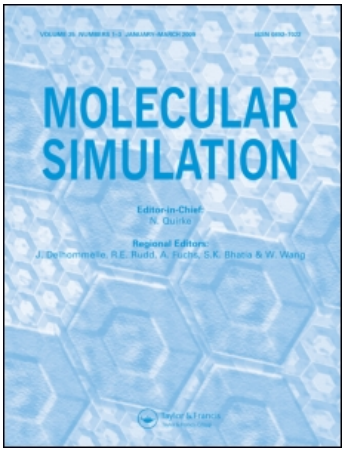

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Perturbation-Relaxation Molecular Dynamics Simulations as a Tool to Explore Conformational Space. Reversible Response of the L3 Loop in Porin Towards Charge Screening Effects

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PERTURBATION-RELAXATION MOLECULAR DYNAMICS SIMULATIONS AS A TOOL TO EXPLORE CONFORMATIONAL SPACE. REVERSIBLE RESPONSE OF THE L3 LOOP IN PORIN TOWARDS CHARGE SCREENING EFFECTS

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A molecular dynamics perturbation-relaxation method is proposed for sensing the response of MD-equilibrated structures to external perturbing conditions. The procedure permits a searching of conformations that are interconvertible with a given structure when the external perturbation is switched off. In this way, it is possible to exploit the intrinsic information of the structure to find new conformations of interest following paths between them that might be accessible at low temperature. In the example reported, the external perturbation is produced by a change in membrane potential. A model is built to mimic this circumstance and the nature of the molecular system, a porin protein. The relaxation time is speeded up by using the GROMOS electroneutral and collisionless MD force field that has been fairly sucessful in representing X-ray and NMR determined structures. Using an electrostatic perturbation we found molecular determinants for a possible mechanism leading to pore closure in porins. The response of the L3-loop correlates well with or previous flexibility studies.

KEY WORDS: MD simulations, Perturbation-relaxation method, Porins, L3 loop, Conformational space search.

INTRODUCTION

Finding conformations that are interconvertible with a given experimentally determined structure when external conditions are changed is an interesting problem that can be treated by molecular dynamics simulations. This is basically a problem of conformational space searching [1]. Exploration of the conformational space of a molecular system is usually done with simulated annealing [2, 3]. This method has been very successful for structure refinement in X-ray crystallography [4, 5] and NMR [6, 7], where system constraints are introduced. For free polypetide chains, however, applications of such methods seem to be limited to lengths of a dozen residues, maybe more in the case of cyclic systems [3]. The maximum number of residues can be increased if constraints and approximations on the molecular system are made [8–11]. Our own experience with the L3 loop from *Rhodobacter capsulatus* porin showed that

simulated annealing was not successful in finding adequate closed states [12]. A large number of structures were found but there was no simple way (except by introducing constraints) to find paths between them and the original structure, or to discriminate between physically "reasonable" (conformations that would be accessible to the loop under physiological conditions) from "unreasonable" states in the framework of the simulation. A step beyond this procedure is required. In fact, the physical situation under which the loop is assumed to move suggests another method to explore the energy hypersurface relevant to a closing–opening process.

The permeability of porins *in vitro* can be voltage regulated and the L3 loop seems to be mechanistically involved. Looking at the X-ray structure one can see that this mainly negatively charged loop and a patch of predominantly positive charged residues situated in the barrel wall opposite it, form the pore "eyelet". Mutations in the L3 loop and the patch of residues in the barrel wall affect channel size and voltage sensitivity [13]. Cowan *et al.* [14, 15] have suggested that this loop could be involved in the voltage gating and that changes in the charge distribution and in the electrostatic potential inside the pore might be the key factors in this process.

The electrostatic forces in the eyelet zone suggest an alternative way for finding closed states. The voltage effect can be envisaged as a change in the distribution of ions in the channel due to the external electric field applied across the pore. Being a flexible object, the L3 loop would then respond to such a change in the electrostatic conditions. Such situation can be effectively simulated at the highest level of detail including water and counter-ions; however, since the electrostatic interaction is screened by the water inside the pore, which also provides a microviscous medium, a simulation including all these factors involve long relaxation times; actually, a 100 ps simulation of such a system shows no significant response towards ion depletion.

One way to go around the slow relaxation time problem is to use a collisionless solvent model (in previous work we have named it as non-inertial solvent (NIS) [16–20] model and this name will be used for the sake of reference in what follows). This GROMOS' parameter set 37D4. This scheme has the adavantage of avoiding explicit inclusion of water and couterions. The latter are assumed to counterbalance the charges of ionizable residues [16]; thus, the protein is globally electroneutral. As collisions between protein atoms and the solvent are not present, the time scale for relaxation from non-equilibrium situations is bound to be shorter than for full solvent models. The NIS force has been used in the simulation of several proteins [16–20], reproducing fairly well the features found in the X-ray structures; the loop L3 proved to be no exception [12]. GROMOS–NIS constitutes the equilibrium simulation engine. The response of this system to an external perturbation mimicking changes introduced by the voltage across the membrane is determined by using molecular dynamics simulations with a time dependent perturbation. This one is switched-off after a while and the relaxation process is followed with the NIS-model. The limit state of perturbation corresponds to fully charged side chains as it is assumed that the counter-ions have been shifted away. Although the fully charged state is an unrealistic situation, the important issue is the relaxation process. Thus, a simple method for exploring the conformational space of the protein is obtained by using such a perturbation-relaxation approach. This has resulted in a possible mechanism for pore closure in porins.

I. MODELING AND METHODS

R. capsulatus porin is a 16-stranded β-barrel with a 44-residue interior loop, called L3 [21–23]. The same fold seems to be shared by *E. coli* porins [14] and porins from other sources [24]. Porins have been shown to be voltage-gated *in vitro* [25]. The channels show at least one open and one closed states. Experimentally, the L3 loop appears to be involved in channel permeability and selectivity; this is shown by sequence alignment of porins from *E. coli* and other cells [26, 27], since mutations in the L3 region affect channel size and voltage sensitivity of porin in gating [13].

Porins are trimeric proteins that define three water-filled channels. Simulation of this explict system (porin + membrane + water) is not feasible with current computer resources. However, simulation of this complete system may not be needed, since at this stage we are interested in studying the dynamics of the L3 loop, which is inside the protein pore, and it is unlikely that the membrane would seriously affect its dynamic properties. On the other hand the membrane certainly has a stabilizing and structural effect on the β-barrel, which has a hydrophobic exterior. The same considerations apply to the presence of the trimer. In order to keep the molecular topology observed in the X-ray structure while simulating one single monomer, all the main chain atoms, except the ones belonging to the L3 loop, were position restrained. The use of position restraints allows the study of the dynamics of L3 without undesirable interference from deformations caused by the absence of the membrane and trimer. This is a standard technique used in conformational studies of protein loops [28]. The restraints do not permit deformation, but allow fluctuations around average positions. In addition, all side chain atoms are allowed to move freely, which in fact means that the L3 loop is studied in the fluctuating field of the whole molecule. This model was previously developed by us to study the plastic properties of this system [12]. These simulations were done within the NIS framework which implicitly accounts for the electrostatic screening effects of water and counter-ions by using a modified set of partial charges on ionizable groups, making them globally electroneutral. As the collision forces with explicit water molecules and ions are absent, plastic properties and conformational changes can be accessed on a shorter time scale than in simulations using explicit water. In a 100 ps reference run at 293 K the L3 loop was very stable around the open state (the state present in the X-ray structure), with a mean r.m.s. deviation of only 1.4 Å from the X-ray structure. In the present work we use this model as the reference open state of the porin.

An important aspect of the situation one wants to simulate is the result of a potential difference across the membrane. The first thing to be affected by would be the ion distribution in the solution around and inside the porin. These species are the most mobile in the solution, thus an ionic gradient would arise, leading, among other things to changes in the equilibrium ionic distribution inside porin. One would expect that such a perturbation disrupts the screening of charged groups by counter ions (the equilibrium situation). If this happens, the effects of an asymmetric charge distribution in the pore would create an attractive force, pulling the L3 loop towards the positive patch. If we bear in mind that the dielectric constant inside the pore, according to Gutman *et al.* [29], has a value of 24, such effects may be even stronger than in bulk water. Putting the two things together, the change in the electric field creates the

conditions for a conformational change in the L3 loop, which would approach the positive patch in the barrel wall and close the pore. This is the hypothesis that we put to test, by studying the effects of changes in the electric field inside the pore by means of an electrostatic perturbation.

Perturbation-Relaxation

The perturbation of the electric field is introduced by changing the atomic charges of the force field. This model is the equivalent to change (decrease) the dielectric constant in a continuum model approach. The screened charges of the NIS force field were changed into their full, unscreened values (see the values in Table 1) as follows: the electrostatic part of the total Hamiltonian, $\mathscr{H}(t)$, was slowly changed according to the formula

$$\mathscr{H}(t) = (1 - \lambda(t))\, \mathscr{H}_{\text{NIS}} + \lambda(t)\, \mathscr{H}_{\text{unscreened}}$$

where λ is an auxiliary time-dependent variable that goes from 0 to 1. This is similar to the technique used in free energy calculations [1], although thermodynamic equlibrium is not assumed here. This particular procedure will be referred to as charge scaling. Only the Asp, Glu, Lys and Arg residues in L3 and the positive patch opposite it were modified. The variable λ was linearly changed over 100 ps at 293 K.

What is actually measured in these simulations is the response of the protein structure (loop) to diverse degrees of perturbation by returning the system to the condition controlled by the NIS hamiltonian.

Permeability Measurement

A measure of permeability was specifically developed for the present problem, and it is based on an approach where a large number of atomic-sized probes (radius of 1.86 Å) were released at one end of the pore (which was covered at both sides by an irregular boundary) and their trajectories monitored, counting how many reach the other side of the pore. The protein is treated as a set of static spheres with sizes corresponding to the van der Waals radii of each individual atom. This can be likened to bouncing tennis balls inside the pore and seeing how many can get through in a certain time. Particles follow hard-sphere dynamics [31, 30] but do not see each other, only the protein; this is similar to the time-dependent Hartree approximation previously used to study carbon dioxide diffusion in myoglobin [32]. The procedure takes into account the possibility of curved paths for passage of substances, thus simulating permeability of non-charged solutes, although the protein is kept static. For each conformation studied, 100 spherical probes were released at the periplasmic side of the pore with a Maxwellian distribution of velocities corresponding to the temperature of 293 K and their trajectory was monitored during 220 ps. During the first 20 ps, probes were confined to the periplasmic side of the pore using a rigid wall; this allowed them to distribute themselves homogeneously prior to release. The rigid wall was then removed and the probes could start to penetrate the pore. The percentage of probes that reached the bottom of the pore is counted.

Technical Details

The GROMOS program package [33] was used in all the simulations. Except when stated otherwise the SHAKE alogrithm [34–36] was used to constrain bond lengths. The 100 ps equilibrated conformation [12] in the NIS field was used in the molecular dynamics simulations. Crystal water and calcium ions are not included. Backbone atoms of selected regions were restrained with force constants of 400 kJ/(mol nm^2), the reference coordinates being those of the energy-minimized X-ray conformation. Integration was performed with a time step of 2.0 fs. The temperature was kept constant by coupling the system to a thermal bath [37] with a temperature coupling constant of $\tau = 0.1$ ps. Non-bonded forces were calculated using the twin-range charge-group technique [33] with cutoffs of 8 Å and 13 Å. The molecular pair-list was updated each 10 steps. The X-ray conformation was energy minimized for 400 steps using the steepest descents method and then used in the energy minimizations involving perturbation. These energy minimizations were done without SHAKE, with the conjugated gradient method and with position restraints in selected backbone atoms, in the same way as in the molecular dynamics.

For trajectory analysis, programs from the NICE [38] packages were used. The mdFRODO program [38] was used for graphical analysis. R.m.s. deviations were calculated for main chain atoms only.

II. RESULTS

With the method of electrostatic perturbation trajectories of 100 ps. were run, where the charges of ionizable residues inside the pore were slowly changed from the NIS field to their screened values. The procedure led to a localized conformational change, where the loop segment Ile 102 to Ala 113 moved towards the positive patch opposite it, as can be observed in the comparison presented in Figure 1. The results of one representative trajectory are given here.

In Figure 2 the r.m.s. deviation from the average (90–100 ps) structure of the equilibration simulation is plotted along the pertubation simulation. The Ile 102 to Ala 113 reaches a r.m.s. deviation of approximately 5.5 Å, whereas the rest of the loop reached an r.m.s. deviation of about 1.6 Å. This shows that the conformational change was very localized affecting less than 30% of the residues of the loop. The conformational change closed the pore to one sixth of its original size, according to the measure of permeability shown in Figure 3.

At several points along the trajectory, charges on ionizable residues were returned to their NIS values and the system was allowed to relax. Up until 80 ps of charge scaling (80% of total charge) the segment Ile 102 Ala 113 returned to its original topology. This conformational change leads from a closed to an open channel in few picoseconds. This can be seen in Figures 2 and 3. The relaxed structure is compared with the 90–100 ps average structure of the equilibration simulation in Figure 4. The structures are very similar showing that the loop returned to the conformational space region of the open state.

The rapid relaxation to the open state could suggest that the closed state is not stable when the perturbation is removed; however, reversibility was detected until the 80 ps

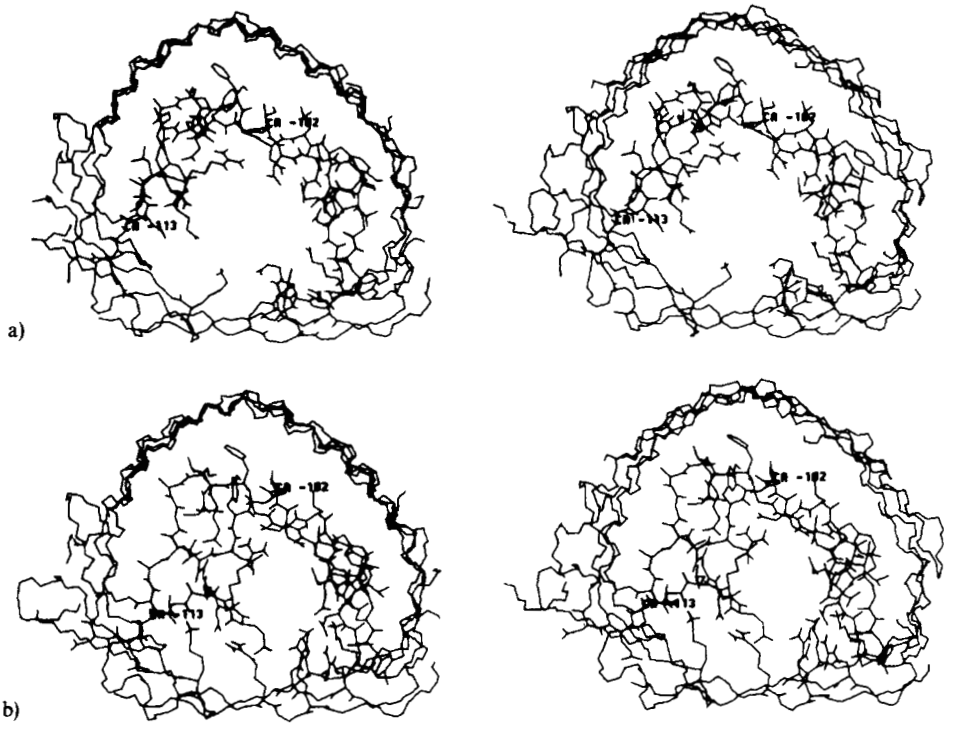

Figure 1 Cross section stereo renditions of porin conformations. Only side chains of the L3 loop (seen on the upper half of the pore) and the positive patch (seen on the lower half of the pore, "attached" to the barrel wall) are represented. The rest of the protein is represented with main chain atoms. The beginning and end of the of the Ile 102–Ala 113 segment are labled (C_α marked). The images are clipped in the Z-direction at the level of the L3 loop for better comprehension. a) Top-Average $\langle$ 90–100 ps $\rangle$ structure of the NIS simulation seen in cross section. b) Bottom-Conformation obtained at 80 ps of the perturbation simulation seen in cross section. The Ile 102–Ala 113 which makes the upper left half of the loop moved towards the positive patch.

point of charge scaling simulation, but not from the conformation 100 ps, even though these two conformations are not noticeably different. This result suggests that a closed state can be stable in itself, even upon removal of the perturbing conditions. Depending on electrostatic determinants, the L3 loop can thus exist in two distinct states. A small perturbation is enough to effect the conformational change; studies were made where the charge scaling was stopped at certain percentages of the total charge and proceeded constant until the end of the 100 ps trajectory. Down to 30% charge increase (charge scaling stopped at 30 ps) the behavior of the loop was the same and the closed state reached was similar.

III. DISCUSSION

The perturbation-relaxation method presented here permits sensing the structural response of the *L3* loop to external perturbations. Such a response involves a localized

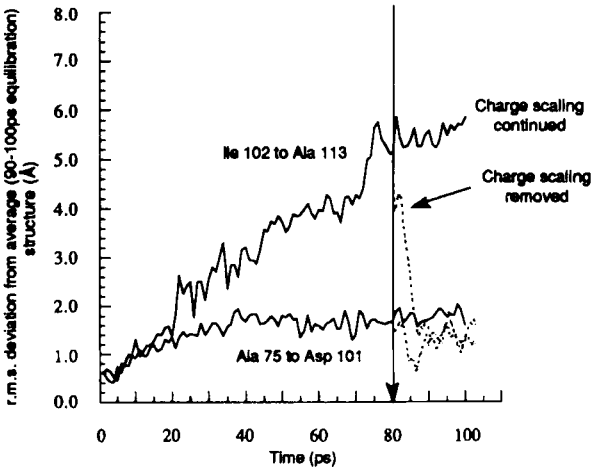

Figure 2 R.m.s. deviation determined for selected zones of the L3 loop (the stable one, Ala 75-Asp 101, and the perturbed one, Ile 102–Ala 113) along the charge scaling simulation (curves plotted in solid lines). The reference structure is the average structure (90–100 ps) of the equilibration run, and the r.m.s. deviation is calculated for main chain atoms. The curves plotted with dashed lines describe the evolution of the system (the upper one corresponds to the Ile 102–Ala 113 zone) after the perturbation was removed (from 80 ps of the perturbation simulation, a time marked in the plot with a vertical arrow corresponding to the beginning of the reversibility test); the r.m.s. deviation of the Ila 102–Ala 113 segment goes sharply down from the high value of the perturbed state to a value close to the one of the equilibrated state. The 80 ps conformation was the last point from which reversibility could be achieved. Even though this is not the final (100 ps) conformation in the trajectory, it was very similar to it.

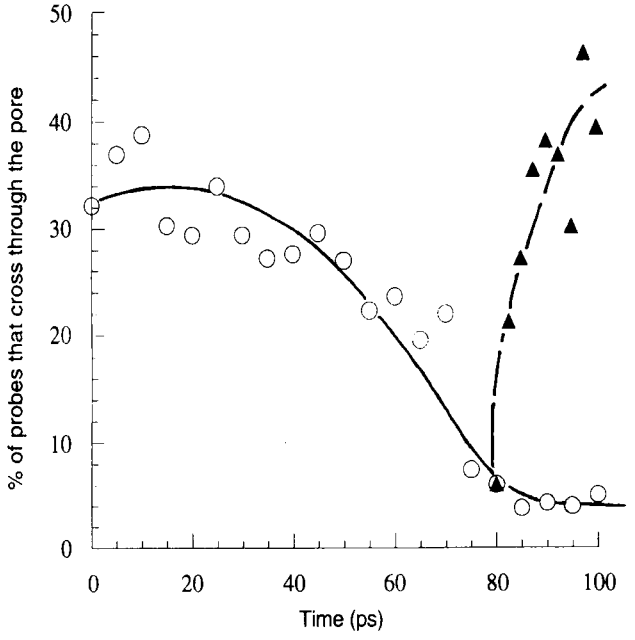

Figure 3 Permeability measure of the porin pore in different conformations. Each data point corresponds to one conformation at a certain time in the trajectory. The percentage of these probes that reached the bottom of the pore is shown on the ordinate. The circles correspond to the 293 K charge scaling simulation; the loop segment Ile 102 to Ala 113 moves with time, gradually decreasing the size of the pore. When charges were scaled down to their original values, the pore opened again (triangles).

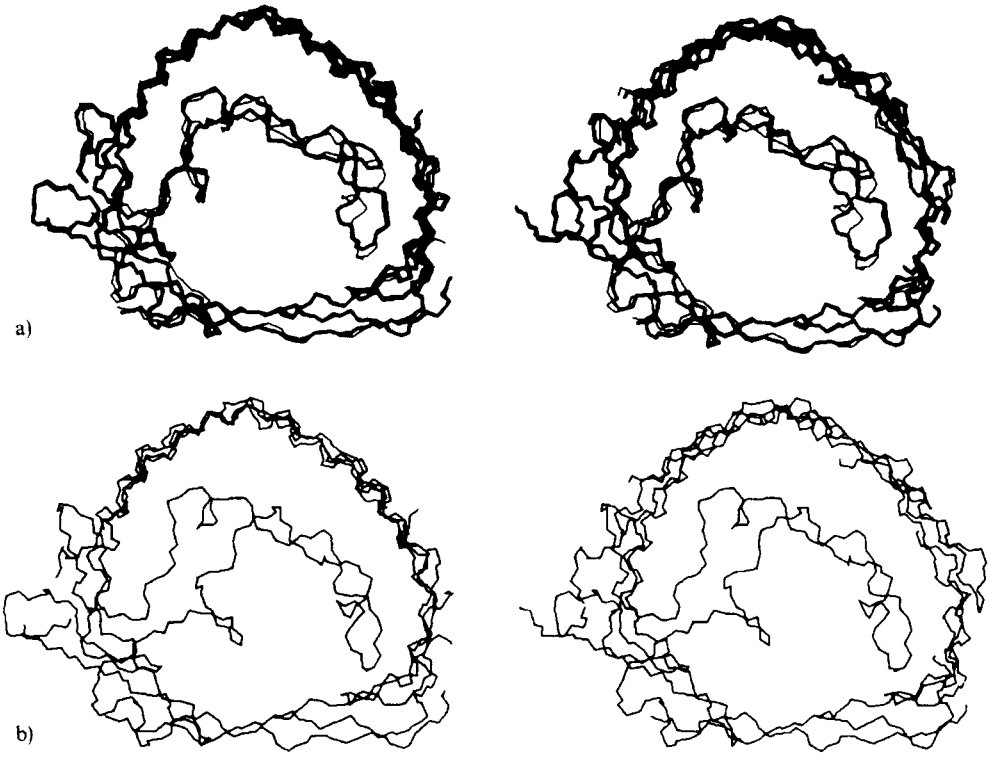

Figure 4 a) Top–Cross section stereo renditions of an overlay of the average structure (90–100 ps) of the equilibration simulation (in thin line) and of the final structure of the reversibility study started at 80 ps perturbation (in thick line). The structures are rendered with main chain atoms only. The images are clipped in the loop for better comprehension. b) Bottom–Same type of rendering, but representing the conformation obtained at 80 ps of the perturbation simulation (closed state), for comparison with the above.

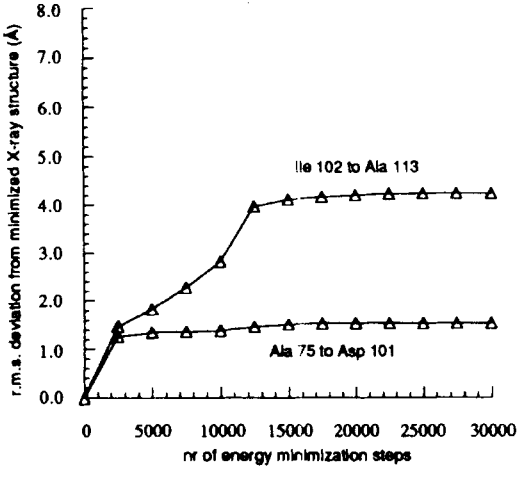

Figure 5 R.m.s. deviation of main chain atoms of the L3 loop from the minimized X-ray structure, in the charge scaling energy minimization, started from the minimized X-ray structure.

conformational change, which is reversible when the perturbing conditions are removed. The zone involved in the conformational change contains one of the two zones previously found to be more flexible than the rest of the loop [12] using high temperature simulation techniques. This result is quite natural since one would expect that conformational changes might take in potentially flexible zones of the protein. The convergence obtained with two different techniques renders more plausible the conclusion that these residues constitute the molecular determinants for a mechanism of pore closure/opening.

The results obtained here are in contrast with the ones obtained with the simulating annealing technique, where conformational changes were much more extensive and the system could never go back to the open state. The present method fully uses the intrinsic information and tries to mimic the process that leads to porin closure. This results in finding new conformations of interest, following paths between that are accessible at low temperature. Simulations carried out under slightly different initial conditions to test for statistically significant results show that the present results are not accidental. A possible candidate for a closed state of porin follows from this study.

The hypothesis of counter-ion diffusion needs some qualifications. Full depletion is not a central issue to the method presented. It is a speculation on a possible mechanism of pore closure which would explain experimental results of voltage gating. In the equilibrium situation, counter-ions would screen the charged side chains of the protein. An electric field can partially upset this equilibrium. The charge scaling (with its consequent unscreening) required to effect the conformational change can be very small. Actually, Partenskii and Jordan [39] have found that the dielectric constant of water in a channel may significantly be altered during the process of ion diffusion through it, and its effective value becomes much lower than the bulk value. The model used by these authors to describe water dipole orientation by ions introduces a charge-dependent effective dielectric constant for water in the channel. In our case, the displacements of counter ions in the pore may be seen as decreasing the effective dielectric constant. The model used here, of increasing the charge to simulate such effects is compatible with the continuum model where the presence of ions in the water channel decreases its dielectric constant. Furthermore, from the results obtained, it appears to be good enough to give a reasonable description of structural determinants for the process of pore closing/opening under external variable conditions.

Perturbation approaches were previously used in very elegant studies of fast,photo-induced processes [40–43]. However, in these papers what was actually studied was the dynamics and relaxation of the perturbed system i.e. the state obtained immediately after the excitation). Our approach is different: the "excitation" process is simulated and the relaxation back to the initial (or "ground") state is studied.

The perturbation-relaxation concept behind our approach is quite general. It can well be used in other contexts such as the protein folding problem. In this case, one is dealing with a massively large conformational space which might be locally searched using relevant determinants (such as hydrophobic or electrostatic screening effects) of the folding process. The inclusion of hydrophobic and solvent polarization effects can be done following ideas from Cramer and Truhlar [44]. The solvent accessible surfaces are obatined from GEPOL [45]. Generalized Langevin equation for the system of interest are used to define an appropriate theoretical framework [46]. An important

aspect of the perturbation method presented here is that it can be adapted to simulate denaturing conditions. the equilibrium MD model will be again the NIS-force field thereby leading to rapid relaxation effects (obviously, other force fields such as CHARMM [47] with distance dependent dielectric functions can equally be used.) One target for this type of simulations is to find out a point where the memory of the folded state is lost. Once such structures-if they exist-are identified, simulations under more realistic conditions can be used to determine their fluctuations properties. This technique has been applied to explore some aspects of the protein folding-unfolding problem for the zinc finger protein Zif-268 [48]. The denaturing forces act along the normal to the surface atoms that are accessible to the solvent via a screening of the protein van der Waals and electrostatic forces acting on the particular accessible atom. The results are fairly promising.

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