Calculation of the Potential of Mean Force for the Binding of Glucose to Benzene in Aqueous Solution

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Molecular dynamics simulations employing umbrella sampling techniques have been used to calculate the potential of mean force (pmf) for the binding of a β-D-glucopyranose molecule to a benzene molecule in aqueous solution, as a model for the binding of sugar substrates to phenylalanine residues in proteins. The interaction of these two molecules was found to be strongly affected by hydration, as expected, with their nonpolar faces pairing by hydrophobic association to minimize the exposure of apolar groups to water. The pmf for the approach of these molecules is oscillatory in character, with two primary low-energy minima separated by a high free energy barrier, and with the net binding energy being approximately 1.1 kcal/mol and the intervening barrier being almost 2 kcal/mol. A third, weaker minimum was observed between 11 and 12 Å. The oscillatory nature of the pmf results from the successive removal of water layers between the two molecules as they approach one another.

I. Introduction

It has become increasingly clear in recent years that carbohydrates play a role in a variety of important molecular recognition processes. Often, recognition involves a carbohydrate molecule binding very specifically to a particular site in a protein which has been designed by natural selection to be highly complementary to the positions of functional groups in the carbohydrate. This precise design of carbohydrate binding sites is necessary for two reasons: (1) the simple sugars are all structural isomers of one another, differing only in their stereochemistry at the various asymmetric carbon atoms; (2) most sugars are quite soluble in water due to their hydrogen bonding hydroxyl groups. The highly favorable interactions with water, and the clear loss of translational and rotational entropy on the part of the sugar upon binding, mean that a protein binding site must also have very favorable interactions with the sugar molecule. The relatively small differences in the various sugars means that where specificity is required, precise positioning of the receptor functional groups is necessary.

The availability in recent years of high-resolution crystallographic structures for proteins which bind carbohydrates has made it possible to make certain generalizations about the characteristics of carbohydrate binding sites, as has been done by Quiocho for a series of sugar-binding proteins.[1−4] From the crystal structures, it can be seen that proteins use hydrogen bonding side chains to take the place of hydrogen bonds to the solvent. However, topological constraints make it impossible for the protein to provide three such hydrogen bond partners for each hydroxyl group, as is possible in water,[5] so the majority of protein−sugar hydrogen bonds involve acid side chains. These charged groups make hydrogen bonds which are much stronger than those made by neutral groups,[6] compensating for the smaller overall number of bonds.

Protein binding sites also use hydrophobic interactions to increase their affinity for carbohydrates. The hydroxyl groups of carbohydrates are held at approximately fixed relative orientations by the complex stereochemistry of each sugar molecule and by the rigidity of sugar pyranose rings. For glucose, with all of its hydroxyl groups in equatorial positions in the $^4C_1$ conformation,[7] the "top" and "bottom" of the molecule are hydrophobic, made up of the aliphatic C−H surfaces, while the equatorial periphery is quite hydrophilic.[8] The binding sites of proteins which recognize this sugar thus also contain hydrophobic residues such as phenylalanine or tryptophan to provide an extended hydrophobic surface for the nonpolar faces of the sugars to dock against. A sugar which binds in such a site thus either suffers no loss in hydrogen bonding energy, or very likely experiences a strengthening of these interactions, while simultaneously removing its hydrophobic surfaces from contact with water.

As might be expected, enzymes which have carbohydrate substrates generally have binding sites which exhibit many of these same features. This is also true for enzymes such as glycosidases, including the amylases and cellulases, which have polysaccharide substrates, as is illustrated in Figure 1 for a cellulase.[9] In these cases, there is often an extended binding region containing subsites which interact with successive residues in the polymer chain. A particularly recognizable feature of many such binding sites is a series of hydrophobic residues, approximately parallel and aligned along the length of the binding groove, as in the cellulases E1 from *Acidothermus cellulolyticus*,[9] E2 and E4 from *Thermomonospora fusca*,[9−11] and CBHII from *Trichoderma reesei*.[12] Designing improved versions of these enzymes through mutagenesis[13] would be greatly facilitated by both a quantitative and qualitative understanding of the role of various interactions in substrate binding. In particular, it would be useful to determine the effect on binding energy of a point mutation changing one of the aromatic residues of the active-site groove to some other residue, such as alanine.

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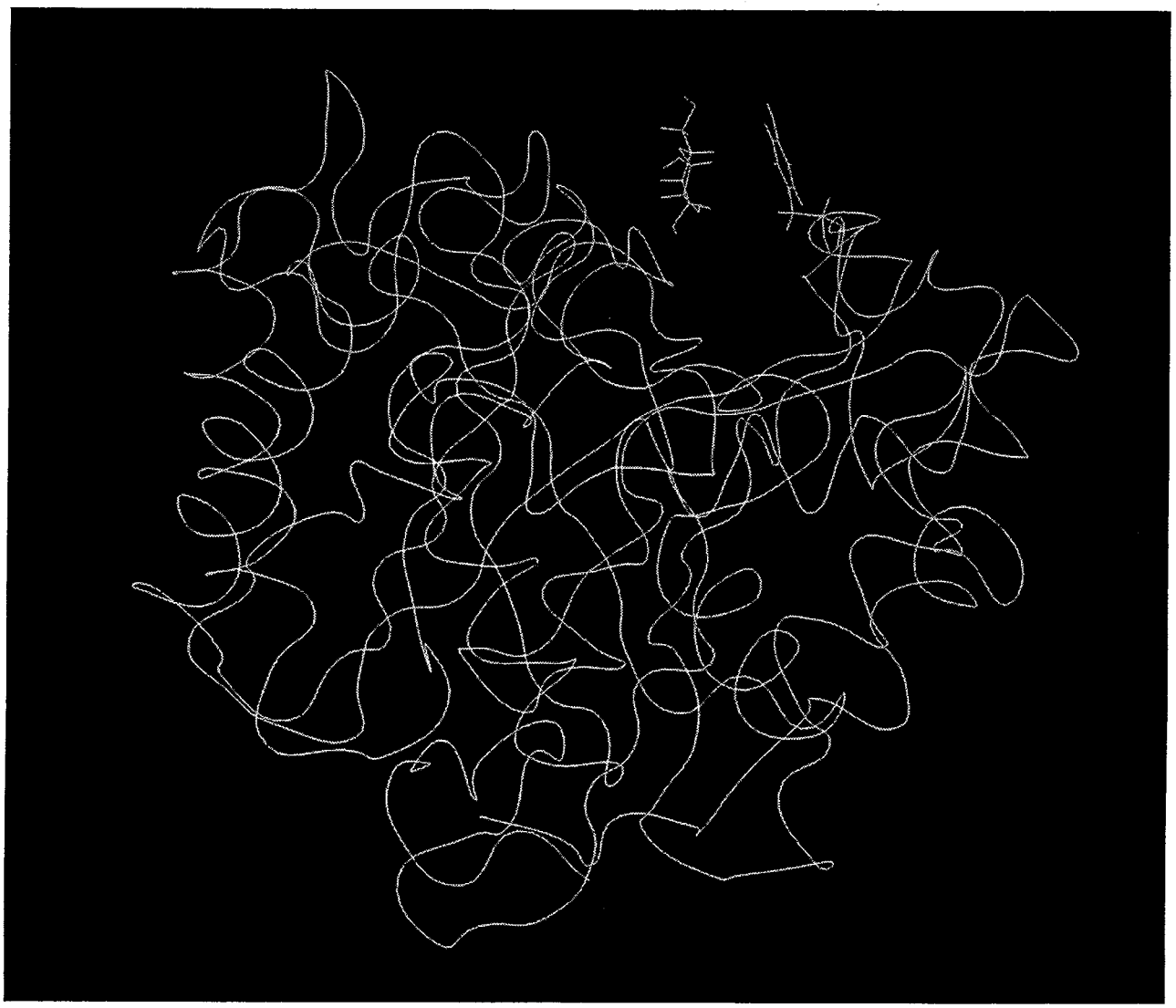

Figure 1. The crystal structure of the cellulase enzyme E1 from *Acidothermus cellulolyticus*, showing the terminal sugar residue (on the reducing end) of a tetrasaccharide substrate stacked against the aromatic ring of Tyr 245. Apart from residue 245, the protein is shown only as a backbone trace.

In principle, it is possible to calculate the change in binding free energy for such a mutation from molecular mechanics (MM) computer simulations, as in free energy perturbation calculations of point mutants using Monte Carlo (MC) or molecular dynamics (MD) simulations.[14] It is also possible to calculate the free energy change as a function of separation distance, called the potential of mean force (pmf), using techniques such as umbrella sampling. Using this approach, to calculate the differences between point mutants, it is necessary to perform the pmf calculation twice, once for each amino acid, and then to take the difference between the two functions. The principal advantage of calculating pmf's is that the detailed information they provide allows an in-depth analysis of the factors contributing to the overall process, thus potentially facilitating rational design decisions. In this paper, we report the calculation of the potential of mean force for the binding of a glucose molecule to the face of a benzene molecule in aqueous solution as a model for the binding of sugars to phenylalanine residues in protein binding sites.

It is reasonable to expect that the presence of solvent water molecules will significantly affect the pmf for molecules in aqueous solution. It is generally understood that water plays an important part in determining the conformational structure and properties of biopolymers, causing globular proteins, for example, to assume their characteristic folded shapes, or maintaining the bilayer structure of lipid membranes. For this reason, it is often necessary to include the effects of hydration in theoretical simulations of such systems.[15,16] Liquid water is a highly structured fluid due to its extensive hydrogen bonding, and the inability of aliphatic and aromatic species to make strong hydrogen bonds causes these molecules to impose considerable structure on adjacent water molecules as they reorganize to make all of their hydrogen bonds to other water molecules. This reorganization can have profound effects on the free energy of interaction of hydrophobic species.

Potentials of mean force have been calculated previously for the approach of two small, spherical nonpolar molecules such as neon or methane in aqueous solution.[17−19] In a vacuum, as these two spheres approach, the pmf is the same as the potential energy between the two molecules, generally represented with a function like the Lennard−Jones or Buckingham potentials, which have steep repulsive walls at close separations, and approach zero for large separations, but which exhibit a single well at intermediate distances up to the point of contact. The

pmf for the approach of such molecules in aqueous solution, however, is much more complex due to the averaging over the behavior of the surrounding water molecules. In solution the pmf was found to have a narrow primary minimum at the contact separation and a broad but shallower minimum at a distance large enough to allow solvent separation between the two spheres.[17,18] The barrier between the two minima, not present on the vacuum pmf, can be thought of as arising from the energy necessary to "squeeze" the last solvent from between the two spheres as they approach.

The present problem in some ways resembles the problem of the interaction of two benzene molecules. In the gas phase these molecules experience an attraction due to dispersion forces. In aqueous solution, however, they will be driven together by hydrophobic forces, since benzene cannot strongly hydrogen bond to the water, a process which eventually leads to phase separation. (The extent and strength of benzene−water "hydrogen bonds" is still a matter of debate, but all estimates find these interactions to be weaker than water−water interactions, and thus unable to compete for hydrogen bond partners in aqueous solution).[20,21] It thus would be expected that the potential of mean force for the association of two benzene molecules would be very different in vacuo and in aqueous solution, as in the case for the methane molecules in solution.

Several potentials of mean force have been calculated for benzene molecules associating in water using either MD or MC simulations.[22−25] Calculations have been reported for both the case where the benzene rings are parallel and for the case where they are perpendicular to one another. As in the pmf's for nonpolar spheres, these pmf's exhibited two minima, one at the contact distance and one at much larger distance, measured as the distance between the centers of the two rings, and corresponding to a solvent-separated dimer pair. In each of these calculations, however, the pmf indicated a net attractive interaction relative to infinite separation, reflecting the tendency of benzene to self-associate in aqueous solution and to separate out as a distinct phase. A pmf has also been calculated for α-D-glucopyranose adsorbing onto a graphite surface, which also exhibits a similar two-minimum character and a net tendency for adsorption from aqueous solution.[26] Considering the results of these studies, it would be reasonable to expect similar behavior in the pmf for glucose approaching benzene in water.

II. Methods

The pmf for the approach of β-D-glucopyranose and benzene in aqueous solution was calculated from the separation distance probability distribution function as observed from molecular dynamics simulations. Because standard MD simulations rarely sample high-energy configurations, statistically meaningful results would in general require impossibly long simulation times to fully explore all of the conformations of interest, such as the saddle regions of barriers between low energy wells. To overcome this problem, a number of related free energy simulation techniques have been developed.[27−29] In the present simulations, we employed simple harmonic umbrella potential functions of the separation distance, as implemented in the general MM program CHARMM,[30] to increase the probability of sampling high-energy regions.[31,32] The pmf without the biasing umbrella energy function, $W(R)$, can be calculated from the probability density distribution function observed from the MD trajectory calculated with umbrella potentials, $P^*(R)$,

$$W(R) = -kT \ln P^*(R) - V_U(R) + C$$

where V_U is the potential augmented by the umbrella function, $\langle\ \rangle_U$ indicates averaging over the ensemble augmented by the umbrella function, and C is an arbitrary constant.[33,34]

The umbrella potential functions were applied to an otherwise conventional series of MD simulations of the sugar and benzene in aqueous solution. In these MD simulations, the solute pair was modeled surrounded by water molecules in a cubic box 24.6481 Å in length subject to periodic boundary conditions.[35] The simulations were initiated by placing the sugar in contact with a benzene molecule, aligned in a "stacked" or parallel arrangement, with the glucose molecule oriented such that the face making contact with the benzene is the one made up of the axial C1, C3, and C5 protons. This dimer was then placed in the center of a previously equilibrated box of 512 TIP3P water molecules,[36] and those solvent molecules which overlapped with the solute heavy atoms were removed, which produced a primary system containing 494 water molecules and the two solute molecules. The benzene force field was taken from the new CHARMM23 parameter set,[37] and the sugar molecule was modeled using a CHARMM-type force field developed for use with sugar molecules.[38] A typical "snapshot" configuration of these molecules in the primary water box is illustrated in Figure 2.

The free energy profile was calculated through a series of simulations in which the solute pair was restrained to remain at a particular distance through the application of umbrella potentials. These umbrella potentials consisted of a harmonic restraining force placed on the intermolecular distance R calculated between the respective centers of mass, and of additional harmonic restraining potentials placed on the distances between the H1, H3, and H5 atoms of the sugar molecule and the C1, C3, and C5 atoms, respectively, of the benzene molecule. These latter terms were needed to keep the two molecules in the parallel stacked arrangement, since the simulations were not long enough to adequately sample over all possible alternative orientations. At each selected distance, beginning with 3.25 Å, 100 ps of dynamics were calculated, with only the last 50 ps used to compute average values. After each simulation, the r_0's of the harmonic umbrella and angular restraint terms were increased by 0.25 Å, and the molecules were then allowed to diffuse toward one another under the influence of these forces, which is the reason for disregarding the first 50 ps as equilibration. The force constant for the umbrella term was selected to be 25 kcal mol^{-1} Å^{-1}, and that for the angular restraints was 10 kcal mol^{-1} Å^{-1}. Fifty separate "windows" were used over the distance range studied, corresponding to fifty individual 150 ps simulations, with good overlap between adjacent windows.

The simulations were performed in the (NVT)-ensemble using a Langevin equation of motion, with a bath temperature of 300 K. Langevin simulations were employed because these trajectories equilibrate more quickly with fewer long-lived, anomalous energy inhomogeneities than microcanonical ensemble trajectories. The trajectories were integrated with a Verlet integration algorithm[39] and a step size of 1 fs. Chemical bond lengths involving hydrogen atoms, and the geometries of the water molecules, were kept rigid using the constraint algorithm SHAKE.[40] The trajectories were initiated by assigning starting atomic velocities from a Boltzmann distribution at 300 K. The large number of nonbonded interactions in the solution simulations required that long-range interactions be smoothed to zero between 10 and 12 Å using switching functions applied on a neutral group basis. This treatment of long-range interactions

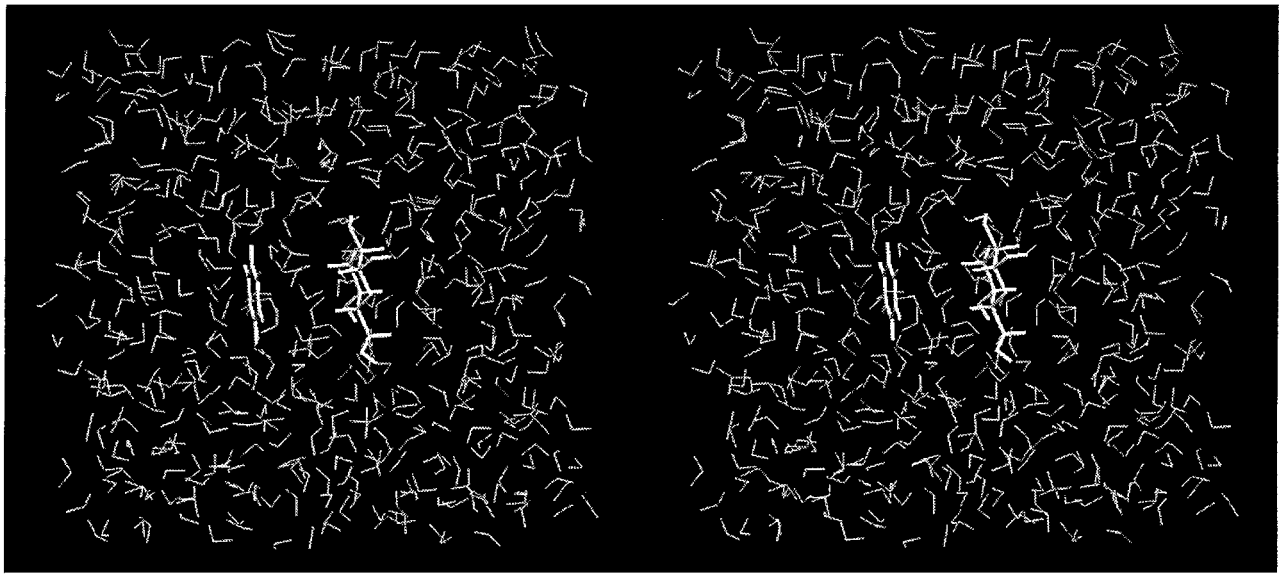

Figure 2. A stereopair illustrating a typical orientation for the solute molecules during the simulation.

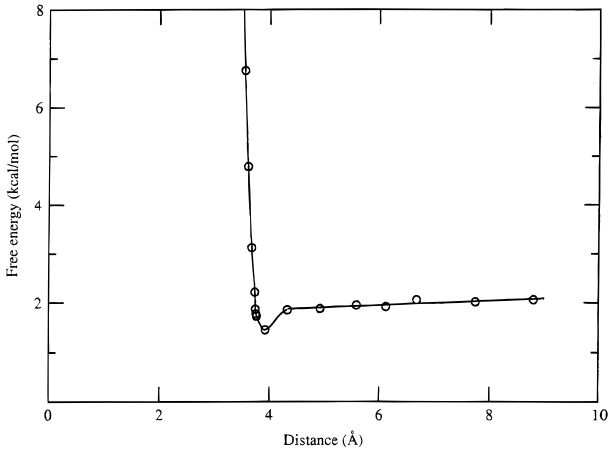

Figure 3. The calculated potential of mean force for β-D-glucopyranose approaching a benzene molecule in a vacuum in the parallel stacked arrangement. The distance R is calculated between the centers of the two rings. The continuous curve in this figure represents two eighth-order polynomials fitted to the data and arbitrarily splined together.

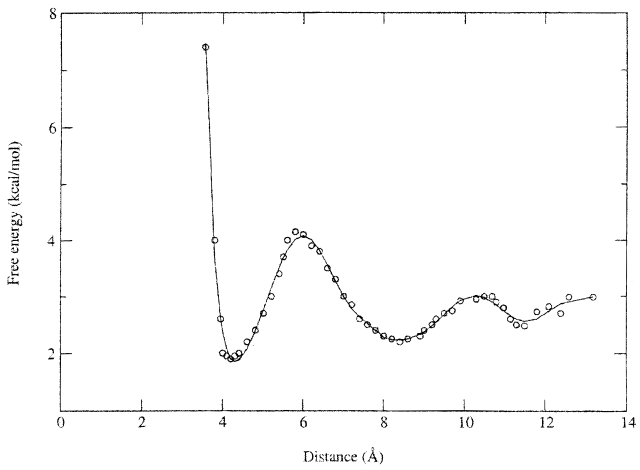

Figure 4. The calculated potential of mean force between stacked β-D-glucopyranose and benzene in aqueous solution. The continuous curve in this figure represents two eighth-order polynomials fitted to the data and arbitrarily splined together.

has previously been demonstrated to reduce truncation artifacts to negligible levels.[30,41]

III. Results and Discussion

Figure 3 displays the calculated pmf for the glucose/benzene pair in a vacuum as a function of the distance between the centers of the two rings. As can be seen, this function essentially reflects the summed van der Waals interactions of the two molecules, with a shallow minimum at 3.9 Å. This distance is slightly larger than the vacuum minimum-energy distance in the previously reported benzene dimer studies, due to the axial projection of the aliphatic sugar protons. The depth of the attractive well is only about half that previously reported for stacked, or parallel, benzene dimers in a vacuum,[25] presumably also due to the axial projection of the sugar protons keeping the rings apart. Thus, unlike the benzene−benzene case, in which 24 atoms in two molecules are approximately at their optimal contact distances, maximizing their favorable van der Waals interactions, in the present case only three proton atoms from the sugar make such optimal interactions with the benzene molecule, while the rest of the sugar atoms are farther away from the benzene atoms. The previous benzene study found that a "T-shaped" perpendicular arrangement of the molecules produced an even stronger binding energy, more than 2 kcal/mol using the AMBER force field. As noted in the Methods Section above, the present study did not consider nonstacked arrangements.

The presence of water around the two solute molecules has a major effect on their free energy of association, as has been previously seen for two benzene molecules in water and for glucose approaching a graphite surface through an aqueous medium. As expected, the structuring of water over the two hydrophobic faces of these ring-shaped molecules leads to a greater free energy of association than found in a vacuum, and also to an oscillatory character to the free energy profile as a function of distance, as seen in Figure 4. The continuous curve in this figure represents two eighth-order polynomials fitted to the data and arbitrarily splined together, and is not the result of an analytical theory. The calculated pmf exhibits a large barrier splitting the broad shallow minimum of the vacuum function into two deeper minima (Figure 4). The pmf exhibits a primary minimum at 4.2 Å, essentially the contact distance, and a weaker secondary minimum at a distance of 8.4 Å, with the rings

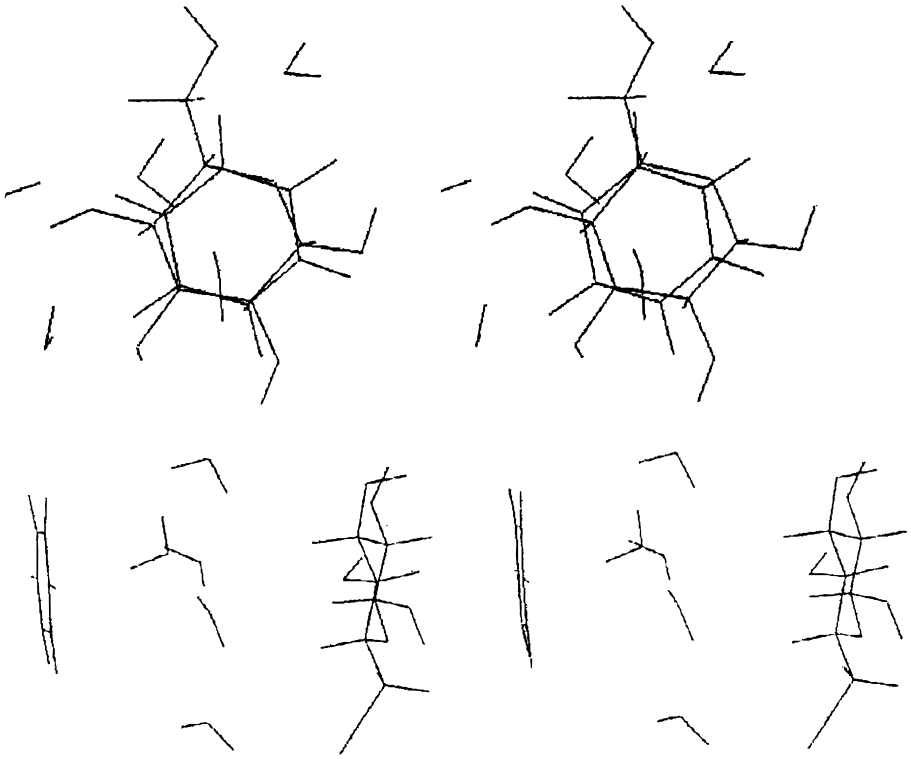

Figure 5. Two different stereoviews of a typical dynamics "snapshot" showing the orientations of those water molecules between, or close to being between, the two solute molecules for the separation distance of 8.4 Å, corresponding to the second minimum in the calculated pmf.

separated by a layer of specifically structured solvent. The approximate depths of these minima and the height of the barrier separating them are similar to, but again somewhat smaller than, those seen for glucose on graphite, calculated using the AMBER force field.[26] The binding affinity of glucose for a benzene molecule was found to be about the same as that calculated for two stacked benzene molecules for one another in aqueous solution, and less than that for perpendicular "T"-shaped dimers.[22,23,25] The barrier between the secondary and primary minima is almost 2.0 kcal/mol, centered at a distance of 5.8 Å, but the primary minimum is only 0.3 kcal/mol lower in energy, and only 1.1 kcal/mol lower in energy than the completely separated pair. Because it is broader, the secondary minimum is more probable than the primary one, referenced to the energy zero. A weaker third minimum centered around 11.5 Å corresponds to the two rings separated by two water layers. The barrier between this minimum and the secondary minimum is only 0.52 kcal/mol. This energy profile must significantly affect the kinetics of binding; as the molecules approach, they would be fairly stable at the solvent separated distance, and would presumably remain at this distance for some time before actually surmounting the barrier and making direct contact. Considering both wells, the contact geometry would not be significantly more stable than the solvent-separated arrangement because this secondary minimum is less narrow.

The oscillatory character of the pmf is typical for surfaces approaching one another in liquid solutions, where the distance between minima roughly corresponds to the solvent molecular diameter and where the oscillations typically damp out after a few periods.[42] In the present pmf the distance of 4.2 Å between the first and second minima is somewhat larger than the dimensions of a water molecule, while the distance of 3.1 Å between the second and third minima is quite close to the diameter of water. The second minimum of the pmf corresponds to a separation in which a layer of water most favorably structures itself between the two molecular surfaces (see Figure 5), and the large barrier between the first two minima can be thought of qualitatively as resulting from the energy needed to "squeeze" this layer of water out from between the two molecules as they approach. As can be seen from Figure 5, which represents a typical snapshot of the water configurations between the rings at the solute separation distance of 8.4 Å, the waters interposed between the two faces are oddly structured, with one pointing a proton at the benzene ring but with others almost parallel to the planes of the two rings as they make hydrogen bonds to water molecules which are not between the solutes. This snapshot configuration is very typical of those examined in that there is little water density along the center line directly between the two solutes, with most of the intervening water molecules residing along a circle above the carbon ring of the benzene. Once the last of these intervening water molecules has been removed, the two solute molecules can move together without resistance, and in fact are driven together by the solvent pressure on their reverse sides as they fill the small vacuum left between them by the removal of the water, so that the energy decreases until they are in van der Waals contact. The third minimum at 11.5 Å roughly corresponds to two layers of structured water between the two solutes, and the small barrier can be thought of as coming from the "squeezing" out of one of these layers.

It is difficult to evaluate the error in MD simulations, which includes both systematic errors such as inaccuracies in the force fields and statistical errors which result from failure of the calculations to converge to the thermodynamic limiting averages. These statistical errors result from inadequate sampling, primarily due to simulations of insufficient length, which is often a problem in calculations of large systems where the total integration time is limited by practical considerations. In an attempt to assess the statistical error in these simulations, one point, at 11.3 Å, was repeated 6 times starting with different

random assignments of atomic momenta and water arrangements. The standard deviation in the free energy assigned to this point was only 0.1 kcal/mol, but another source of error lay in an rms deviation of the actual average separation coordinate of 0.17 Å (the molecules were not fixed at each selected separation, but were merely constrained to the desired distance with harmonic forces (see above); thus, there is an additional scatter in the resulting data points due to the small variations in the actual average separation observed, where the point is placed at the position of maximum probability). The increase in the scatter of the points at larger distances is apparently due in part to a larger angular range sampled at a specified distance as the uniform constraint on the parallel stacking of the rings becomes relatively less restrictive on the angle between the rings, allowing greater deviations away from planarity, and in part to a tendency for the rings to "slip" relative to one another so that they are less perfectly stacked.

It would be highly desirable to perform this calculation without the constraints on the relative orientations of the two rings. Unfortunately, this is not immediately feasible due to the enormous size of the calculation. Unlike the benzene dimer case, the sugar molecule is highly asymmetric in its structure, and it would surely make a difference which functional groups were directed toward or away from the benzene molecule. Calculating a meaningful unrestricted pmf would thus require averaging over all of these possible distinct orientations (at each separation distance). Due to the slow rotation rate for the sugar in solution, adequate averaging would require more computer time than is presently available. Since sugar and phenylalanine rings are usually stacked approximately parallel in crystal structures of protein−carbohydrate complexes, the geometry used here would seem to be the most biologically relevant.

Specific $\pi-\pi$ interactions play a role in the interactions of benzene molecules with one another and with other species such as ions.[43,44] It has been suggested that hydrogen bonds might be possible to the π electrons of benzene,[20,21,45] perhaps with a structure which would be only approximately reproduced by conventional semiempirical molecular mechanics force fields, which do not explicitly include orbital structure. It is thus possible that such interactions play a role in the binding of sugar molecules to phenylalanine groups in proteins. This possibility might be further supported by the observation that in many of the protein structures in which a sugar group is seen bound to a phenylalanine side chain, the sugar is tilted at an angle relative to the plane of the aromatic ring. Since the MM energy function used in the present simulations does not include such interactions, further studies will be required to investigate any possible role such π-hydroxyl hydrogen bonds might have in the interactions of sugars with protein side chains.

IV. Conclusions

The present simulations confirm that the β-D-glucopyranose units of cellulose should exhibit a binding affinity for the aromatic rings of phenylalanine residues in proteins. From comparisons with the vacuum pmf for the approach of benzene and this sugar, it can be seen that this binding affinity is increased by hydration effects due to hydrophobic binding between the nonpolar faces of the two molecules. These results are in excellent agreement with similar previous calculations of pmf's for nonpolar spheres in aqueous solution and for benzene dimers[22,23,25] and α-D-glucose sorbing onto graphite.[26] To use such a calculation to make an estimate of the difference in binding affinity for glucose to a protein in which a phenylalanine residue is mutated into an alanine, for example, it would be necessary to calculate a similar pmf for the interaction of glucose with a methane molecule, and take the difference of the two functions, a calculation which is presently underway. More generally, it should be possible to perform such pmf calculations for the binding of glucose to particular residues in specific protein binding sites, such as the cellulase E1, and thus to be able to predict the outcome of actual mutagenesis experiments.

It should be remembered that actual experimental binding affinities do not restrict the orientation of the two rings. It would thus be highly desirable to be able to extend this type of study to allow averaging over nonstacked arrangements. In many of the reported crystal structures of proteins bound to sugar molecules, while the interactions with the numerous tryptophan residues are often in a nearly stacked or parallel arrangement, in those cases where a sugar binds to a phenylalanine side chain, the aromatic ring is sometimes interacting with the sugar ring at a small angle. In other cases, the rings may not be lined up directly over one another, as in the binding site of the E1 cellulase from *A. cellulolyticus*,[9] where the exocyclic primary alcohol of sugar 4 is positioned over the aromatic ring of Phe 29, rather than the sugar ring itself, and only one of the sugar axial protons is in contact with the side-chain ring. In cases such as these, the geometry simulated here would be only a first approximation for the interaction of the two rings. It would therefore be most desirable to extend these calculations to consider alternate geometric arrangements such as those found in these proteins.

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