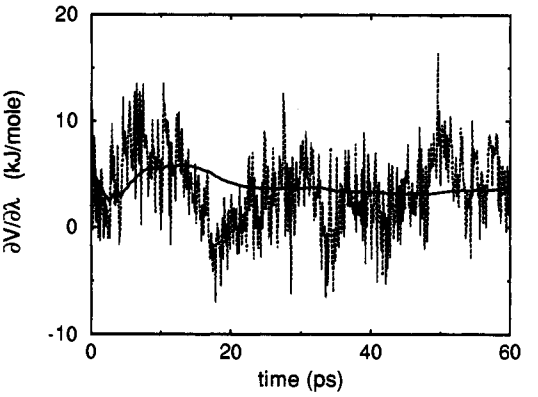

Figure 5. $\partial V/\partial\lambda$ (kJ mol^{-1}) at $\lambda = 0.5$ as a function of simulation time for the mutation of *p*-chlorophenol to *p*-methylphenol complexed to α-cyclodextrin. The dashed line corresponds to the instantaneous derivative, and the solid line, to the accumulative average.

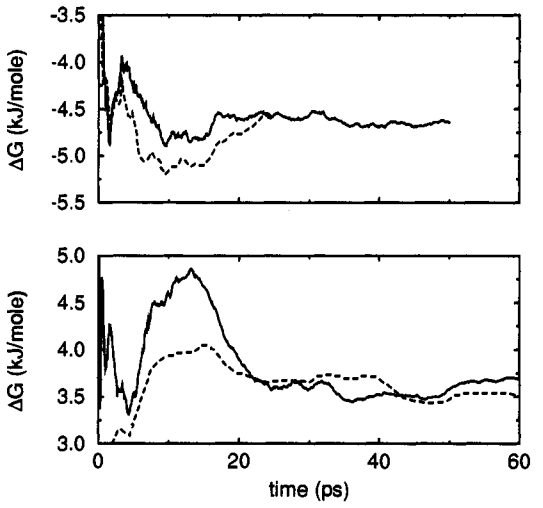

Figure 6. Change in free energy (kJ mol^{-1}) as a function of sampling time for the mutation of *p*-chlorophenol to *p*-methylphenol (top) in water and (bottom) complexed to α-cyclodextrin. The solid lines correspond to determining the integral in (1) using three Gaussian points, and the broken lines, using five Gaussian points.

fluctuations in $\partial V/\partial\lambda$ are observed. Fluctuations on the order of 10 kJ mol^{-1} that occur on a sub-picosecond time scale are superimposed on slower modes with a similar magnitude. The rapid fluctuations correspond to angle vibrations and short time librations of the molecule. The longer time fluctuations which occur with a period on the order of 10–20 ps most likely correspond to the rotation of the guest molecule inside the α-CD. It is clear that at least 30 ps is required for the average value of $\partial V/\partial\lambda$ to converge in this case. The convergencies of the integrals using three and five quadrature points for the free guest and the complex are shown in Figure 6, top and bottom, respectively. From Figure 6 it can be seen that the value of the integral determined using three (—) and five (- - -) Gaussian points converges to within 0.2 kJ mol^{-1} of the final value within 20 ps in the case of the free guest and 40 ps in the case of the α-CD complex. It should be noted, however, that the same simulation for $\lambda = 0.5$ was used to calculate the integral using both three and five Gaussian points. For this reason, the curves shown in Figure 6 appear correlated and the apparent convergence may not be representative of two independent runs.

(3) Convergence of Closed Cycles. The mutation of *p*-chlorophenol to *p*-methylphenol represents a minor perturbation to the system. To test the convergence of mutations involving the creation of new atoms and changes in angle and dihedral angle parameters, we considered all possible mutations among *p*-chlorophenol, *p*-methylphenol, *p*-cyanophenol, and *p*-methox-

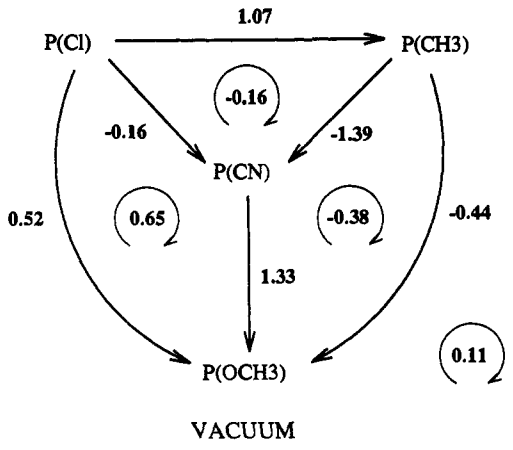

Figure 7. Change in free energy (kJ mol^{-1}) for all possible mutations among *p*-chlorophenol, *p*-methylphenol, *p*-cyanophenol, and *p*-methoxyphenol in vacuum. The arrows indicate the direction of the mutation. The residual free energies for each of the four three-membered closed cycles are also indicated.

Table 5. Ensemble Averages of the Partial Derivative, $\langle \partial V/\partial \lambda \rangle_\lambda$, in Vacuum at Selected λ-values[a]

mutation	λ = 0.0	λ = 0.25	λ = 0.5	λ = 0.75	λ = 1.0	$\Delta G_{5\,point}$	$\Delta G_{9\,point}$
Cl → CH_3	0.94	1.02	1.08	1.12	1.17	1.07	1.07
Cl → CN	–0.06	–0.12	–0.17	–0.21	–0.22	–0.59	–0.16
Cl → OCH_3	–46.29	–3.85	–1.28	7.94	30.80	0.14	0.52
CH_3 → CN	–1.34	–1.38	–1.40	–1.40	–1.40	–1.39	–1.39
CH_3 → OCH_3	–39.30	–6.19	–1.37	7.26	31.87	–0.49	–0.44
CN → OCH_3	–41.41	–3.72	1.59	10.17	27.31	1.24	1.33

[a] The integral over the interval λ = 0 to 1.0 for five and nine points using a Simpson's rule approximation are also shown. Values are in kJ mol^{-1}.

yphenol in vacuum, water, and in α-CD. The mutations were performed in vacuum to assess the importance of internal terms when determining the integral. Figure 7 shows each of the possible three-membered cycles together with the calculated free energy for each mutation and the sum for each closed cycle in vacuum. The free energy values were obtained using nine equally spaced λ-values, (λ = 0.0, 0.125, 0.25, 0.375, 0.5, 0.625, 0.75, 0.875, and 1.0) 20-ps equilibration, and 20-ps sampling. The results were integrated using a standard Simpson's rule approximation. From Figure 7 it can be seen that the internal contributions to the free energy for each mutation in vacuum are on the order of 1.5 kJ mol^{-1} or less and that each of the four three-membered cycles closes within 1 kJ mol^{-1}. The total free energy change for each mutation in vacuum is small. This does not mean, however, that the partial derivatives at each λ-value of the internal terms are also small and can be ignored in the integration of the free energy. Table 5 lists the partial derivatives for each of the mutations in vacuum at λ = 0.0, 0.25, 0.5, 0.75, and 1.0. The mutations of *p*-cyanophenol, *p*-chlorophenol, and *p*-methylphenol to *p*-methoxyphenol, which each involve modification of the angle parameters of the N atom (or dummy N atom), have large compensating contributions at λ = 0.0 and 1.0.

The results of performing the same mutations in water and complexed with α-CD are given in Table 6. Again results correspond to simulations performed at λ = 0.0, 0.125, 0.25, 0.375, 0.5, 0.625, 0.75, 0.875, and 1.0 by using, for the isolated guest molecule, an equilibration period of 10 ps and by sampling the partial derivatives $\langle \partial V/\partial \lambda \rangle_\lambda$ over the subsequent 30 ps at each λ-value. For the complex an equilibration period of 20 ps and a sampling time of 40 ps were used at each λ-value. Simpson's rule was used to integrate from λ = 0 to 1 using either all nine points or for comparison a subset of five points (λ = 0.0, 0.25, 0.5, 0.75, and 1.0). Using numerical quadrature it is important to consider both the convergence of the free energy with respect to the sampling time and whether the points chosen adequately determine the integral. The accumulative average of ΔG for each mutation using nine-point integration as a function of sampling time is shown in Figure 8. As expected the calculated ΔG converges faster for the isolated guest than for the complex. The results in water have converged to within 0.5 kJ mol^{-1} of the final answer after 20 ps. The results for the complex still show fluctuations between 1 and 2 kJ mol^{-1} after 30 ps. In water and in the complex the mutation from *p*-chlorophenol to *p*-methylphenol converges significantly faster than the other perturbations. This would suggest that considerably longer simulations than indicated in Figure 2 would be required to obtain precise values for the free energy changes in these cases using slow growth. When comparing Figures 2 and 8 it is important to note that Figure 8 shows the convergence when integrating over nine fixed values of λ after 10 (water) or 20 ps (α-cyclodextrin) of equilibration at each point. The averages after 20 ps shown in Figure 8 (top) correspond to a total simulation time of 270 ps. During slow-growth calculations the value of λ is constantly changed. Thus, in Figure 2 (top), the mutation of *p*-chlorophenol to *p*-methylphenol over 50 ps in water which shows the maximum hysteresis corresponds to an approximately 5-ps sampling for each 0.1 change in λ.

Although the integral is converged to within approximately 1 kJ mol^{-1} (Figure 8 (top)), the difference in taking nine as opposed to five points to determine the integral for the mutation of *p*-methylphenol to *p*-methoxyphenol in water is 4.8 kJ mol^{-1}. The reason for this is illustrated in Figure 9 which shows the partial derivative $\langle \partial V/\partial \lambda \rangle_\lambda$ at each of the λ-values used in the integration together with the curve which is integrated when using a five- (– – –) and nine- (- - -) point Simpson's rule approximation. The large positive values as λ approaches 0.0 result from the Lennard–Jones interaction and are a consequence of taking a linear dependence of the potential on the coupling parameter λ. Large fluctuations in the value of $\langle \partial V/\partial \lambda \rangle_\lambda$ at λ = 0.0 also adversely affect the convergence of the integral. Clearly the integration and convergence would be improved by taking additional points as λ approaches 0.0. Three additional simulations at λ = 0.005, 0.02, and 0.0625 were therefore performed for the mutations that involved atom creation. The integral from λ = 0 to 1.0 was determined using a trapezoidal approximation (the solid line in Figure 9), and the results are listed in Table 6. The values of the calculated free energies change significantly in comparison with taking nine evenly spaced points ranging from 1.3 kJ mol^{-1} for the mutation of *p*-chlorophenol to *p*-cyanophenol to 4.7 kJ mol^{-1} for the mutation of *p*-methylphenol to *p*-methoxyphenol in the complex. Although the sign of the correction is the same in water and in the complex, the magnitudes differ, leading to different net effects for each of the four mutations.

The total change in free energy for each of the four three-membered closed cycles is listed in Table 7. Results are shown using five, nine, and 12 points to perform the integration in water and in α-CD as well as for the difference α-CD – water. Values were derived from Table 6 using the entries for the nine-point integration to complete the 12-point cycles for the mutation of *p*-chlorophenol to *p*-methylphenol and the mutation of *p*-cyanophenol to *p*-methoxyphenol. Table 7 illustrates two important features. First, as the integration is improved by using more λ-values, which in turn also improves the sampling, the residual free energy decreases. Second, there is a compensation of errors leading to the difference in the free energy residual ($\Delta\Delta G_{\alpha\text{-CD–water}}$) being often smaller than the one for the α-CD complex.

(4) Effect of the Mutation and Integration Scheme on Cycle Closure. Using a five-point integration scheme, the cycles in water close with a maximum residual of 2.0 kJ mol^{-1} (Table 7). The difference between using five or 12 points to perform the integration for the mutation of *p*-methylphenol to *p*-methox-

Table 6. Calculated ΔG Values for Mutations between Four Para-Substituted Phenols Free in Solution and Bound to α-Cyclodextrin[a]

	water			α-CD			α-CD – water			
mutation	$\Delta G_{5\ point}$	$\Delta G_{9\ point}$	$\Delta G_{12\ point}$	$\Delta G_{5\ point}$	$\Delta G_{9\ point}$	$\Delta G_{12\ point}$	$\Delta\Delta G_{5\ point}$	$\Delta\Delta G_{9\ point}$	$\Delta\Delta G_{12\ point}$	$\Delta\Delta G_{expl}$[b]
Cl → CH_3	-3.2	-2.8		5.3	5.3		8.5	8.1		3.9
Cl → CN	-9.7	-12.1	-13.4	-7.2	-10.1	-11.4	2.5	2.0	2.0	1.0
Cl → OCH_3	-3.2	-8.2	-9.8	1.6	-5.6	-8.8	4.8	2.6	1.0	3.8
CH_3 → CN	-4.5	-8.8	-10.3	-12.5	-14.7	-16.6	-8.0	-5.9	-6.3	-2.9
CH_3 → OCH_3	1.3	-3.5	-6.8	-0.8	-7.7	-12.4	-2.1	-4.2	-5.6	-0.1
CN → OCH_3	4.7	4.6		3.9	3.8		-0.8	-0.8		2.8

[a] Values correspond to integrating from λ = 0 to 1 using five or nine equally spaced points and a Simpson's rule approximation or 12 points (λ = 0.0, 0.005, 0.02, 0.0625, 0.125, 0.25, 0.375, 0.5, 0.625, 0.75, 0.875, and 1.0) and a trapezoidal approximation. Values are in kJ mol^{-1}. [b] Experimental values taken from Hilbers et al.[30]

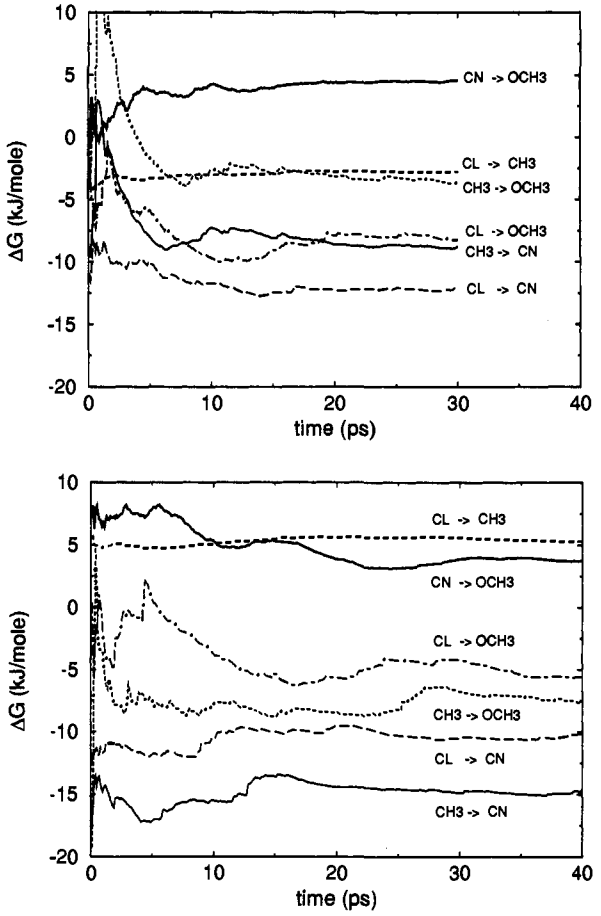

Figure 8. Change in free energy (kJ mol^{-1}) as a function of sampling time for each of the six mutations shown in Figure 9 (top) in water and (bottom) complexed with α-cyclodextrin. The integral from λ = 0 to 1 was determined using nine equally spaced points and a Simpson's rule approximation.

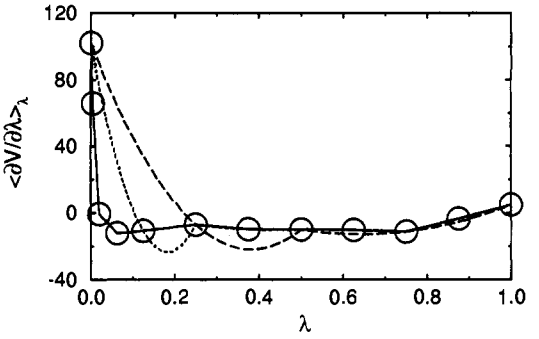

Figure 9. Illustration of the points integrated to determine the change in free energy (kJ mol^{-1}) for the mutation of *p*-methylphenol to *p*-methoxyphenol in water. The lines show the curves that are integrated when using a five- (- - -) and nine- (- · -) point Simpson's rule approximations and a 12-point trapezoidal approximation (—).

yphenol in water is, however, 8.2 kJ mol^{-1} (Table 6). The observed closure results from cancellation of errors from the other legs of the cycle that involve atom creation. The importance of the design of the mutation in regard to the cancellation of potential errors can be illustrated by a comparison of an initial investigation of free energy changes within this system using a different mutation and integration scheme[29] to the current study.

When atoms are annihilated during a simulation the nonbonded terms are reduced to zero. Either associated bonded terms can be left unchanged or the relevant force constants can be reduced. In the limit that the force constants go to zero, the atom becomes uncoupled from the system. Within a closed thermodynamic cycle both pathways are equivalent. Work is, however, required to change the bonded terms, and the change in free energy for individual legs will not be the same. Where possible in the current study the bonded terms were held constant. A dummy atom was included in the mutation from *p*-chlorophenol to *p*-methylphenol with bond and angle parameters equivalent to those of the N atom in *p*-cyanophenol. In the initial investigation of van Helden[29] on this system, no dummy atom was included in the *p*-chlorophenol to *p*-methylphenol mutation and the force constants of the bond and angle terms with dummy atoms in mutations involving atom annihilation were scaled by λ to zero. The integration was performed using Gaussian quadrature. Three points were used where the number of atoms remains unchanged and five points for atom annihilation. It was anticipated that the Lennard–Jones contribution would contribute significantly to the free energy when atoms were removed during the mutation. Because a linear dependence of the potential on the coupling parameter λ leads to a highly nonlinear response with respect to the Lennard–Jones contribution, a transform was applied to obtain Gaussian points and weights appropriate for a $\lambda^{1/6}$ dependence for atom annihilation. The appropriate λ-values and weights are given in Table 4. It should be stressed that, in hindsight, this choice of transform is far from optimal and we do not recommend its use in future studies. Separate simulations were performed at each λ-value using 10-ps equilibration and 20-ps sampling in water and 20-ps equilibration and 40-ps sampling for the complex. The convergence of the free energy was comparable to the convergence shown in Figure 8. The free energy for each of the six mutations is shown in Table 8. It should be noted that the direction in which all mutations except from *p*-chlorophenol to *p*-methylphenol were performed is the inverse of that used in the current study and that, to permit direct comparison of the results in Table 8 to those of Table 6, the order of the mutations and the signs of the corresponding free energy have been inverted. The differences between Table 8 and Table 6 are dramatic. For mutations involving *p*-methoxyphenol some of the difference can be attributed to differences in the force field parameters (see Tables 1 and 2). However, the differences for the mutations of *p*-chlorophenol and *p*-methylphenol to *p*-cyanophenol, which are on the order of 20 kJ mol^{-1}, are purely a consequence of the different mutation and integration schemes. In the context of a

(29) van Helden, S. P. Ph.D. Thesis, University of Utrecht, Utrecht, The Netherlands, 1992.

Table 7. Total Change in Free Energy for Each of the Possible Three-Membered Closed Cycles Involving Mutations between *p*-Chlorophenol, *p*-Methylphenol, *p*-Cyanophenol, and *p*-Methoxyphenol[a]

	water			α-CD			α-CD – water		
	$\Delta G_{5\ point}$	$\Delta G_{9\ point}$	$\Delta G_{12\ point}$	$\Delta G_{5\ point}$	$\Delta G_{9\ point}$	$\Delta G_{12\ point}$	$\Delta\Delta G_{5\ point}$	$\Delta\Delta G_{9\ point}$	$\Delta\Delta G_{12\ point}$
$Cl \rightarrow CH_3 \rightarrow CN \rightarrow Cl$	2.0	0.5	0.3	0.0	0.7	0.1	−2.0	0.2	0.1
$Cl \rightarrow CN \rightarrow OCH_3 \rightarrow Cl$	1.8	0.7	1.0	−4.9	−0.7	1.2	−6.7	−1.4	0.2
$Cl \rightarrow CH_3 \rightarrow OCH_3 \rightarrow Cl$	1.3	1.9	0.2	2.9	3.2	1.7	1.6	1.3	1.5
$CH_3 \rightarrow OCH_3 \rightarrow CN \rightarrow CH_3$	1.1	0.7	−1.1	7.8	3.2	0.4	6.7	2.5	1.5

[a] Values are in kJ mol^{-1}.

Table 8. Calculated Free Energy Changes Taken from van Helden[29]

mutation	ΔG_{water}	$\Delta G_{\alpha\text{-CD}}$	$\Delta\Delta G_{\alpha CD-water}$	$\Delta\Delta G_{expt}$[a]
$Cl \rightarrow CH_3$	−4.6	3.1	7.7	3.9
$Cl \rightarrow CN$	9.4	9.4	0.0	1.0
$Cl \rightarrow OCH_3$	10.2	13.7	3.5	3.8
$CH_3 \rightarrow CN$	13.0	11.5	−1.5	−2.9
$CH_3 \rightarrow OCH_3$	17.5	16.6	−0.9	−0.1
$CN \rightarrow OCH_3$	18.0	17.1	−0.9	2.8

[a] Experimental values taken from Hilbers et al.[30]

Table 9. Residual Free Energy for Each of the Possible Three-Membered Closed Cycles Taken from van Helden[29]

mutation	ΔG_{water}	$\Delta G_{\alpha\text{-CD}}$	$\Delta\Delta G_{water-\alpha\text{-CD}}$
$Cl \rightarrow CH_3 \rightarrow CN \rightarrow Cl$	−1.0	5.2	6.2
$Cl \rightarrow CN \rightarrow OCH_3 \rightarrow Cl$	17.2	12.8	−4.4
$Cl \rightarrow CH_3 \rightarrow OCH_3 \rightarrow Cl$	2.7	6.0	3.3
$CH_3 \rightarrow OCH_3 \rightarrow CN \rightarrow CH_3$	−13.5	−12.0	1.5

thermodynamic cycle, both mutation schemes should be equivalent and the residual free energies for each of the different cyclic mutations given in Table 9 should approach zero. In contrast, the residual for the mutation of *p*-chlorophenol → *p*-cyanophenol → *p*-methoxyphenol → *p*-chlorophenol in water is 17.2 kJ mol^{-1}. This is much larger than the expected error due to insufficient sampling. The discrepancy stems from the fact that the transform that was used assumes a given dependence of the derivative of the potential energy function on λ. The Coulombic and angle terms do not have the same dependency and in fact dominate the total free energy leading to a poor estimate of the free energy using the points chosen. The mutation of *p*-chlorophenol → *p*-methylphenol is well integrated using three points. The cycle p-chlorophenol → *p*-methylphenol → *p*-cyanophenol → *p*-chlorophenol has a small residual due to the cancelation of errors between the mutations of *p*-chlorophenol → *p*-cyanophenol and *p*-methylphenol → *p*-cyanophenol. In the cycles *p*-chlorophenol → *p*-cyanophenol → *p*-methoxyphenol → *p*-chlorophenol and *p*-methylphenol → *p*-methoxyphenol → *p*-cyanophenol → *p*-methylphenol, the same cancellation does not occur. As the mutation of *p*-cyanophenol → *p*-methoxyphenol is in both cycles, it might be concluded that the error in integration is due to this mutation. The results of taking five and nine points to perform the integration shown in Table 6 indicate, however, that three points would probably still give a reasonable approximation of the integral and that the difference for this mutation between Tables 6 and 8 is a consequence of the difference in molecular topology. Therefore, the failure of the cycles to close is more likely due to large systematic errors in the mutations of *p*-chlorophenol → *p*-cyanophenol and *p*-methylphenol → *p*-cyanophenol which do not cancel within the cycle. That the errors that lead to failure of the cycles to close in water and in α-CD are primarily systematic is confirmed by the fact that the residuals for closed cycles involving the difference in free energy ($\Delta\Delta G_{\alpha\text{-CD-water}}$) are small.

(5) Comparison with Experiment. Experimentally derived free energy differences for the binding of the inclusion compounds to α-CD[30] are listed in Tables 6 and 8. The differences between the calculated free energy differences in both the current study and the initial study of van Helden[29] compared to the experimental results range from 0.3 to 5.5 kJ mol^{-1}. The apparent agreement with experiment for the different mutations can, however, be highly misleading in regard to the reliability of the calculation and the reliability of the underlying force field. This can be best illustrated in regard to the study of van Helden. The residual free energy difference for the cycle *p*-chlorophenol → *p*-methylphenol → *p*-cyanophenol → *p*-chlorophenol from Table 9 is 6.2 kJ mol^{-1}. This represents the minimum combined error in the three mutations. For the mutation of *p*-chlorophenol → *p*-methylphenol, the difference between the calculated and experimental free energies is 3.8 kJ mol^{-1}. For the mutations of *p*-chlorophenol → *p*-cyanophenol and *p*-methylphenol → *p*-cyanophenol, this difference is only 1.0 and 1.4 kJ mol^{-1}, respectively. The uncertainty in the calculated free energy for the mutation of *p*-chlorophenol → *p*-methylphenol is, however, far smaller than for all the other mutations. The agreement with experiment for the other two legs of the cycle is simply fortuitous as is the very close agreement between the experimental and calculated values for the mutation of *p*-chlorophenol → *p*-methoxyphenol. Of course in cases such as the mutation of *p*-chlorophenol → *p*-methoxyphenol the calculated free energy may indeed reflect the true difference resulting from the force field. This would be true if the components that are not integrated correctly make equivalent contributions in the bound and unbound states and cancel within the thermodynamic cycle. This is, however, not always the case. From the results of the current study, shown in Table 7, it can be seen that increasing the number of points used to determine the integral results in much better convergence of closed cycles. The discrepancy between the calculated and experimental free energies for each of the mutations involving a change in the number of atoms is, however, significantly larger (Table 6) than determined by van Helden[29] (Table 8). For the mutations of *p*-chlorophenol → *p*-methoxyphenol and *p*-methylphenol → *p*-methoxyphenol, the difference between the calculated and experimental free energies systematically increases as the integral is determined with higher precision.

We may have a high degree of confidence that the calculated free energy changes for the mutations of *p*-chlorophenol → *p*-methylphenol and *p*-cyanophenol → *p*-methoxyphenol represent the true free energy differences due to the force field. In both cases no significant difference was observed using nine as opposed to five points to perform the integration. The results for these mutations correspond closely to those obtained by van Helden[29] (Table 8). This illustrates an important feature in regard to the design of the mutation. In order to achieve cycle closure in the current study, a dummy atom was included in the mutation of *p*-chlorophenol → *p*-methylphenol. By comparing the results in Tables 6 and 8, it can be seen that for the individual legs in water and in α-CD the inclusion of the dummy atom resulted in a difference in ΔG of approximately 2.0 kJ mol^{-1}. The effects of the dummy atom cancel, however, within the thermodynamic cycle, and there is close agreement for the ΔΔG values. The effects of the dummy atom arise from the fact that, though the dummy atom is not changed in the mutation and has no nonbonded interactions with the rest of the system, it is coupled to the mutated atom by bond constraints, bond angle bending terms, and a

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dihedral angle term. Changing the Cl–C bond length during the mutation will change, for example, the C–Cl–dummy angle vibrational frequency, requiring work to be done on the system.

The differences between the calculated and experimental free energies for the mutations of *p*-chlorophenol → *p*-methylphenol and *p*-cyanophenol → *p*-methoxyphenol are 4.2 and 3.6 kJ mol^{-1}, respectively. These differences can be primarily attributed to inadequacies in the force field. The experimental free energy differences range from 0.1 to 3.9 kJ mol^{-1}. Thus, though the calculation predicted the correct sign of the free energy change in all but one case and reproduced the experimental free energy differences to within 5.5 kJ mol^{-1}, the force field that was used cannot be claimed to be capable of discriminating between the different inclusion compounds.

Though the differences between the calculated and experimental free energies are most likely attributable to inadequacies in the force field, other factors may also contribute. In particular it has been assumed that all the inclusion compounds bind with the same orientation within the cyclodextrin cavity. The reversal of the guest within the cavity is not possible during the time frame of the simulation of the complex. Thus the calculation is only representative of one particular state which may or may not be experimentally relevant.

(6) Force Field Correction. Since the start of this study a correction to the GROMOS force field has been proposed (Aqvist, Personal communication). In the GROMOS force field, the Lennard–Jones potential, V_{LJ}, is expressed in terms of C6 and C12 parameters where $V_{LJ} = C12/r^{12} - C6/r^6$. The C6 and C12 parameters for the pairwise interaction between two atoms i and j are derived using the combination rule $C12(i,j) = C12^{1/2}(i,i)C12^{1/2}(j,j)$, where different values for $C12^{1/2}(i,i)$ and $C12^{1/2}(j,j)$ can be chosen for a given i or j depending on the nature of the interaction. The rationale for choosing different $C12^{1/2}(i,i)$ and $C12^{1/2}(j,j)$ values depending on the nature of the interaction is 2-fold. First, though the above combination rule yields a very good estimate for $C6(i,j)$, this combination rule has been shown to significantly overestimate the well depth for interactions between dissimilar atom types.[31] This can be compensated for by choosing alternative $C12^{1/2}(i,i)$ parameters for specific interactions. Second, different $C12^{1/2}(i,i)$ parameters can be used to correct for the short-range distorting effects of point charges in order to better reproduce experimentally determined interatomic distances. For the interaction between a neutral carbon and SPC water, a value for the repulsive term in the Lennard–Jones potential of $C12^{1/2}(OW,OW) = 421.0$ (kcal mol^{-1} Å^{12})$^{1/2}$ is used in the standard GROMOS force field.[8] Calculation of the free energy of solvation of CH_4 (results not shown) suggests, however, that this results in a free energy of hydration that is too favorable and that a value of $C12^{1/2}(OW,OW) = 793.3$ (kcal mol^{-1} Å^{12})$^{1/2}$ is more appropriate. A value of $C12^{1/2}(OW,OW) = 793.3$ (kcal mol^{-1} Å^{12})$^{1/2}$ is used for the water oxygen to water oxygen interaction in SPC water.[20] The lower value used in the GROMOS force field had been selected by analogy to the carbonyl oxygen to neutral carbon interaction. The difference in the free energy of hydration ($\Delta\Delta G_{hydr} = \Delta G_{water} - \Delta G_{vacuum}$) for the mutation of *p*-chlorophenol → *p*-methylphenol from Tables 5 and 6 calculated using the original GROMOS value is −3.9 kJ mol^{-1}. The value of this free energy difference can be estimated, from the experimental free energies of hydration of *p*-bromophenol and *p*-methylphenol and from the difference in group contributions of Br and Cl given by Cabani et al.,[32] to be approximately 3 kJ mol^{-1}. This supports the suggestion that the interaction of the CH_3 group with SPC water is too favorable. The mutation of *p*-chlorophenol → *p*-methylphenol was repeated using the modified water–CH_3 parameters. For the same sampling and equilibration periods as before and using nine points to perform the integration, the changes in free energy are $\Delta G_{water}(Cl \rightarrow CH_3) = 5.5$ kJ mol^{-1} and $\Delta G_{\alpha\text{-CD}}(Cl \rightarrow CH_3) = 7.2$ kJ mol^{-1}. The vacuum simulations are unaffected. The revised $\Delta\Delta G_{hydr}(Cl \rightarrow CH_3) = 4.4$ kJ mol^{-1}. The difference in binding free energy $\Delta\Delta G_{\alpha\text{-CD–water}} = 1.7$ kJ mol^{-1}, which differs from the experimental value by 2.2 kJ mol^{-1}. The modification of the force field has only been tested for the mutation of Cl → CH_3. Clearly this modification will affect the calculated free energy for all mutations involving *p*-methoxyphenol and *p*-methylphenol and is in the process of being further tested. Nevertheless, the proposed modification of the water–CH_3 interaction clearly improves the agreement with experiment for the mutation of Cl → CH_3 and it is recommended that this modification be incorporated into the GROMOS force field.

Conclusions

The accuracy of free energy calculations is dependent on (1) the force field and (2) the degree to which the relevant configurational space can be sampled. As access to large-scale computing facilities becomes more available there will be an increasing trend to apply free energy calculations to ever more complex and less defined systems. The danger is that protocols in terms of equilibration or sampling time will be directly transferred from successful calculations on simple systems. The difficulty in establishing the reliability of a given calculation is that no single criterium can be used to determine if the results have converged and that the calculated free energy represents the true free energy according to the force field used. This has been clearly illustrated in the current study. By considering all possible mutations among four para-substituted phenols in vacuum, water, and complexed to α-cyclodextrin, we have been able to separate the effects of incomplete sampling from those caused by force field quality and other factors. We have demonstrated the utility of forming closed cycles in each environment as a means of detecting errors due to insufficient sampling and inaccuracies due to the integration scheme.

A minimum set of criteria for judging if the results from a given free energy calculation have converged would be the following: (1) The change in free energy for a closed cycle is zero. (2) Increasing the sampling or equilibration time does not affect the calculated free energy. (3) The introduction of additional points to determine the integral does not effect the calculated free energy. These are of course necessary but by no means sufficient conditions. There is naturally a strong temptation to publish the results of free energy calculations which show good apparent agreement with experiment.[33] Such agreement can, however, be highly misleading both in regard to the reliability of the calculation, in terms of implications for the methodology and force field, and in respect to the understanding of the system.

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