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Computer simulation of the dynamics of hydrated protein crystals and its comparison with x-ray data

(protein dynamics/solvent effects/fluctuations/structure factors/crystallographic refinement)

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ABSTRACT **The structure and dynamics of the full unit cell of a protein (bovine pancreatic trypsin inhibitor) containing 4 protein molecules and 560 water molecules have been simulated by using the molecular dynamics method. The obtained structure, atom positional fluctuations, and structure factors are compared with x-ray values. A way of calculating the motional contributions to structure factors is proposed.**

From early crystallographic studies a picture of a globular protein in its native conformation emerged as a well-defined more or less static structure. In recent years light has been shed on the dynamics and flexibility of protein molecules, both by experimental and theoretical studies (1–4). There appears to be considerable local motion within proteins at ordinary temperatures, both in solution and in the crystalline state. In addition, significant displacements of chain segments or even complete domains have been linked to the activity of proteins (5–7). Differences in activity between precursor enzymes and their native counterparts have been related to the increased mobility of substantial parts of the precursor molecules (8, 9). A detailed analysis of the mobility of the polypeptide chain, and of the surrounding solvent, has become an integral part of the description of a protein structure.

From a theoretical point of view, the description of the dynamics of a protein in terms of simple interactions between atoms is a classical many-particle problem. Because of the large number of atoms, the variety of essentially nonlinear interactions involved, and the fluid-like character of the system of atoms, the usual theoretical methods, such as the harmonic approximation, are not very promising, if applicable at all. Therefore, one is led to attack the dynamic problem by brute force—that is, by solving numerically the classical equations of motion for all atoms that are considered simultaneously for a chosen period of time. This yields trajectories for all atoms in the system, from which physical quantities can be calculated.

Only a few proteins have been studied by computer simulation (10–22). Various approximations have been made in order to decrease the complexity of a simulation including all degrees of freedom of both protein and solvent. Molecular dynamics (MD) simulations of pancreatic trypsin inhibitor (PTI) (10–15), cytochrome *c* (16–18), and rubredoxin (13) have been performed *in vacuo*—namely, ignoring the effect of the solvent or crystalline environment on the dynamics of the protein. Hagler and Moult (19) have performed a Monte Carlo (MC) simulation of the unit cell of a lysozyme crystal, decreasing the number of degrees of freedom by keeping all protein atoms in fixed positions and allowing only the water and counterions to move. Hermans and Vacatello (20) went one step further in a MC simulation of the asymmetric unit of a PTI crystal by allowing the protein side chain atoms to move as well. Recently it has been shown that inclusion of an atomic solvent or of a static crystal environment in a MD simulation of PTI does improve the agreement with x-ray data considerably (22).

Here, we report a MD simulation of the full unit cell of a PTI crystal, involving 4 protein molecules and 560 water molecules. The crystalline state has been simulated, rather than PTI in solution, in order to make a detailed comparison with x-ray data possible. On the one hand, this provides a test on the validity of the potential functions and approximations that were used. On the other hand, we discuss how information on the anisotropy and the anharmonicity of the atomic motions that is present in the simulated atomic trajectories can be directly used for the calculation of structure factors and R values.

Model and computational procedure

The protein PTI consists of 454 heavy atoms. Hydrogen atoms attached to carbon atoms are incorporated into the latter, whereas the other 113 hydrogen atoms, which may form hydrogen bonds, are explicitly treated. The empirical interaction function, which will be published elsewhere, is of a similar type as used in other protein studies (13–15). It is a pair potential consisting of a sum of terms associated with bond angles, dihedral angles, out-of-plane torsion angles, and electrostatic and van der Waals interactions. No explicit hydrogen bonding interaction is applied, because this interaction can be adequately modeled by electrostatic and van der Waals forces (23). Atomic partial charges are used without modification; a dielectric constant of 1 is used. To decrease the computing costs nonbonded interactions beyond 8 Å are neglected, without using switching functions to smooth the cutoff effects. All bond lengths are kept rigid during the simulation by using the SHAKE method (24, 25). It has been demonstrated that freezing these degrees of freedom does not change the equilibrium properties of the protein (15). The water molecules are modeled by a simple rigid three-point charge model (SPC model), which adequately describes the properties of bulk water at ordinary temperatures (26). Interactions between water and protein atoms are obtained from combination rules (unpublished data).

The initial configuration was taken from a MC run, covering 300,000 moves, equivalent to 1,500 successful MC steps per moving atom, of the PTI asymmetric unit, which contains 1 protein molecule and 140 water molecules, allowing side chain atoms and water molecules to move. The protein structure for initiating the MC was the x-ray structure; water molecules were placed and statistically replaced without reference to the crys-

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Abbreviations: MC, Monte Carlo; MD, molecular dynamics; PTI, pancreatic trypsin inhibitor; EM, energy minimization.

tallographically determined water positions. Subsequently, the $P2_12_12_1$ symmetry transformations were applied to the final MC configuration to construct the full unit cell. Thus, the assembly of atoms that is simulated consists of 4 PTI molecules plus 560 water molecules in the orthorhombic unit cell, involving 11,844 degrees of freedom. Before starting the MD simulation, 50 steepest-descent energy minimization (EM) steps were performed (27). Initial velocities for the atoms were taken from a maxwellian distribution at 300 K, independently for each of the four molecules. In order to avoid slow temperature drift, the system was weakly coupled to a thermal bath of $T_0 = 300$ K, when integrating the equations of motion with time step $\Delta t = 0.002$ ps. This is done (unpublished data) by rescaling all atomic velocities $v_i(t)$ of the N atoms which determine the temperature at time t

$$T(t) = \sum_{i=1}^{N} 1/2\, m_i v_i^2(t)/(3/2\, Nk) \quad [1]$$

at each MD step with the relation

$$\frac{dT(t)}{dt} = \tau^{-1}\{T_0 - T(t)\}. \quad [2]$$

The strength of the coupling to the heat bath is determined by the temperature relaxation time τ, which has been taken equal to 0.1 ps. Periodic boundary conditions corresponding to the crystal translational symmetry were applied. The MD run covered a time span of 20 ps, which took about 140 central processing unit (CPU) hours on a Cyber 170/760. The computation is roughly 10 times more time-consuming than a simulation of similar length on four molecules *in vacuo*. The averages of various quantities are only calculated over the final 12 ps, allowing for transient dynamical effects associated with the initial conditions to decay.

Protein structure and dynamics

The potential energy was monitored during the simulation in order to judge the equilibration of the system. It appears that from 8 ps onwards the potential energy has stabilized within the limits of its stationary fluctuations. Thus, the equilibration period was limited to the relatively short period of 8 ps, as in ref. 22.

In Table 1 the rms differences between simulated structures at various times and the x-ray structure (28, 29) are shown. The initial structure, taken from a MC run with static backbone, is close to the x-ray structure. EM changed the conformation only a little. During the MD run the four protein molecules in the unit cell wander away more and more from the x-ray structure and, as can be seen from the right-hand side columns, also from each other. Although the data averaged over 1 ps—namely, ⟨ 8–9 ps ⟩ and ⟨ 19–20 ps ⟩—do suggest that the MD structure drifts steadily away from the x-ray structure, this is contradicted by the observation that the MD structure averaged over 12 ps (⟨ 8–20 ps ⟩) is in some cases for the C_α atoms closer to the x-ray structure than are both the ⟨ 8–9 ps ⟩ and the ⟨ 19–20 ps ⟩ MD structures. Because the atom positional fluctuation relaxation times are of the order of 1 ps (22), it is not surprising that the 1-ps averages in Table 1 differ from the 12-ps averages.

The average dynamical structure of each of the four molecules is significantly closer to the x-ray structure than those obtained from earlier simulations (10, 13, 22). Moreover, averaging over the four molecules in the unit cell brings the MD structure still closer to the x-ray one; the rms difference for the C_α atoms is 0.8 Å and for all atoms it is 1.2 Å. This means that the accuracy of this simulation (evaluating the average structure) is almost 3-fold better than that of the MD simulation *in vacuo* (22), due to the inclusion of the solvent and crystalline environment.

The difference between the average structures of the four molecules in the unit cell appears to be of the same magnitude as the difference between each of those four molecules and the x-ray structure (Table 1). Averaging over the four molecules decreases the difference with the x-ray positions. This suggests that there is no appreciable systematic deviation of the average structure from the x-ray structure. If the four molecules were random samples from a gaussian distribution around the x-ray positions, the average structure $\overline{1\text{–}4}$ would be expected to deviate half as much from the x-ray structure as the individual molecules do: 0.81 Å (all atoms) and 0.53 Å (C_α atoms). The observed deviations (1.19 Å and 0.82 Å, respectively) are somewhat larger. Interestingly, the agreement with the x-ray structure virtually does not change when extending the MD run from 9 to 20 ps, but it does when averaging over the four proteins in the unit cell. This means that it is more efficient to start repeatedly from the initial configuration, taking the velocities from

Table 1. rms differences for all (C_α) atoms between various structures

Method	Time, ps	1/x-ray (28)	2/x-ray	3/x-ray	4/x-ray	$\overline{1\text{–}4}$/x-ray	1/2	2/3	3/4
MC		0.93							
EM		0.93 (0.26)	0.93 (0.26)	0.93 (0.26)	0.93 (0.26)		0.01 (0.0)	0.01 (0.0)	0.01 (0.0)
MD	1	0.94 (0.37)	1.06 (0.44)	0.99 (0.42)	1.16 (0.48)		0.65 (0.44)	0.67 (0.44)	0.75 (0.43)
	3	1.25 (0.80)	1.26 (0.73)	1.31 (0.75)	1.41 (0.73)		1.04 (0.77)	1.06 (0.78)	1.39 (0.78)
	5	1.33 (0.94)	1.43 (0.82)	1.32 (0.84)	1.44 (0.77)		1.32 (0.95)	1.24 (0.88)	1.46 (0.82)
	7	1.54 (1.01	1.49 (1.01)	1.36 (0.95)	1.59 (0.92)		1.38 (0.98)	1.39 (1.02)	1.71 (1.09)
MD	⟨ 8–9 ⟩	1.51 (1.14)	1.50 (1.02)	1.44 (1.05)	1.62 (0.99)	1.16 (0.80)	1.44 (1.06)	1.55 (1.09)	1.83 (1.18)
MD	⟨ 19–20 ⟩	1.94 (1.27)	1.99 (1.59)	2.11 (1.38)	1.77 (1.07)	1.31 (0.88)	2.14 (1.70)	2.43 (1.67)	2.56 (1.33)
MD	⟨ 8–20 ⟩	1.66 (1.13)	1.57 (1.12)	1.62 (1.06)	1.63 (0.94)	1.19 (0.82)	1.57 (1.18)	1.69 (0.99)	2.08 (0.94)
MD*	25*	1.94 (1.35)							
MD†	25†	2.12 (1.52)							
MD‡	25‡	3.02 (2.20)							

The rms difference $\overline{([\langle \vec{r} \rangle_1 - \langle \vec{r} \rangle_2]^2)}^{1/2}$ between two structures is given in Å. The time average (MD) is denoted by ⟨ ⟩ and $\overline{\ }$ denotes averaging over all atoms or all C_α atoms (between parentheses). The four molecules in the unit cell are denoted by 1, 2, 3, and 4 and their average structure, by $\overline{1\text{–}4}$.

MC, initial structure, taken from MC simulation with static backbone of the asymmetric unit.

EM, structure after 50 EM steps.

*MD, in van der Waals solvent (no crystal environment), averaged over 25 ps (22).

†MD, in static crystal environment (no solvent), averaged over 25 ps (22).

‡MD, *in vacuo*, averaged over 25 ps (22).

a maxwellian distribution, than to extend the MD run over a long period of time. It also means that 12 ps is an insufficient length of time to achieve an ergodic average. The average of four molecules over 12 ps is not expected to be equivalent to the average of one molecule over 4 × 12 ps, because each of the molecules has gone through EM and equilibration.

Another check on the reliability of the simulation is a comparison of the positional fluctuations of protein atoms with values obtained from experimental temperature factors by the relation $\langle (\Delta r)^2 \rangle^{1/2} = (3B/8\pi^2)^{1/2}$. Generally, the accuracy of temperature factors emerging from the refinement of an x-ray structure is an order of magnitude smaller than the accuracy of the resulting structure itself. Thus, the comparison is not as reliable a test as the comparison of structures.

In Fig. 1*C* the rms fluctuations of the side chains (atom fluctuations averaged over each side chain) derived from a set of temperature factors (J. O. Deisenhofer, personal communication) are plotted as a function of residue number. There is a correlation between the x-ray values and the MD fluctuations per molecule (Fig. 1*B*). Although not yet satisfactory, the correlation is better than in previous simulations (10, 13, 22) of bovine PTI. The variation along a side chain agrees reasonably well; for the C_α, C_β, C_γ, and C_δ carbons, the MD values averaged per molecule are 0.62, 0.68, 0.74, and 0.84 Å, respectively, whereas those obtained from experimental temperature factors are 0.67, 0.68, 0.80, and 0.94 Å, respectively. When calculating the fluctuations by averaging over time and over all four molecules in the unit cell, much larger values are found (Fig. 1*A*). This corresponds to the fact that the four average structures differ by more than 1.5 Å. For example, the large rms fluctuation of the Arg-42 side chain reflects the different conformations of this side chain in the four proteins. We think that the use of a cutoff radius in a simulation that contains the full electric interaction of charges allows structural deviations and enhances structural fluctuations. This effect can be avoided by including long-range electrostatic interactions in the simulations (30). The too large fluctuations might also be explained by the use of too small van der Waals repulsive parameters or by an insufficient number of water molecules in the crystal. This point must be investigated by repeating the simulation at constant pressure.

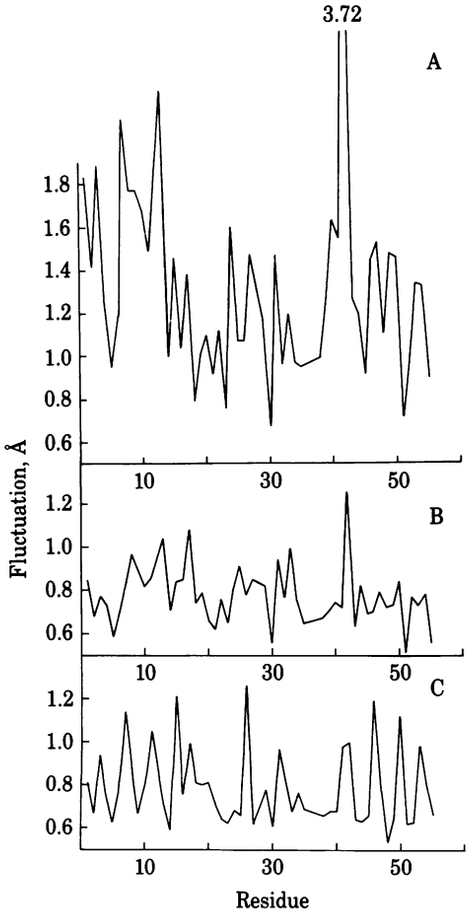

FIG. 1. The rms positional fluctuations for the PTI side chain atoms averaged over each residue. (*A*) Fluctuations around the average structure obtained by averaging over time *and* over all four molecules in the unit cell; (*B*) mean (over four molecules) fluctuations *per molecule*; and (*C*) fluctuations from a set of x-ray temperature factors.

Water structure and dynamics

When comparing the positions of the 47 observed x-ray waters (28) with the simulated ones, only 9 waters are reproduced within 1 Å, when measured in the crystal coordinate system. However, if one considers the local environment of the water molecules, the agreement of the simulated and the x-ray data is considerably better, as is illustrated in Table 2. Three hydrogen bonds of the one isolated internal water W1 are reproduced by the simulation; its fourth hydrogen bond to N of Cys-14 (x-ray) is replaced in the MD structure by one to N of Gly-37, lying at 3.1 Å from this water molecule. For water molecules at the protein surface comparable agreement between x-ray and MD local structure is observed. For example, water W5 has three neighbors within 4.1 Å in the x-ray structure, which are also found in the MD structure. But, the latter also contains a fourth neighbor, water W7, which is missing in the x-ray data. We conclude that the local water structure—that is, the position relative to neighboring protein atoms—seems reasonably well reproduced by the MD simulation, although further analysis is required.

The distribution of the mobility of simulated waters is plotted in Fig. 2. The diffusion constant of SPC water (26) is D = 0.36 $Å^2$ ps^{-1}, which yields a rms fluctuation of 5.1 Å for bulk water over a period of 12 ps. In the present study only a few water molecules exhibit such bulk water mobility. About 10–20 waters show a low mobility, comparable to that of protein backbone atoms; about 100–150 waters possess a mobility like that of side chain atoms. The distribution gives no clear indication of the occurrence of distinct mobility classes for crystalline water. The mobility of water molecules gradually increases with their distance to the protein. In the crystalline state the motion of nearly all water molecules seems to be affected by the presence

Table 2. Nearest neighbor distances for various water molecules

Water W1			Water W2			Water W5		
	Distance, Å			Distance, Å			Distance, Å	
Atom	X-ray	MD	Atom	X-ray	MD	Atom	X-ray	MD
O of Thr-11	2.8	2.9	O of W3	2.6	2.8	O of Ala-16	3.0	2.9
O of Cys-38	2.9	2.8	O of Asn-43	2.7	2.6	O of W6	3.1	2.9
N of Cys-14	3.1	3.7	O of W4	2.8	2.7	C_{γ_2} of Val-34	3.8	3.7
N of Cys-38	3.2	3.1	N of Tyr-10	3.0	3.0	O of W7	—	2.6

The four internally hydrogen-bonded water molecules are denoted by W1–W4. Water W7 has not been identified in the x-ray electron density map. The Protein Data Bank (28) coordinates of the water molecules are: W1, 10.7, 15.7, 14.4; W2, 16.2, 18.5, 7.0; and W5, 5.0, 13.0, 10.9.

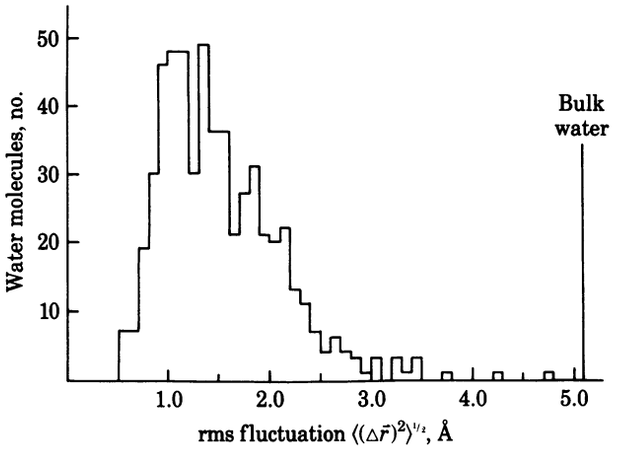

FIG. 2. Distribution of the water positional fluctuations (MD) of the 560 waters in the unit cell.

of the protein molecules. A more extensive analysis of the water structure and dynamics will be given elsewhere.

Temperature factors employed in protein crystallography

Up to now thermal motion of protein atoms has been described virtually only by isotropic temperature factors. This implies the assumption that the motion of each atom is harmonic and of equal magnitude in all directions. This is obviously wrong for nearly all atoms, but the limited number of observations even in high-resolution studies up to, say, 1.5 Å prevents the application of more sophisticated procedures, because of the large increase in the number of parameters involved. Even the use of anisotropic temperature factors (refs. 3, 31, and 32; T. L. Blundell and D. Moss, personal communication) involves an implicit assumption of harmonic motion; it is clear that many side chains do not move harmonically at all (4, 18, 22).

The motions of solvent atoms near the protein surface pose additional problems. Their mobilities vary widely, some sites are not fully occupied, and the displacements from the apparent equilibrium positions are in many cases far from harmonic. The use of relative occupancy factors, in addition to temperature factors, is probably an improvement, although the high correlation between these quantities is a serious difficulty.

At larger distances from the protein molecule, the solvent atom mobilities approach that of bulk water. At present these atoms cannot be described properly with equilibrium positions, temperature, and occupancy factors. As the bulk solvent regions of a protein crystal mostly affect the low-order reflections, the data are usually ignored in refinement procedures and *R*-factor calculations.

MD simulations of proteins and surrounding liquid in the crystalline state provide, in principle, an elegant method to alleviate all of these shortcomings in describing the complex motions of side chains, "bound" solvent molecules, and bulk water. The result of a MD simulation is a large number of configurations, which together describe the trajectories of all atoms in the unit cell. These configurations can be used to generate, by well-known procedures (33, 34), an electron density map which includes then the motions of all protein and solvent atoms, without any restrictions as to their complexity. Fourier transformation of the electron density distribution yields structure factors that can be directly compared with the observed structure amplitudes. The value of the resulting *R* factor will depend on the accuracy of the simulation—i.e., on details of the interaction potentials, such as the treatment of the long-range electrostatic interactions, etc.

The present simulation allows a comparison of calculated structure factors incorporating general thermal motions of atoms with results from x-ray crystallography.

Structure factors calculated directly from MD simulations

The average deviations of about 1 Å between the x-ray and the average MD positions (Table 1) signal a high *R* factor if the procedure just described is followed. Structure factors calculated with 100 unit cell configurations, separated by time intervals of 0.01 ps, taken from the 9th ps of the MD run yield an *R* factor of 52% (curve ■——■ in Fig. 3). A similar number is obtained when using 100 configurations from the 20th ps of the run. The interesting point concerns the low resolution data. As can be seen in Fig. 3, the *R* factor for these reflections is considerably better than the one calculated from the x-ray coordinates (curve □——□ in Fig. 3). This is a result of a proper treatment of the bulk water molecules in the calculation based on the MD results. It is obviously also possible to obtain good *R* factors for the low-order reflections by the use of Fourier-inversion of continuous solvent regions (see e.g., ref. 35). In the procedure proposed here, this good agreement for the low-order reflections is simply a spin-off of the simulation of the motions of all atoms in a hydrated crystal. With more refined MD techniques, it is envisaged that eventually also the partially ordered water molecules in the second or third layers of hydrations will be described properly. These solvent molecules are difficult to parameterize with the usual atomic temperature and occupancy factors and fall obviously beyond the scope of the solvent flattening techniques.

Although the absolute positions of the atoms in the MD sim-

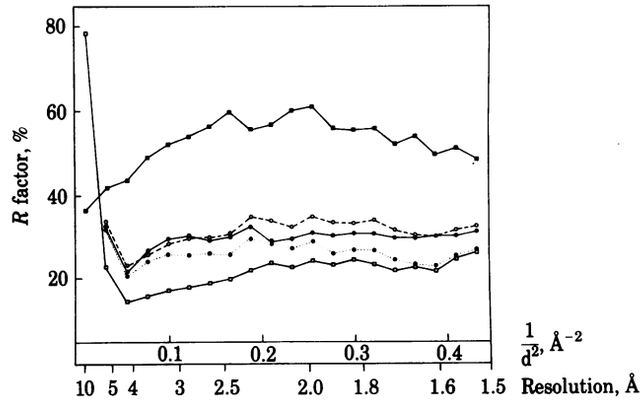

FIG. 3. Reliability factors, *R*, as a function of resolution. The structure factors, F_c, were calculated by fast Fourier methods (35). Before calculating *R* factors [$R = (\Sigma|F_{obs} - F_{calc}| \times 100)/\Sigma|F_{obs}|$], first an overall temperature factor, B_{WILSON}, obtained from a relative Wilson plot of the data between 6.65 and 1.50 Å, was applied to the F_c values. ■——■, Structure factors obtained by summing the F_cs of 100 configurations of the 9th ps of the MD run. B_{WILSON} = 3.2 Å^2; *R* = 52.2% for 8,079 reflections between 50.0 and 1.50 Å. □——□, Structure factors obtained from the x-ray coordinates, including individual temperature factors and 47 water molecules with relative occupancies. Twelve protein atoms, for which no temperature factors were known, were omitted. B_{WILSON} = 0.8 Å^2; *R* = 22.3% for 8,079 reflections between 50.0 and 1.50 Å. ○---○, Structure factors from x-ray coordinates without individual temperature factors or water molecules. All 454 protein atoms were included. B_{WILSON} = 11.8 Å^2; *R* = 30.0% for 7,963 reflections between 6.652 and 1.50 Å. ●·····●, Structure factors from x-ray coordinates with individual temperature factors. For the 12 atoms for which no temperature factor was known, a value of 30 Å^2 was assumed. B_{WILSON} = 0.7 Å^2; *R* = 25.8% for 7,963 reflections between 6.652 and 1.50 Å. ●——●, Structure factors from 1,200 configurations of the 8- to 20-ps part of the MD simulation. B_{WILSON} = 0.5 Å^2; *R* = 29.0% for 7,963 reflections between 6.652 and 1.50 Å.

ulation have shifted from the x-ray positions, by about 1 Å on average, a detailed analysis of the data shows that the local structure in the PTI molecule has been conserved to a much greater extent. This suggests the use of MD structural fluctuations around the x-ray positions in the structure factor calculation. The resulting *R* factors, obtained by shifting the average MD positions back to the x-ray positions, are given as a function of resolution in Fig. 3 (curve ●——●). Because x-ray positions are not available for all water molecules, the water was omitted in this structure factor calculation. We note that this procedure of transferring the vibrational motions to the x-ray positions is very crude. A better procedure would be to restrain certain atoms to their x-ray positions during the simulation. The *R* factor for the data between 6.65 and 1.5 Å resolution is 29.0%. The x-ray coordinates, when omitting the water molecules and utilizing an overall temperature factor, yield an *R* factor of 30.0% (curve ○---○ in Fig. 3). Inclusion of the x-ray temperature factors for the protein atoms, but still omitting all water molecules, lowers the *R* factor to 25.8% (curve ●·····● in Fig. 3).

From these results it can be concluded that the individual x-ray temperature factors still give a better result than the thermal motions derived from the MD simulation. This is not very surprising as, after all, the x-ray temperature factors are the result of a best fit to the observed data, allowing some 450 temperature factors to vary, whereas the MD fluctuations are obtained completely independent from the observed structure amplitudes. The comparison of 8,079 independent x-ray reflections (29) with those calculated from the MD simulation appears to be a very sensitive test of the accuracy of the latter.

Conclusions

The results of a 20-ps MD simulation of the full unit cell of the PTI crystal, including water, show a considerable improvement over previous MD simulations of proteins. The inclusion of water and of the crystalline environment provides the simulation with a positional accuracy comparable to the structural fluctuations that occur at room temperature, although these fluctuations are larger than suggested by x-ray temperature factors. The MD average structure deviates only about 1 Å from the x-ray structure. This is a considerable improvement over previous simulations with 2- to 3-Å deviation and a significant step towards the presumed accuracy of 0.1–0.2 Å of x-ray coordinates.

The direct calculation of structure factors from a MD simulation provides an excellent test for the accuracy of the simulation. It is an opportunity to check simulation results with a very large number (thousands) of observations. Use of the results of the present MD simulation for the description of the highly anharmonic and anisotropic motions of the atoms around their x-ray positions yields a *R* value of 29%, slightly better than when using an overall temperature factor (30%) and slightly worse than when using individual atom temperature factors (26%) in the x-ray refinement.

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