The Conformational Free Energy of Carbohydrates

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Abstract: Although free energy calculations have been extensively employed to explore the conformational space of proteins, to date carbohydrates have received considerably less attention. This review provides a survey of recent computational free energy studies of the preferred conformations and characteristic dynamics of carbohydrate molecules in solution. The article comprises a motivation for employing expensive free energy calculations for carbohydrates, an outline of the principal computational methods and techniques employed for free energy determinations, together with a summary of the development of the field in recent years and the principal findings to date. Finally, possible future directions in free energy investigations of this type are discussed.

Keywords: Free energy, carbohydrates, polysaccharides, dihedral angles, ring conformations, hydroxymethyl.

1. INTRODUCTION

Carbohydrates perform a plethora of biological roles, ranging from familiar functions as central energy storage metabolites and structural support molecules, to less widely appreciated roles in complex molecular recognition and signalling events, such as cell–cell adhesion and immunological protection.

As a molecular class, carbohydrates are distinguished by the heterogeneity of their cyclic monomer units, their potential for a variety of possible linkages between their monomer units and their marked conformational flexibility, characteristics which are intrinsic to their diverse biological functions. The dynamic behaviour of carbohydrates in solution encompasses inter-conversion between conformers and the possible association of carbohydrate solute molecules with each other. The variety of possible relaxation processes for poly- and oligosaccharides range from pseudorotation inter-conversions between different linear and ring forms (relaxation time $\approx$ 1–100 μs), inter-conversion between the various pucker formations of the sugar ring ($\approx$ 100 ns), rotation about the glycosidic linkage dihedral angles ($\approx$ 10 ns) and rotations of the primary ($\approx$ 1 ns) and secondary ($\approx$ 100 ps) hydroxyls [1-3].

The extreme flexibility of carbohydrates results in the frequent difficulties experienced with NMR and many other experimental probes of solution structure. Oligo- and polysaccharide structures often occupy an ensemble of rotational isomeric states under physiological conditions and, as few experimental techniques probe the nanosecond time scale, the observed signal of many spectroscopic or electrochemical techniques often represents a "virtual" structure, comprising the dynamic average of several structures [4-7]. Further, X-ray structures of carbohydrates in the solid state are often of poor quality: surveys suggest that about one-third of carbohydrate data in the Protein Databank (PDB) contain significant errors with regards to their stereochemistry, nomenclature, and consistency with electron density maps [8, 9] and can exhibit non-biological ring pucker conformations [10]. Therefore, determination of the tertiary structures of oligosaccharides continues to present a considerable challenge to glycobiology.

A further key aspect of carbohydrate chemistry is the vexed question of the interaction of carbohydrates with water and, potentially, ions. Carbohydrates have considerable potential for specific interactions with water through their numerous pendant hydroxyl groups. However, the extent to which this affects carbohydrate structure is still a matter of investigation [11-15]. In the absence of sufficient experimental data, complementary computational simulations have been used to provide atomic detail on the molecular structure and conformational dynamics of carbohydrates and the effect of water on these molecules.

Advances in methodology and computer architecture have allowed computationally expensive quantum mechanical calculations to be applied to small carbohydrate structures. However, while accurate, quantum mechanical calculations typically consider exclusively single point energies of representative conformations of the molecule of interest in the gas phase and preclude the inclusion of solvent molecules [16-20]. Calculations of this sort have limited value in representing the state of a dynamic saccharide in a solvent environment.

The conformational dynamics of specific carbohydrates in solution are most commonly simulated with Molecular Dynamics (MD) methods using empirical force fields with explicit solvent models [21-32]. The drive to understand the dynamic motions and the range of possible conformations for particular carbohydrate classes has promoted much of the work in this area. Results from MD simulations can be compared with experimental thermodynamic and dynamic properties. Of particular interest are conformational free energies, which are fundamental to obtaining accurate theoretical estimates of the energetics of conformational changes and the characterisation of dynamic behaviour of at the atomic-level. Free energy calculations differ as to whether the result is a pure free energy difference (such as free energies of solvation or differences between ligand–host binding energies) or rather a potential of mean force (PMF) describing a complete free energy pathway (such as that between a reactant and product or for a change in a coordinate). In most cases, free energy calculations are aimed at relative free energies: the change in free energy as a function of either an external parameter or an internal coordinate. The principal advantage of calculating PMFs, as opposed to simple free energy differences, is that a detailed description of barrier heights and energy changes along a complete free energy pathway allows for precise characterisation of a structural change. Free energy calculations for the important degrees of freedom in a carbohydrate allow for identification of the number of low-energy conformers, the flexibility of each conformer, the time spent in each conformation and the rate of conversion between conformers. In solution, a PMF incorporates not only contributions from entropy, but also the averaged solvent–solute interactions. Free energy calculations also provide extremely valuable information for refinement of carbohydrate empirical force fields, highlighting where the force fields produce conformational populations inconsistent with experimental data and hence require adjustment [33-35].

In principle, a potential of mean force may be obtained from a single standard MD simulation which is run long enough to ensure that all conformational minima for the molecule are adequately sampled. However, in practice, representative sampling is often prevented by high energy barriers - simulations become trapped in local energy minima for long periods of time and there is the addi-

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tional problem of poor sampling in the barrier regions themselves. Although advances in methodology and computer architecture have allowed for recent microsecond length Molecular Dynamics simulations of small oligosaccharides carbohydrates in explicit solution [36], thorough sampling of the neither the various pucker conformations of a single monosaccharide ring in solution nor the glycosidic linkage conformations is currently practical for standard MD simulations.

There are a number of techniques to enhance conformational sampling to the extent that free energy calculations become tractable. However, free energy calculations remain extremely costly and efficient calculation of free energy remains one of the major challenges to computational chemists [37-39]. In addition, carbohydrates have not been subject to the full range of computational methods applied to the more frequently simulated proteins and nucleic acids [40] and complex free energy calculations for carbohydrates are infrequently attempted.

This review offers an overview of recent free energy calculations performed on free (unbound) carbohydrate molecules in vacuum and solution with the aim of charaterising the structure and dynamics of these flexible molecules. We consider neither hydration free energies nor the vast field of prediction of the relative and absolute free energy of non-covalent protein-carbohydrate ligand binding, which was a primary focus of a review by De Marco and Woods in 2008 [41]. Rather, we focus on the principal degrees of freedom for an oligosaccharide: ring conformations and rotations about the various glycosidic linkages.

2. METHODS FOR FREE ENERGY CALCULATION

Driven primarily by research into protein structure and dynamics, a number of strategies have been developed to increase the conformational sampling in Molecular Dynamics simulations and thus enable calculation of PMFs from trajectories. Most techniques require equilibrium simulations, including thermodynamic integration (TI) and free energy perturbation (FEP), and the related biasing techniques: umbrella sampling, adaptive umbrella sampling, parallel tempering, metadynamics and adaptive biasing force. In addition, free energy calculations have recently been extended to non-equilibrium simulations, such as steered molecular dynamics (SMD) [39].

The methods of thermodynamic integration (TI) and free energy perturbation (FEP) can be used to calculate free energy differences for both conformational and chemical changes. Depending on the calculation required, FEP and TI require a series of simulations along either a reactant–product mutation pathway (free energy differences) or a reaction coordinate (PMF). As free energy is a state function and both of these simulation techniques invoke reversible paths, the mutation pathway chosen need not be an actual physical pathway. However, each simulation along the path must be run for long enough to ensure thorough equilibration of the hybrid system.

The more generally applicable class of biasing methods have a common strategy in achieving accelerated conformational sampling by the addition of a biasing potential to help the system overcome free-energy barriers. If the biased dynamics still generate a well-defined distribution in configurational space, the simulation results can in principle be reweighted so as to obtain so as to produce exact thermodynamical information (in the limit of infinite sampling) for the real (unbiased) system. Biasing methods are usually employed in order to calculate a potential of mean force.

In keeping with the usage of Hansen and Hünenberger, we use the term "umbrella sampling" to encompasses all methods where an arbitrary (time-independent) biasing potential is added to the physical potential energy function in order to enhance sampling in the high energy regions that are unexplored by regular simulations [42]. Umbrella sampling requires both a choice of the configurational degrees of freedom along which the biasing potential will be applied and identification of a form of the biasing potential. In the traditional multiple-windows variant of umbrella sampling, a set of MD simulations is performed using different harmonic biasing potentials to restrict the sampling locally to conformational specific regions, whereas the "adaptive umbrella sampling" approach applies biasing potentials that "adapt" with successive simulations to converge on a potential that results in complete coverage of the configurational coordinates in a single simulation. In both methods, the data from overlapping subregions is combined, typically with the Weighted Histogram Analysis Method (WHAM) in order to obtain the PMF [43-45]. Umbrella sampling is most efficient when the applied biasing potential is close to the negative of the free energy surface in the specified configurational sub-space, but this free energy surface is seldom known in advance as it is generally the expected outcome of the calculation. It has been found advantageous to use other methods to estimate the free energy surface before commencing the rather slowly adapting umbrella sampling routines. For example, Hansen and Hünenberger recently developed a method combining umbrella sampling with local elevation conformational searching - Local Elevation Umbrella Sampling (LEUS) - which they used to study applied to glucopyranose ring interconversion [42]. In this approach, a relatively short LE search phase is used to construct an optimized biasing potential, which is then applied in a subsequent umbrella sampling phase.

There are a variety of efficient related adaptive methods for free energy calculation which adjust the biasing potential for the current simulation based on the history of exploration of conformational space, in order to prevent the continuous revisiting of previously discovered configurations. Examples are the local elevation, conformational flooding, adaptive biasing force [46] metadynamics [39] and filing potential methods. In the metadynamics method, reviewed in Park *et al.* [39], the bias potential is applied as a penalty function on a suitably chosen set of collective variables, which reduce the dimnasionality of the problem. The bias potential is formulated as the sum of history-dependent Gaussians which accumulate throughout the system to energetically disfavour (or "flood") previously visited free energy minima of the system. Metadyamics methods have recently been employed in a number of free energy simulations to model the ring puckering conformational path in pyranose sugars [47-49].

Dickson *et al.* developed an efficient sampling and free energy calculation technique within the adaptive biasing potential (ABP) framework [50]. The adaptive biasing force method [46, 51] follows the same basic principle, but relies on the build up of a biasing force as opposed to a biasing potential. Integration of the mean force measured along a chosen reaction coordinate yields the PMF. The biasing force is continuously updated at every step during the simulation. The mean force is estimated from an average of the instantaneous force exerted on the system.

For calculation of PMFs, it is often difficult to find appropriate collective coordinates that represent a specific conformational change. In order to be efficient, the baising potential has to be the function of a small number of collective variables (interatomic distances, angles, torsions and others) which must be appropriately chosen for successful calculation of the free energy. For example, Sega *et al.* have shown that the use of the wrong coordinates to define the saccharide ring effectively prevents ergodic sampling when high energy barriers are present [52]. Spiwok at al. proposed an approach that combines an extraction of collective motions of a molecular system with metadynamics sampling of its free energy surface. They used essential coordinates determined by essential dynamics (principle component analysis) as collective variables in metadynamics [53, 54].

2.1. Empirical Force Fields

There are a number of recently developed Molecular Mechanics force fields that have been specifically adapted for carbohydrates, including the GLYCAM series [55] of force fields for AMBER, OPLS-AA [56], GROMOS [57], and, for CHARMM, CSFF [33] and a new, redesigned comprehensive force field for cyclic and acyclic carbohydrates under active development by MacKerrel and co-workers [58-61]. Therefore, the choice of which force field to use for carbohydrate modelling is less of a concern than was previously the case [62]: a recent comparative study showed that the GROMOS, GLYCAM06, OPLS and CSFF force fields give reasonably consistent results and predict conformational equilibria more consistently than general organic force fields [63]. In addition, a free energy study of β-D-glucopyranose ring interconversion in water for three different carbohydrate force fields found all the three to describe the conformational behaviour of relatively accurately [49]. New coarse-grained models for carbohydrates [64] or new methods for continuum solvation [40] may also be used to increase the length- and time scale of the practicable simulations. Interestingly, comparison of Car-Parrinello molecular dynamics simulations with classical molecular dynamics simulations has suggested that CPMD simulations are necessary for studying the structural properties of carbohydrates in aqueous solution because of the much stronger intermolecular hydrogen bonding with surrounding water molecules shown by *ab initio* MD simulations [65, 66].

3. RING CONFORMATIONS

Monosaccharides, being at once the building blocks of larger chains and more tractable because of their small size, have received the bulk of attention for free energy studies of carbohydrates. Every carbohydrate residue has a variety of configurational options: D- or L-isomers, α- or β-anomers and one of a number of possible ring forms (e.g. pyranose or furanose rings). However, as pseudo-rotation interconversions between anomers and different linear and ring forms require bonds to be broken (precluding Molecular Dynamics) and are comparatively slow processes (on the order of seconds), simulation of these processes is out of the reach of current computational methods. Even calculation of the relative free energies and interconversion barriers of ring conformers in water for each of the various carbohydrate ring forms presents a considerable computational challenge, as saccharide rings typically present a number of possible metastable conformations separated by high energy barriers.

In general, carbohydrates in solution primarily have their ring conformations in the low-energy chair conformation. The pyranose form of the hexoaldose sugars in predominant in aqueous solution and the $^4C_1$ conformations is the prevalent state for all but D-idose. However, higher energy boat/skew boat conformations can be stabilised under different conditions, such as under application of an extension force [67-69] or the upon binding to a protein, examples being lectins [10, 70] or glycosidases [71].

All conformations of *N*-membered rings may be conveniently mapped by (*N*–3) puckering parameters, which therefore present suitable collective coordinates for the purpose of free energy calculations. Most frequently used are the long established Cremer-Pople puckering coordinates [72], although the equivalent Hill-Reilly puckering parameters have the possible advantage in that they follow the principle of least motion: puckered rings that are close in conformation are also close in terms of sum of the change in each of the puckering coordinates [73]. For six–membered rings, the Cremer-Pople polar coordinates (Θ,ϕ,Q) map all ring conformations onto the surface of a sphere, with the radial coordinate, Q, representing the total puckering amplitude (the degree of puckering) and the Θ and ϕ angles the type of puckering. Polar positions on the sphere ($\Theta = 0$ or π) correspond to chair conformations, and the equatorial positions ($\Theta = \pi/2$) to alternating boat ($\phi = n\pi/3$, n=0,..,6) and skew–boat ($\phi = n\pi/3 + \pi/6$, n=0,..,6) forms.

For the purposes of free energy calculation, Sega *et al.* have shown that the orthogonal Θ and ϕ Cremer-Pople coordinates are sufficient for a quasi-two dimensional potential of mean force calculation, as the puckering amplitude is tightly bound by the limited extension of chemical bonds [52]. Further, they show that biasing forces have to be applied tangent to the sphere surface in order to achieve sampling of the entire configuration space. With polar parameters, applied biasing forces are always tangent to the polar sphere surface, while with the equivalent Cremer-Pople Cartesian parameterization the biasing forces are applied parallel to the equatorial plane, which effectively prevents ergodic sampling when high energy barriers are present. Insufficient sampling because of incorrectly chosen forces leads to errors in barrier estimation, as is the case with the study of Biarnés *et al.* [47] where the use of two Cartesian Cremer-Pople parameters resulted limited exploration of the pucker space. However, most ring free energy calculations have avoided the ergodicity problem raised by Sega *et al.* by employing a computationally expensive three-dimensional reaction co-ordinate comprising three puckering parameters, analysing the free energy profile across isosurfaces of this volume [42, 48, 49, 74].

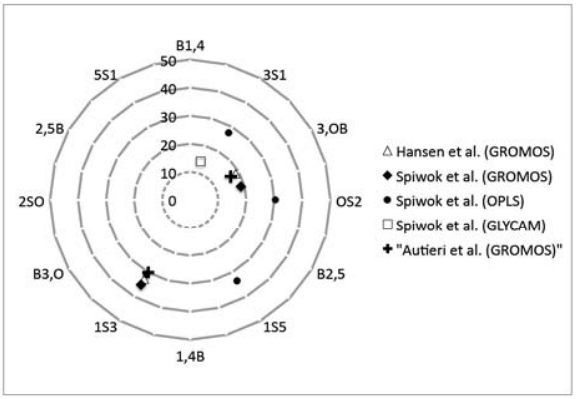
Polar Cremer-Pople puckering coordinates were recently used effectively by Sega and co-workers in the first systematic calculation of the puckering free energy landscapes for the series of α- and β-aldohexoses using the GROMOS 45a4 parameter set and a combined metadynamics-umbrella sampling approach [35]. The conformational free energy landscape of β-D-glucopyranose has been the most intensively explored of all carbohydrate rings, in both vacuum and solution, and hence comparisons of the calculated stable states of the distorted ring conformations, their energies, and the transition states of the ring interconversion among the distorted ring conformations can be made most easily for this system. There are 14 canonical puckering states of the pyranose ring that minimize the angle strain: 6 possible distinct boat forms ($^{1,4}B$, $^{2,5}B$, $^{3,O}B$, $B_{1,4}$, $B_{2,5}$ and $B_{3,O}$), 6 skew-boat forms ($^1S_3$, $^1S_5$, $^3S_5$, $^3S_1$, $^5S_1$, $^5S_3$) and 2 chair forms ($^4C_1$ and $^1C_4$).

Biarnés *et al.* used *ab initio* metadynamics to compute the conformational free energy landscape of β-D-glucopyranose in vacuum as a function of two Cremer-Pople Cartesian puckering coordinates [47]. Barnett and Naidoo modelled the glucose molecule in vacuum only using CHARMM and the PM3CARB-1 semi-empirical potential and the Hill-Reilly puckering parameters [74]. Hansen and Hünenberger calculated a 3D free energy volume for β-D-glucopyranose using a novel local elevation umbrella sampling (LEUS) method using GROMOS with the GROMOS 45a4 force field in vacuum, SPC water solution and three out-of-plane Pickett dihedrals to define the ring pucker [42]. Spiwok *et al.* calculated the conformational free energy surface using a metadynamics method in GROMACS 3.3.3 in the space of three Cremer-Pople ring-puckering coordinates with the GLYCAM, OPLS and GROMOS 45a4 carbohydrate-tuned force fields in both vacuum and explicit SPC water [49].

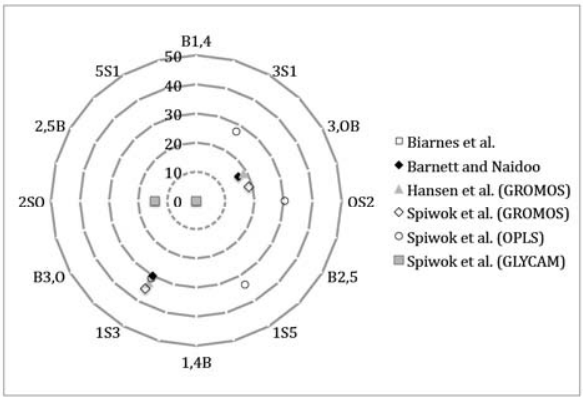
Reassuringly, all free energy studies of β-D-glucopyranose to date [42, 47, 49, 74] identify the $^4C_1$ chair as the global free energy minimum in aqueous solution, in agreement with empirical evidence [2, 75]. The three GROMOS 45a4 calculations are in close agreement, both with respect to the minima identified and their relative free energies, indicating that the free energy surface calculated is not sensitive to the puckering parameters or the method employed to enhance sampling (Table **1** and Fig. **1**). The calculated free energy for the $^1C_4$ conformation differs most in the work of Hansen *et al.*, suggesting a possible lack of convergence in this calculation.

Table 1. Comparison of Reported Relative Free Energies for β-D-glucopyranose Ring Conformational Minima in Water Solution and Vacuum (italics). For Hansen *et al.*, the Values Quoted are for their MS Scheme. GROMOS Refers to GROMOS 45a4 in all Cases. The GLYCAM Results Quoted Refer to the Study with the requisite1-4 Scaling

	ΔG (kJ.mol^{-1})									
	Biarnés et al.[47]	Barnett et al.[74]	Hansen et al.[42]		Spiwok et al.[49]					Autieri et al.[35]
Conf.	*ab initio*	PM3CARB-1	GROMOS		GROMOS		GLYCAM		OPLS	GROMOS
$^4C_1$	*0*	*0*	*0*	0	*0*	0	*0*	0	*0* 0	0
$^1C_4$		*20.3*	*12.2*	16.2	*7.7*	11.6	*48.3*	40.2	*11.8* 26.8	10.0
$B_{3,O}$	*10.9*	*8.4*	*25.3*							
$B_{3,O}/^2S_O$	*12.6*						*14.4*			
^2S_O								14.2		
$^{2,5}B$										
$^{2,5}B/^5S_1$	*37.7*									
$^5S_1$										
$B_{1,4}$	*33.1*	*33.1*								
$B_{1,4}/^3S_1$									*36.9*	
$^3S_1$									27.5	
$^{3,O}B$	*30.1*	*25.3*		18.7	*33.0*					16.2
$^{3,O}B/^OS_2$						18.7				
$^OS_2$		*22.9*							*34.0* 30.4	
$B_{2,5}$	*23.0*									
$^1S_5$	*24.3*	*23.6*							33.5	
$^{1,4}B$										
$^{1,4}B/^1S_3$	*26.4*									
$^1S_3$			*26.1*	32.8	*29.1*	35.1			*25.5* 31.0	≈ 30



(a)



(b)

Fig. (1). Radar plots comparing the ring boat and skew-boat conformational minima and associated energies (kJ.mol) for various free energy studies on β-D-glucopyranose in (**a**) vacuum and (**b**) water, projected onto Stoddart's pseudorotational map of glucose conformers.

However, differences between force fields are considerable, both in the number of secondary minima identified (ranging from 3 to 9), their identity, order and relative energies (Table **1**). Both the GROMOS force field and OPLS considerably favour the $^4C_1$ chair over any boat conformations, with calculated ΔG relative to $^1C_4$ chair in the ranges 7-13 kJ.mol (vacuum) and 11-27 kJ.mol (water) [42, 49]. However, both the GLYCAM force field (vacuum or solution) and the vacuum ab initio and semi-emiprical free energy calculations identify boat/skew-boat conformations in the region $B_{3,O}$ – ^2S_O as the primary secondary minimum (ΔG in range 8-15 kJ.mol), with the $^4C_1$ chair a ΔG in range 20–50 kJ.mol [47, 49, 74]. (Note that in the work of Biarnés *et al.* the $^1C_4$ region was not sampled). In particular, Barnett and Naidoo 's relatively energy value of 20.3 kJ.mol for the $^1C_4$ conformation is markedly higher than those calculated with molecular mechanics force fields, but in closer agreement with Quantum Mechanical lowest energy $^1C_4$ conformational energies in the literature (27.8 kJ.mol) [18].

Additional calculations by Sega and coworkers has further highlighted this over-stabilization of the $^1C_4$ conformer by the GROMOS parameter set, which fails to reproduce proper free energy differences between chair conformers for many of the monosaccharides- particularly the galactose, mannose and allose monosaccharides, where the experimentally non-detectable inverted chair conformers are substantially populated [35, 52]. For idose, which is the only experimentally known aldohexose that shows equilibrium between chair and inverted chair, there was reduced population of the inverted chair conformation. These free energy calculations were used to suggest a modification to the GROMOS 45a4 parameter set, which improves the match between simulation results and theoretical and experimental estimates of puckering free energies. A similar modification is expected to be necessary for the OPLS parameter set.

The GLYCAM parameter set is an interesting case. The GLYCAM force field produces relative energies for the closest to QM results in vacuum, identifying the $B_{3,O}$ boat conformer as the lowest energy minimum after $^4C_1$. However, when the 1-4 interaction scaling standard for AMBER-type force fields is removed, GLYCAM shows similar behaviour to the other force fields, with the $^1C_4$ chair favoured over boat conformations (values not reported here) [49]. However, amore comprehensive study is required to determine the

accuracy of the GLYCAM force field for representation of ring conformer populations: unconstrained molecular dynamics simulations of the monosaccharide 2-O-sulfo-*alpha*-L-iduronic acid (IdoA2S) showed that, while the GROMOS96 force field predicted the skew-boat to chair conformational ratio in good agreement with the experiment, GLYCAM06 showed worse agreement [76].

Another interesting point is that, although the $B_{1,4}$ conformation has been proposed to form part of the stretching response of glucans [67, 68] only the vacuum studies identify this as a minimum, and then at a high energy value of 33.1 kJ.mol.

All studies show the barrier heights for inter-conversion between primary and secondary ring pucker minima to be high - a range of 20-40 kJ.mol is reported, in agreement with recent QM calculations [20].

Solvation effects also vary across the force fields. Solvent was found to destabilize the $^4C_1$ chair relative to the $^1C_4$ conformation in all but the GLYCAM force field. Solvent has a considerable stabilizing effect on the related skew-boat/boat conformations $^3S_1$, $^{3,O}B$ and $^OS_2$ of 5 to 13 kJ.mol and a destabilizing effect of about 6 kJ.mol on the related skew-boat/boat conformations $^1S_3 - B_{3,O}$ in the GROMOS and OPLS force fields. Consensus across the studies appears to be that, while the $B_{3,O}$ boat is preferred in vacuum, in water the $^{3,O}B$ boat becomes more stable. The free energy surface of ionized N-acetylneuraminic acid in the GLYCAM06 force field calculated by Spiwok *et al.* showed a high effect of a water environment on its conformation [48] as compared to β-D-glucopyranose.

Free energy calculation on other sugar rings have been very limited, providing little scope for comparison. Two studies of glucoronic acid using GROMOS [52] and GLYCAM [77] agree on the $^4C_1$ conformation being considerably more stable than the $^1C_4$, but differ as to the tertiary minima. A study of N-Acetylneuraminic (sialic) acid in vaccum and explicit solvent with the GLYCAM force field [48], reproduced the experimentally observed global minimum $^2C_5$ chair conformation, which is unsurprising as the molecule formed part of the training set for GLYCAM06.

4. GLYCOSIDIC LINKAGES

The heterogeneous sugar residue building blocks can be linked in a variety of ways to generate very diverse, potentially branching, oligo- and polysaccharide structures. Each linkage usually has a number of possible low energy conformations, which are difficult to determine experimentally. If a single ring pucker conformation is assumed (such as the preferred $^4C_1$ conformation in pyranose rings), oligosaccharide 3D structures and conformation can be assumed to be determined only by their glycosidic linkage geometries, as a function of composition, linkage type, and solvation [78]. Hence, a direct approach to 3D structure determination is to build a 3D model of an oligosaccharide from known monosaccharide structures together with calculated preferred conformations of the constituent disaccharide fragments [79]. The use of solution conformations of isolated disaccharides to build the carbohydrate moiety of glycoproteins has been shown to be an effective approach to building models of oligosaccharides [80-82]. For these procedures, it is essential to have reliable estimates of the conformational space available to a glycosidic linkage.

A straightforward approach to evaluating the conformational preferences of a disaccharide is to construct an energy contour map, where glycosidic linkages are described in terms of relevant dihedral angles - ϕ, ψ and, in the case of O_6-linkages, ω. A simple ϕ, ψ Ramachandran plot for a disaccharide gives some insight into preferred conformations and can, with care, be extrapolated to the prediction of polysaccharide structures [14, 78, 80, 81, 83]. Adiabatic Ramachandran Maps have been calculated for many disaccharides [84-87]. However, the original Molecular Mechanics methods for determination of a Ramachandran Maps have been largely superceded by more accurate quantum mechanical calculations of the relative energies of carbohydrate conformtions [19]. However, these remain potential energies and, as a global search is not performed, the question remains of whether a global energy conformation has been identified. Calculated free energy potential of mean force (PMF) maps are inherently more reliable as they incorporate entropic effects. In addition, if performed in solution, PMF calculations reveal the effect of solvent on a glycosidic linkage.

The ubiquitous α-(1→4) linkage was the first to be subject to free energy calculations. A ϕ, ψ PMF for a disaccharide - sucrose- in solution was calculated as early as 1995 [88]. However, this procedure used very short simulation times (a total of 3.9 ns) and it is therefore unlikely that the calculations converged sufficiently. Both maltose (4–*O*–α–D–glucopyranosyl–α–D–glucopyranose) and dixylose (4–*O*–α–D–xylopyranosyl–α–D–xylopyranose) were initially investigated with partial PMF calculations restricted to a central ϕ,ψ region because of sampling difficulties [89-91]. We followed these initial studies some years later with a complete conformational free-energy map for solvated maltose [69], using the CSFF force field. Encouragingly, our prediction of an equilibrium between the *syn* and the *anti* conformer for this molecule were later supported by ultrasonic absorption spectrometry measurements of dilute maltose solutions [3]. We later extended this work to the Glc-α-(1→4)-Glc linkage in trehalose [15].

Of primary interest in calculated PMF's for glycosidic linkages is the location of the minima and the effect of solvation, which differs dramatically for different disaccharides. For example, Ueda *et al.* found a considerable solvent shift from the potential energy map in vacuum for neocarrabiose [92]. This new global minimum-energy solution conformation was found to correspond to the experimental value obtained from NMR-NOE measurements and the experimental crystal structure. However, we found maltose to have a small conformational shift on solvation and trehalose very little at all. For globobiose (α-D-Gal-(1→4)-β-D-Gal), substitution of a high dielectric constant for explicit water solution to be a valid approximation for reproducing the minimum energy conformation of this glycosidic linkage - showing that expensive explicit solvation is not necessary. However, others have found that the lowest-energy structure in implicit solvent does not usually correspond to the free-energy minimum and that explicit hydrogen bonding to the solvent is fundamentally important [80, 81].

However, the range of PMF's calculated for disaccharides is by no means comprehensive. The important cellulose Glc-β-(1→4)-Glc linkage has received very little attention [93] and, to date only a few free energy studies on β- or α-linked galactans have been reported [92, 94]. There have been few attempts at systematic studies, as have been done for Ramachandran maps [85]. Campen *et al.* calculated the relative free energy of a series of 12 disaccharides was determined in vacuum using replica exchange molecular dynamics simulations, but this study does not allow for any estimation of solvent effect [95].

There have also been very few attempts to compare the glycosidic linkage PMFs across various carbohydrate force fields. In addition, none of these studies explored different ring conformations.

5. OLIGOSACCHARIDES

If free energy studies on the glycosidic linkages are rare, those attempting to directly investigate the potential of mean force along a collective coordinate for larger polysaccharides are even rarer. Some years ago,we calculated the end-to-end distance-free energy profile for a maltohexaose oligosaccharide as a function of rotation about the central dihedral angle. This calculation was integral to explaining the force-extension behaviour of the amylose polysaccharide [69].

A related recent study by Banavali and Mackerrel probed the behaviour of all localised torsions in a DNA duplex simultaneously in the background of the overall structural change involved in single base flipping, using umbrella sampling with restraints along a pseudo-dihedral reaction coordinate [34]. The free energy landscapes for backbone torsion and sugar pucker degrees of freedom in the DNA assisted in describing its behavior in response to the base flipping perturbation: a free energy difference of up to 14 kcal/mol can be attributed to the flipped state relative to the stacked Watson-Crick base paired state. This two-state classification allows precise evaluation of the effect of base flipping on local backbone degrees of freedom. A similar studies on the affect on ring flipping on backbone dynamics in polysaccharides are likely to be very informative, but as yet none have been attempted.

6. CONCLUSIONS

The small range of free energy calculations performed to date have provided valuable insights into carbohydrate conformation and dynamics and the effect of solvent, while also exposing the limitations of some carbohydrate force fields.

The continuing rapid year-on-year increase in computational power combined with the parallelization of Molecular Dynamics simulation codes is expected to render free energy studies ever more accessible. There is thus great potential for more systematic, comprehensive studies of the conformations of the variety of saccharide rings and glycosidic linkages to effectively map the conformational space available to these highly variable molecules. This would also allow for more extensive validation of the existing and new carbohydrate force fields. For example, the issue of ring puckering has been too long ignored - comprehensive ring puckering studies should be performed for all carbohydrate force fields to ensure that they produce ring conformer populations consistent with experimental data.

Ultimately QM free energy studies will be within reach, allowing for simulation of bond-breaking reactions and hence facilitating a much deeper understanding of of carbohydrate conformation, dynamics and reaction mechanisms than we have today.

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