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Table II
Rate Constants for Reduction of DTNB at 30 °C[a]

reducing reagent	reaction rate constant, $M^{-1}\ s^{-1}$
L-cysteine	1.6×10^3
D,L-homocysteine	1.3×10^3
D,L-homocysteine	1.5×10^3 [b]
D,L-homocysteine thiolactone	0.29
N-acetyl-D,L-homocysteine thiolactone (*N*-Ac-D,L-HCTL)	0.13
N-Ac-D,L-HCTL	6.3×10^2 [c]
N-Ac-D,L-HCTL	6.2×10^2 [d]

[a] pH 7.7, [HEPES] = 5 mM. [b] A homocysteine solution was acidified to pH 1.3, and the solution was incubated at 30 °C. A total of 1 h later the solution was neutralized and mixed with DTNB solution quickly. [c] A *N*-Ac-D,L-HCTL solution was alkalinized to pH 12.7, and the solution was incubated at 30 °C. A total of 1 h later the solution was neutralized and mixed with DTNB solution quickly. [d] A *N*-Ac-D,L-HCTL solution, alkalinized to pH 12.7, incubated at 30 °Cfor 1 h, and neutralized, was further acidified to pH 1.5, and the solution was incubated at 30 °C. A total of 1 h later the solution was neutralized again and mixed with DTNB solution quickly.

the hydrophobicity of the *N*-methylacryloylhomocysteine residues is much higher than under basic conditions. Consequently, the blocks of *N*-methacryloylhomocysteine residues penetrate into the lipid bilayers quickly and deeply upon reduction of pH and as a result perturb the packing of the lipids substantially, thereby inducing the permeation of Eosin Y.

These results show that, though the thiolactone formation is not the real reason for the high pH sensitivity observed here, polymerized liposomes containing the homocysteine moiety are useful for a construction of pH-responsive devices.

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Absolute Free Energies in Biomolecular Systems

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ABSTRACT: We investigate a technique for convenient, accurate, and numerically efficient determination of absolute free energies of biomolecular conformations, regardless of mechanical stability and applicable to systems in vacuo or in solution, under any conditions of temperature and volume. It is anticipated that this free energy approach will aid elucidation of free energy surfaces in phase space and thermodynamic consequences to changes in structure and environment.

Introduction

Polypeptides and proteins can exist in a variety of metastable conformational states, corresponding to minima in some postulated potential energy function.[1,2] Determination of native conformations of these systems and investigation of kinetic processes responsible for conformational transitions between nearby free energy minima are important goals. In addition to these small structural fluctuations, the problem of large structural rearrangement in computer modeling needs to be solved.[3] This involves, ideally, elucidation of the entire free energy surface for the system of interest. Consequently, evaluating absolute free energies for particular configurations is an important theoretical goal allowing, in principle, construction of free energy surfaces in these systems. Additionally, relative values of absolute free energies from different thermodynamic states describe their relative stability.

The search for the optimum configuration has had a long history and still receives considerable experimental

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and theoretical attention.[4] Molecular dynamics, Monte Carlo, and molecular mechanics have been used extensively to study the energetics, structures, and dynamics of stable conformational states.[5] Initially, study of protein structure involved consideration of only lowest energy (enthalpy) configurations, although it was well-known that a proteins native structure (which minimizes absolute free energy) does not necessarily coincide with the minimum-energy structure.[6] The study of minimum-energy conformers is still an active area of research. For example, Gibson and Scheraga[7] have recently extended their buildup procedure for finding low-energy conformers of polypeptides. This procedure was previously applied in a more restrictive form to enkephalin.[8] Levitt[9] has studied BPTI (bovine pancreatic trypsin inhibitor) using restrained energy minimization by taking 25 random starting configurations in torsion space and finding the lowest energy conformers, which he considers to be a first approximation to the lowest free energy state.

These seminal works found zero-temperature structures, since they expediently ignored configurational fluctuations from local potential minima into which the system had been quenched. Attempting to study biomolecules at different temperatures and to assess the effect of local configurational entropy on conformational stability, investigators studied harmonic fluctuations about minimum-energy conformations. Specifically, Go and Scheraga[10] calculated the entropy of polypeptides undergoing harmonic fluctuations about their stable states and introduced the concept of "important" and "unimportant" variables. Hagler et al.[11] used Monte Carlo to determine relative vibrational free energies of different minimum-energy conformations for several hexapeptides and ascertained the role of local configurational entropy in relative conformational stability. They found large harmonic entropy effects on conformational stability. Subsequently, Karplus and Kushick[12] extended the approach of Go and Scheraga by calculating the covariance matrix of internal coordinates during molecular dynamics (rather than using normal mode analysis). By harmonically approximating the potential in internal variable space, they related local configurational entropy to fluctuations and correlations in internal variables, finding that internal variables other than torsions contribute significantly to configurational entropy. Thus, bond lengths, bond angles, and other degrees of freedom must be considered in calculations of free energy.

These methods could not address the large-scale conformational flexibility known to exist in these peptides, nor could they address the well-known[13] influence of anharmonic contributions to free energy. Several investigators recently presented first steps in understanding anharmonic effects on free energy for particular minimum-energy conformations. Meirovitch et al.[14] studied the thermodynamic stability of hairpin and α-helical structures of decaglycine, without solvent, using Monte Carlo to generate conformations in the vicinity of stable states, which are then used to estimate sampling probabilities needed in entropy calculations. Previously, this probability was difficult to estimate, explaining the use of enthalpy, not free energy, as a criterion of peptide conformational stability. Although the method does not limit free energy calculations to harmonic or quasi-harmonic states, it has thus far been applied to backbone torsional states. Consequently, the results may not reflect the true native state.[12] Although extension of this method to treat side chain torsional states is progressing, a more complex set of required local states will increase calculational difficulty.

An exciting first attempt at global conformational searching for minimum free energy structures has been reported.[15] In this and subsequent[16] studies, the multiple minima problem in potential surfaces of proteins is addressed by adopting the Monte Carlo method of Lepage,[17] which searches conformational space for free energy minima by iteratively searching areas within which the integrand of the partition function is largest. Monte Carlo searching and adapting of the probability distribution function are continued until a converged probability distribution function obtains. Free energy maps for each residue of the protein are generated in torsional space. It is assumed that low free energy conformations for a protein can be built from low free energy fragments (residues). A statistical coil treatment of protein free energy has also recently been presented,[18] in which the entire phase space has been explored.

The literature cited indicates that description of the structure, dynamics, and thermodynamics of proteins, polymers, and peptides has considered only low-enthalpy configurations, obtained with molecular mechanics or with molecular dynamics annealing to low-energy conformations, followed by evaluation of harmonic, quasi-harmonic, or anharmonic free energies (all of which sample states near low-energy structures). Higher energy states have been ignored; yet, in general, these play a major role in the determination of configurational entropy of biopolymers (since configurational entropy derives in part from local fluctuations about these well-defined structures and from large scale conformational rearrangement among structures) and in understanding the kinetic processes of conformational transitions between potential minima. Incorporation of these states into a description of structure and dynamics is therefore essential.

The present method can investigate these higher energy structures and their thermodynamic stability by evaluating free energies of stable and/or unstable, constrained states in a general and transparent manner.

Theory

This molecular dynamics approach for calculating absolute free energies of arbitrary biomolecular configurations is motivated by density functional methods for free-energy evaluation. In density functional theory, the nonuniform density of the system is written as a sum of Gaussians and parameterized by atomic mean-squared displacements. By changing mean-squared fluctuations from zero to infinity, solid-phase properties can be obtained from the reference uniform system. In a Hamiltonian formulation of free energy, the assumption of harmonic displacements in density is equivalent to an added harmonic site potential, which, in our model, represents the reference state of the biomolecule, the free energy of which is known. This structure can be a low- or high-energy conformation and need not be mechanically stable. Both the reference state and the actual system are connected by the well-known "charging strategy", wherein harmonic site potentials that fix atoms to their fiducial sites are "switched on" under the hamiltonian $H(\lambda)$. This parameterized hamiltonian is formed by perturbing the full interaction potential of the biomolecule, V, with Einstein independent oscillator site potentials, V_H.

A simple parameterization is used

$$H(\lambda) = K + (1-\lambda)V + \lambda V_H \tag{1}$$

with

$$V_H = \sum k_i(\mathbf{r}_i - \mathbf{r}_i^0)^2$$

where K is kinetic energy, V is the full interaction potential of the biomolecule, $0 < \lambda < 1$, k_i is the ith atom spring constant, $\mathbf{r}_i$ is the ith atom instantaneous coordinate, and $\mathbf{r}_i^0$ denotes atomic position in the reference Einstein structure.

When λ is zero, one is simulating the full interacting system, while for $\lambda = 1$, a noninteracting harmonic solid, with spring constants k_i, is being simulated. For intermediate λ values, constrained molecular dynamics is being performed.

From standard thermodynamic identities,[19] the relation between Helmholtz free energy, F, and the canonical partition function, Q, is given by

$$-\beta F = \ln (Q) \qquad (2)$$

where $\beta^{-1} = k_BT$ (k_B = Boltzmann's constant and T is absolute temperature). The new hamiltonian leads to a λ-dependent free energy, after differentiating and integrating eq 2 with respect to charging parameter λ:

$$F_0 = F_1 + \int_0^1 \mathrm{d}\lambda \, \langle (V - V_H) \rangle_\lambda \qquad (3)$$

where $\langle \ldots \rangle_\lambda$ indicates time average over the ensemble of states generated by $H(\lambda)$. The free energy of the $\lambda = 0$ state, F_0, is that which obtains for a system moving under the influence of the potential V. The free energy of the $\lambda = 1$ state, F_1, is that for a system of independent harmonic oscillators, known analytically

$$F_1 = -3Nk_BT/2 \sum \ln (4m_i\pi^2/h^2\beta^2k_i) \qquad (4)$$

where N is the number of atoms in the biomolecule, m_i is the ith atom's mass, and h is Planck's constant.

Molecular dynamics at each Gauss–Legendre quadrature point evaluates the ensemble average in eq 3.

As a measure of error in the free energy, we evaluate

$$\int_0^1 \mathrm{d}\lambda \ \mathrm{adev}\ (V - V_H)_\lambda \qquad (5)$$

where adev (x) is the average absolute deviation of x.[20] Spring constants are arbitrary parameters, set to minimize error in eq 5. This presumably ensures effective spring constants that mimic total interactions on atoms from their surroundings in the biomolecule.

Summarizing, the advantage of this method is its ability to evaluate absolute free energies in a transparent fashion. Free energies include configurational entropy contributions, even from highly anharmonic potentials, since the integral in eq 3 includes contributions from the full interacting system. The present approach addresses valid criticisms made by others[15,16] about the coupling parameter[21] and perturbation[22] methods of free-energy evaluation. The problems addressed are the following: (i) a reference system is required, (ii) the reference free energy must be known, and (iii) the reference system must be similar in structure to the system under study. In the present method, the reference system has a structure similar to that of the biomolecule being studied, and the free energy of this reference state is known analytically. In addition, because the method involves only simple averages of potential energy and mean-squared displacements, it is fast and accurate, with few intermediate states required for convergence. One possible drawback is as follows: the method does not discriminate low- and high-frequency motions. Some investigators may consider this troublesome.[23]

Results and Discussion

The method was implemented into the simulation program DISCOVER[24] and first tested on an fcc (face-centered cubic) lattice of 108 Lennard-Jones atoms. Krypton potential parameters were used (ϵ = 165 K, σ = 3.62 Å). Potential was cut off at 2.34σ. Lattice parameters were consistent with zero-pressure density, in accordance with the Lennard-Jones phase diagram. The system was equilibrated for 1000 steps of 1 fs, followed by 5000 steps of calculational dynamics. Results were compared with those from the quasi-harmonic approximation (QHA)[25] and shown in Figure 1. The present approach agrees well with QHA for $T < 0.5\epsilon$. For larger T, anharmonic effects become evident, and errors in QHA obtain. Others[26] have calculated the absolute free energy of this system, using different approaches. A method based on the free volume theory is being extended to treat the entropy of proteins in a novel manner.[27]

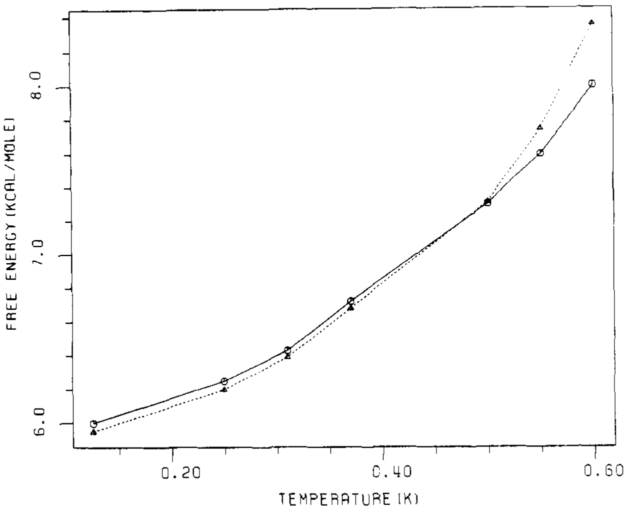

Figure 1. Free energy (eV) versus temperature (ϵ) from the present approach (solid) and QHA (dashed).

The theory was then applied to the model system decaglycine in three states: α-helix, hairpin, and extended. This system (hairpin and α-helix) was recently investigated[14] by using Monte Carlo to extract entropy of polypeptides from samples of conformations. The local configurational entropy included harmonic and quasi-harmonic contributions. The method confined Monte Carlo searching to a conformational region near both stable states by constructing structural transition probabilities from previous steps in generating the polypeptide chain. Their method was applied with rigid geometry (i.e., fixed bond lengths and angles), by using the ECEPP (Empirical Conformational Energy Program for Peptides) potential energy function.[28] In vacuo, at T = 100 K, the α-helix state was found more thermodynamically stable than the hairpin, with a significant entropy effect on stabilization. The α-helix entropy is larger than that of the hairpin state, arising from extra rotational freedom. The extended state was unstable and consequently was not studied.

In our application, conformations of hairpin and α-helix states were built and energy minimized under the valence force field of Dauber–Osguthorpe et al.[29] In building the initial unminimized α-helix and hairpin states, we estimated the ϕ,ψ angles as the midpoint of each $\Delta\phi,\Delta\psi$ used by Scheraga et al.[14] where $\Delta\phi,\Delta\psi$ are differences between minimum and maximum values for the 10 ϕ,ψ pairs found during their Monte Carlo runs. This ensures relaxed structures and time-averaged molecular dynamics structures in configurational space regions presumably similar to those sampled by Scheraga and co-workers. The relaxed structures were used as template coordinates, $\{\mathbf{r}_i^0\}$, for the reference Einstein solid in 100K and

298K molecular dynamics simulations. Bond lengths and angles were not constrained, and torsional states were included. Unlike previous treatments, the method is not limited to systems without side chains, making application to branched peptides and/or polymers tractable.

The absolute free energy for α-helix decaglycine at four temperatures was calculated, by using 5000 timesteps of 1 fs, following a 3000 time step initialization. In Figure 2, comparison is shown with free energy from QHA. Qualitative behavior is correct. Free energies are lower from methods sampling more energy states (larger entropy) and increase with temperature. Average absolute deviations (error) of simulated free energies were consistently <2 kcal/mol.

Spring constants were chosen to minimize the integrand in eq 3, according to

$$k_i = \langle V \rangle_0 / \langle \Delta x^2 \rangle_0 \qquad (6)$$

where $\langle V \rangle_0$ is average potential and $\langle \Delta x^2 \rangle_0$ is the mean-squared displacement, relative to fiducial sites, per atom. Averages are taken in the full interacting system. Each atom is assigned the same k_i value. By construction, this guarantees small differences between average energy and average harmonic energy. Judging from the acceptable errors observed (see table below), this difference presumably remains small in the full harmonic limit ($\lambda = 1$).

Other prescriptions for the spring constant are possible. For instance, the expression

$$k_B T / \langle \Delta x^2 \rangle_0 \qquad (7)$$

can be used as an estimator for k_i, where each atom receives unique values. This expression follows from the definition of the mean-squared displacement under the harmonic site potential, V_H, in eq 1. As a third alternative, one could assign arbitrary spring constants for all atoms, without having to first evaluate quantities in eq 6 or 7. These alternative prescriptions for the spring constant may be needed, depending on the system studied, to optimize error.

Results for simulation of decaglycine in hairpin and α-helix structures on a Convex C1, at $T = 100$ K, using eq 6 for spring constants, are

quantity	α-helix	hairpin
free energy	114.074	120.356
error	1.714	2.59
CPU, min	84	93
k_i	380.991	204.937

Simulations used 1000 steps of initialization, followed by 3000 steps of dynamics, at each of 10 Gauss–Legendre quadrature points. When the number of quadrature points was increased to 14, the percent increase in free energy was ~0.03, with a 1.4% decrease in error. The literature value for the free energy difference[14] between these two states (using the ECEPP potential) is ~0.4 (kcal/mol)/residue. In our simulations (using the VFF force field), we find a difference of ~0.6 (kcal/mol)/residue. Obtaining this free energy difference from relative free energy methods would be difficult, since the conformational perturbation involved is enormous.

As a final illustration of the method, we calculated the absolute free energy of the extended state of decaglycine, a previously impossible calculation. Spring constants of 500 (kcal/mol)/Å^2 were assigned to every atom at $T = 298$ K. From 10 quadrature points, the free energy obtained was $F = 173.69$ (converged to within 0.17%), with an error of 4.5 kcal/mol, using 63 Convex CPU minutes. Initially, spring constants were based on eqs 6 and 7 but gave unacceptable errors, due to large, unconverged mean-squared displacements. By assigning large k_i values, dynamics was constrained to the phase space near the initial extended structure, thereby minimizing errors. This approach is analogous to restricted partition function evaluation. Consequently, mechanically unstable states can be investigated and compared, provided consistency is maintained.

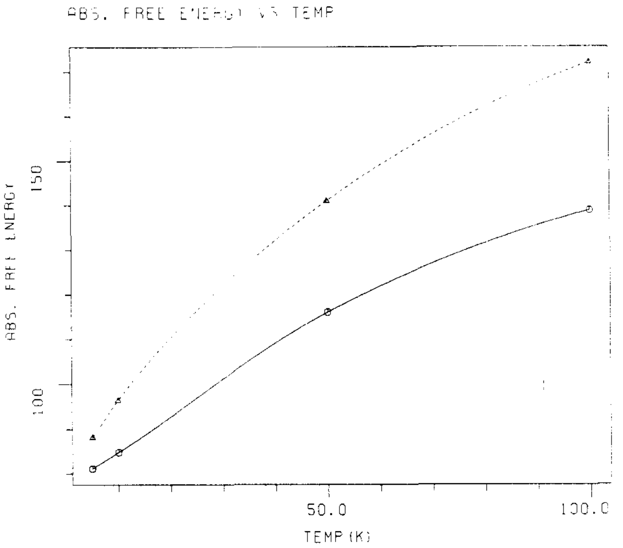

Figure 2. Free energy (kcal/mol) versus temperature (K) from the present approach (solid) and QHA (dashed).

Because simulations are fast and accurate, we will study global search strategies, at least for simple systems (e.g., simple peptides). Torsion angles will be forced over the entire torsional space and absolute free energies for constrained states evaluated. This is based on ideas for studying torsional states presented by Hagler et al.[11] and Paine and Scheraga.[15] A free energy map in torsion space would elucidate not only probable conformations but also transition states (the free energies of which would be known). This would be enlightening vis-à-vis annealing kinetics of suboptimal conformations to the most probable configuration. Future applications to branched polymers and peptides, and to peptides with side chains, will be straightforward and ascertain the influence of entropy on the stability of the native states, on the dynamics of the structural transitions between native states, or even on determination of the predominant conformations in the native state.

Conclusion

The method described has been shown convenient and accurate, easily implemented, quickly convergent (since it involves only averages of potential energy and mean-squared displacements), and applicable to problems of large structural rearrangement in complex systems.

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^{13}C NMR Investigation of Molecular Order in Liquid Crystal Polysiloxanes

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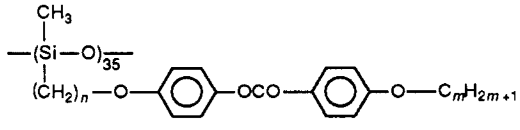
ABSTRACT: High-resolution variable-temperature ^{13}C NMR experiments have been carried out on a series of side-chain liquid-crystal polysiloxanes. The ^{13}C chemical shift dependence on temperature observed in the mesophases is a clear indication that the polysiloxanes orient in the magnetic field. Results have been interpreted in terms of molecular order and local dynamics of the different parts of the molecule: spacer, mesogenic core, and terminal group of the side chain. It has been shown that the spacer and the terminal group do not have the same influence on the order parameter associated with the mesogenic core. The apparent variations of the order parameter along the side chain reflect the existence of several motional processes occurring in the oriented mesophase. Among them are the trans-gauche conformational changes in the spacer, the internal rotation of the phenyl rings about their symmetry axis, the motion of the COO plane associated with the β_2 transition of polysiloxanes, and the overall rotation of the whole side group about its molecular axis.

Side-chain liquid-crystal polymers are interesting materials which combine the properties of polymers and those of liquid crystals of low molecular weight. A deeper understanding of the behavior of these compounds requires the investigation of molecular order and dynamics in the different phases. Some studies have already been performed using X-ray,[1] small-angle neutron scattering[2,3] dielectric relaxation,[4-11] and ^{2}H NMR.[12-14] High-resolution ^{13}C NMR is also a powerful technique which has proven to be useful in the determination of order and dynamics of small liquid-crystal molecules.[15-17] The natural carbon-13 abundance makes it unnecessary to do any kind of labeling, and the selectivity of the technique allows the observation of each magnetically inequivalent carbon independently. In this paper we report a high-resolution ^{13}C NMR investigation of molecular order of a series of side-chain liquid-crystal polysiloxanes. Some results concerning the dynamics of these systems will also be described.

Experimental Section

The polysiloxanes ($P_{n,m}$) of the general formula



$$-(\underset{(CH_2)_n-O-C_6H_4-OCO-C_6H_4-O-C_mH_{2m+1}}{\overset{CH_3}{Si}}-O)_{35}-$$

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