Time-Averaging Crystallographic Refinement: Possibilities and Limitations Using α-Cyclodextrin as a Test System

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Abstract

The method of time-averaging crystallographic refinement is assessed using a small molecule, α-cyclodextrin, as a test system. A total of 16 refinements are performed on simulated data. Three resolution ranges of the data are used, the memory relaxation time of the averaging is varied, and several overall temperature factors are used. The most critical factor in the reliable application of time-averaging is the resolution of the data. The ratio of data to molecular degrees of freedom should be large enough to avoid overfitting of the data by the time-averaging procedure. The use of a free *R* factor can aid in determining whether time-averaging can be reliably applied. Good ensembles of structures are obtained using data up to 1.0 or 2.0 Å resolution. Comparison of electron-density maps from time-averaging refinement and anisotropic temperature-factor refinement indicates that the former technique yields a better representation of the exact data than the latter.

Introduction

Crystallographic refinement is the technique of fitting a model structure to the experimentally observed diffraction intensities. In a perfect crystal at 0 K only one conformation of the molecular system exists. Thus, it is sufficient to represent the structure of the molecules by a single model structure. Often, however, in crystals of biological macromolecules there are regions in the asymmetric unit where the order of the crystal breaks down. Traditionally these regions are characterized by high temperature factors or disordered areas in the electron-density map.

In least-squares crystallographic refinement one structure is fitted within the constraints of chemistry to the experimental X-ray data. This method works as long as the starting configuration of the model is already close to the correct one. With the introduction of molecular dynamics (MD) (Brünger, Kuriyan & Karplus, 1987; Brünger, 1988; Fujinaga, Gros & van Gunsteren, 1989) to crystallographic refinement, the radius of convergence of the refinement increased substantially. By simulating the system at a non-zero temperature the kinetic energy of the system allows the model structure to overcome potential energy barriers and thus to search for a better fit with the experimental data. This method was still only used to search for a single model structure that fits the experimental data. This restriction prohibits an adequate analysis of regions where multiple molecular conformations exist.

The technique of time-averaging crystallographic refinement was introduced by Gros, van Gunsteren & Hol (1990). This method refines an ensemble of structures against the data rather than just a single structure. The ensemble consists of conformations of the molecule at time points along an MD trajectory in which a pseudo potential energy term restrains the molecular configurations such that the deviation of the amplitudes of the time-averaged structure factors from the observed amplitudes is minimal. Time-averaging refinement may distinguish whether a disordered part of the molecule adopts a multitude of configurations throughout the crystal, which would indicate a mobile region, or resides mainly in two or three distinct conformations, which reflects static disorder in the crystal. Better understanding of the flexibility in a molecule will aid in understanding how a particular molecule functions.

Gros *et al.* (1990) applied the time-averaging refinement technique to bovine pancreatic phospholipase A2. The resulting ensemble of protein structures showed a narrow distribution of atomic positions in secondary-structure elements and a wide variation of loop conformations. The latter were

interpreted to reflect the larger flexibility of loops of a protein. The time-averaged refined ensemble of protein structures had a considerable lower R value, 9.8%, than the conventionally refined single protein structure, 17.1%. This result posed the question whether the smaller R value is due to the extension of the number of protein structures used to fit the experimental data, or to a better representation of the average electron distribution by an MD-generated ensemble of protein structures. Probably both effects play a role. When applying time-averaging to the X-ray restraint potential energy term (V_{restr}^{sf}), the physical interaction term (V_{phys}) gets to play a larger role in the refinement process, and its influence on the quality of the resulting ensemble of structures will be enhanced. To separate the effect of the application of time-averaging from the effect of the physical force field (stereochemical restraints) used, Gros & van Gunsteren (1993) examined a nine-atom test system in which no physical force field was used, and analyzed the effect of time-averaging on the resulting electron density when only X-ray restraint forces are applied. The next step is to analyze these effects for a more realistic case.

In time-averaging refinement a term, V_{restr}^{sf}, is added to the physical potential energy function, V_{phys}, which restrains the system to the X-ray data [(1)] just like in traditional MD refinement.

$$V = V_{phys} + V_{restr}^{sf}. \tag{1}$$

The difference to traditional refinement is that V_{restr}^{sf} is a function of an ensemble of structures rather than a single structure [(2)],

$$V_{restr}^{sf} = \tfrac{1}{2}k^{sf}\sum_{s}[|F_{obs}(s)| - k_{sc}|\langle F_{calc}(s)\rangle|]^2 \tag{2}$$

and

$$\langle F_{calc}(s)\rangle_{\tau_x,t} = \{\tau_x[1 - \exp(-t/\tau_x)]\}^{-1}\int_0^t$$
$$\times \exp[-(t - t')/\tau_x]F_{calc}[s;r(t')]dt'. \tag{3}$$

Here τ_x is the (memory) relaxation time over which an ensemble of structures is accumulated and averaged. The exponential decay function in (3) has been built into the standard formula for averaging the structure factor over a time period t,

$$\langle F_{calc}(s)\rangle_{\infty,t} = t^{-1}\int_0^t F_{calc}[s,r(t')]dt', \tag{4}$$

for the following reason. The force due to (2) on the atoms depends on the rate of change of the time-averaged structure factor. This rate of change depends on the length of the averaging period, that is the period t when (4) is used, and the period τ_x when expression (3) is used. The former changes continuously during a simulation, whereas the latter remains constant and therefore the use of (3) is preferred.

The choice of the memory relaxation time τ_x (averaging time) is related to that of the overall B factors, since both parameters affect the spatial distribution of the electron density due to a particular atom. The B factor gives an instantaneous contribution, whereas the averaging contributes as many atom positions as are taken into account within time τ_x. Here, we investigate this relationship. A long τ_x allows for much sampling of configuration space, but is expensive since the length of the MD simulation, τ_{MD}, must be much longer than τ_x in order to obtain sufficient statistics,

$$\tau_{MD} \gg \tau_x. \tag{5}$$

On the other hand, if the real atomic mobility is restricted, a short τ_x should suffice to adequately sample the locally accessible conformations.

Crystallographic refinement of a model structure against the observed reflections may only yield a unique solution when the ratio of the number of independent observations to the number of degrees of freedom of the molecular model is larger than 1. At low resolution insufficient data may be available to determine a structure. When applying time-averaging refinement the ratio of observed data to degrees of freedom decreases, which implies that higher resolution data may be required than for traditional single structure refinement.

In this study we use a test system to investigate the relaxation time necessary to sample conformational space and the resolution of the data necessary to use. We also consider whether the use of an overall temperature factor can help to reduce either the sampling time or the resolution necessary to use time-averaging crystallographic refinement, since increasing the temperature factor effectively increases the width of the instantaneous atomic electron distribution. Thus, in this study we are investigating the versatility of the method of time-averaging crystallographic refinement.

The crystal structure of α-cyclodextrin is chosen as a test system. It is in the space group $P2_12_12_1$. α-Cyclodextrin is a flexible relatively small molecule with 66 non-H atoms and in this crystal form has six water molecules associated with it. For this system we performed 13 time-averaging refinements of 100 ps each to the same set of artificially generated data and three refinements without time-averaging. With these 16 simulations we examine the effects of changing the relaxation time, the resolution and the temperature factor in the simulation.

Generating artificial data

To test the limits of time-averaging crystallographic refinement a set of artificial data was generated to avoid the possibility of ambiguous results due to

experimental errors. Moreover, by using the same force field in structure refinement as when generating 'observed' structure-factor amplitudes, the influence of the force field on the test of the time-averaging methodology is minimized. The crystal structure of α-cyclodextrin with six water molecules (Klar, Hingerty & Saenger, 1980) was used as the initial structure for the molecular system. This structure is in the orthorhombic space group $P2_12_12_1$ with the unit-cell dimensions $a = 14.858$, $b = 34.038$, $c = 9.529$ Å. There is one molecule in the asymmetric unit. The asymmetric unit is the system used in all of the simulations. Crystalline α-cyclodextrin has been studied by Koehler, Saenger & van Gunsteren (1987) using molecular dynamics simulation. The *GROMOS* force field that was used reproduced the crystalline experimental data well (Koehler *et al.*, 1987, 1988*a,b*). Here, the same molecular model, interaction function and computational procedure are used, however, with one exception aimed at a considerable reduction of computing effort. When using periodic boundary conditions the cut-off radius for non-bonded interactions, R_c, must be smaller than the smallest box edge. In order to use the standard cut-off of 8 Å used in the *GROMOS* force field, Koehler *et al.* (1987, 1988*a,b*) simulated a system consisting of four unit cells, the lengths of the edges of the computational box being $2a$, b and $2c$. Use of such a big computational box would make the MD refinement with time-averaging prohibitively expensive for testing a variety of conditions. In the absence of electrostatic interactions a much shorter cut-off radius can be used since the van der Waals interactions are of relatively short range. The use of a 4.0 Å cut-off would allow the use of the asymmetric unit as the computational unit. Therefore, the Coulombic interaction was neglected in the simulations. Since we are interested in testing the time-averaging methodology and use artificial 'observed' structure-factor amplitudes, this degradation of the force field is acceptable in view of the considerable gain in computing efficiency. An MD simulation of the asymmetric unit with its crystal symmetry was run for 100 ps at 300 K to equilibrate the system in the presence of the altered force field.

From this time point the artificial data was generated over a 10 ps MD simulation (with $V^{sf}_{restr} = 0$) at 300 K in which structure factors were calculated from the structure every 2 fs using a 1.0 Å^2 temperature factor on all atoms with the *Gromox* refinement suite (van Gunsteren & Berendsen, 1987; Gros & van Gunsteren, 1993). The grid spacing in the electron-density calculation was 1/3 of the maximum resolution used. After the data-generating simulation these structure factors were averaged [using (4)] into a single set of average structure factors. The generated data was then randomly, within a given resolution range, divided into two parts containing 90 and 10% of the total data. This was to allow for free R values (Brünger, 1992*a*, 1993), R_{free}, to be calculated during the various refinements.

Refinement scheme

All refinements were performed on the α-cyclodextrin structure with *Gromox* (van Gunsteren & Berendsen, 1987; Gros & van Gunsteren, 1993) varying the resolution, relaxation time and temperature factor of the system. In every case the refinement was carried out for 100 ps at 300 K. In every case a constant scale factor [k_{sc}, (2)] of one was used to scale the calculated and observed structure factors. The weight factor [k^{sf}, (2)] which scales the potential energy from the X-ray data to the physical potential energy was varied in each simulation so as to maintain the force generated by the X-ray term to approximately 50% of the force from the physical force field. The structure factors were updated every five time steps of 2 fs or if any atom had moved more than 1 Å.

For every refinement performed the crystallographic R factor and R_{free} were calculated using (3) and monitored as a function of time. A final averaged R factor was calculated for each refinement from the averaged structure factors calculated using (4) over the last 50 ps of the simulation.

Starting from the MD-equilibrated α-cyclodextrin structure the molecule was refined with traditional MD refinement for 5 ps with an overall temperature factor of 1.0 Å^2 and to a resolution of 1.0 Å. The initial crystallographic R factor was 60 2%, after 5 ps of refinement it had been reduced to 44.7% with an R_{free} of 43.4%. At this stage in the refinement a variety of overall temperature factors were tried to see which one would best represent the motions of the atoms in the crystal. Overall temperature factors of 7.5, 5.0 and 2.0 Å^2 were tried for 50 steps of MD each, giving R factors of 33.5, 32.5 and 39.9%, respectively. From these results a further 2 ps of MD refinement was performed using a temperature factor of 5.0 Å^2. The resulting structure had an R factor of 30.8% with $R_{free} = 32.6\%$. This structure then served as the starting point for all the subsequent time-averaging crystallographic refinements. The initial values for the averaged structure factors were taken to be equal to the instantaneous values.

Relaxation time and resolution

The relaxation time, τ_x, of a time-averaging crystallographic refinement is critical to adequate sampling of the fluctuations in the molecular system. The longer the relaxation time the more configurations will contribute to the ensemble. If the relaxation time

is too short, important configurations may be missed. However, if the data for the structure is not highly resolved then refining with an ensemble of structures is overfitting the available data. The use of R_{free} during refinement reflects whether the ensemble generated by the relaxation time is overfitting the data. If R_{free} deviates from the refined R factor overfitting is likely to occur.

In this study six relaxation times, τ_x, were examined 20, 10, 5, 2, 0.5 and 0 ps. Table 1 shows R factors which were obtained from calculated structure factors averaged over 50 ps (except for the case of $\tau_x = 20$ ps when the structure factors were averaged over 70 ps) at the end of a 100 ps refinement trajectory obtained with various relaxation times and resolutions. The simulations with relaxation time $\tau_x = 0$ correspond to a traditional single-structure MD refinement. Over all three resolution ranges examined the R factor for the data used in the refinement decreased significantly as the relaxation time increased. At 1.0 and 2.0 Å resolution R_{free} also decreased significantly as the relaxation time increased. At 3.0 Å, however, R_{free} is significantly higher than the refined R factor and does not change for longer relaxation times. This indicates that at 3.0 Å the ensemble generated by time averaging is overfitting the data and thus is inappropriate to use.

Whether or not it is possible to use time-averaging crystallographic refinement at a given resolution is dependent on the redundancy of the data. In the case of α-cyclodextrin in this crystal form with six water molecules there are 72 non-H atoms in the asymmetric unit. Each of these atoms can move in three dimensions, so there are formally $216 + 1$ (overall temperature factor) unknowns when determining one structure without individual temperature-factor refinement. The stereochemical constraints and restraints reduce this number in reality, whereas it is increased to an unknown degree by the application of time averaging. The number of unknowns is to be compared with the number of reflections in the three resolution ranges given in Table 2. At 3.0 Å the ratio of used data points to unknowns is 0.55, at 2.0 Å it is 1.7 and at 1.0 Å the ratio is 12.

In Fig. 1 the refined R factor and R_{free} are shown during the course of the 100 ps refinements for the three resolutions using relaxation times of 5 ps and temperature factors of 1.0 Å^2. At 1.0 Å resolution every fluctuation in the refined R factor is reflected in R_{free} (Fig. 1a), as indicated by a high correlation coefficient of 0.99 between the two curves. At 2.0 Å resolution, although the separation between the refined and the free R factor is larger, the fluctuations still have a correlation coefficient of 0.88 (Fig. 1b). At 3.0 Å resolution R_{free} is even further separated from the refined R factor, and becomes much less correlated to the refined R factor (Fig. 1c),

Table 1. *Effect of varying the relaxation time and resolution*

R factors were calculated from averaged structure factors using (4) over the last 50 ps of 100 ps trajectories.

Relaxation time τ_x (ps)	Resolution (Å)	Temperature factor (Å^2)	Overall R factor	Refined R factor	Free R factor
5	1.0	1.0	5.23	5.04	6.86
2	1.0	1.0	7.05	6.87	8.59
0.5	1.0	1.0	16.1	15.9	18.4
0	1.0	1.0	44.6	44.7	43.4
20	2.0	1.0	2.01	1.44	6.73
10	2.0	1.0	2.24	1.68	6.86
5	2.0	1.0	2.65	2.01	7.95
2	2.0	1.0	5.01	4.27	11.1
0.5	2.0	1.0	7.76	7.17	12.7
0	2.0	1.0	21.7	20.7	29.6
5	3.0	1.0	2.02	0.841	12.4
2	3.0	2.0*	2.59	1.50	12.2
0	3.0	1.0	15.8	15.1	22.0

* Since time averaging using 3.0 Å resolution data produced inaccurate results, further simulations with alternate temperature factors were unwarranted.

Table 2. *Number of reflections by resolution*

Resolution range (Å)	All observed data	Refined data	Free data
17.0–1.0	2921	2630	291
17.0–2.0	418	367	51
17.0–3.0	135	120	15

with a correlation coefficient of 0.46. The increasing separation of R_{free} and the refined R factor with decreasing resolution is due to the decreasing redundancy of the data. At high resolution there is a high ratio of data points to unknowns, so the data withdrawn for the R_{free} calculation is redundant in determining atomic positions with respect to data that was used in the refinement. Thus, the R_{free} is very close to and tightly correlated with the refined R factor at high resolution and becomes more separated and less correlated at lower resolution. At 3.0 Å there is less data to uniquely determine the structure in one configuration. The stereochemical constraints as embodied by the force field used make the actual number of unknowns much smaller than 216, which explains the correspondence of the refined and free R factor in the case where $\tau_x = 0$ ps. However, these stereochemical constraints do not suffice to determine several distinct conformations in the cases where $\tau_x \neq 0$ ps. Thus, time-averaging crystallographic refinement is least reliable at 3.0 Å resolution and is extremely reliable at 1.0 Å resolution in this test system.

At the higher resolution ranges, where sufficient data are available for the application of time-averaging refinement, the relaxation time for the system should be optimized. Even at very short relaxation times, time averaging can increase the accuracy with which the system is being described. This is seen by the dramatic decrease in crystallo-
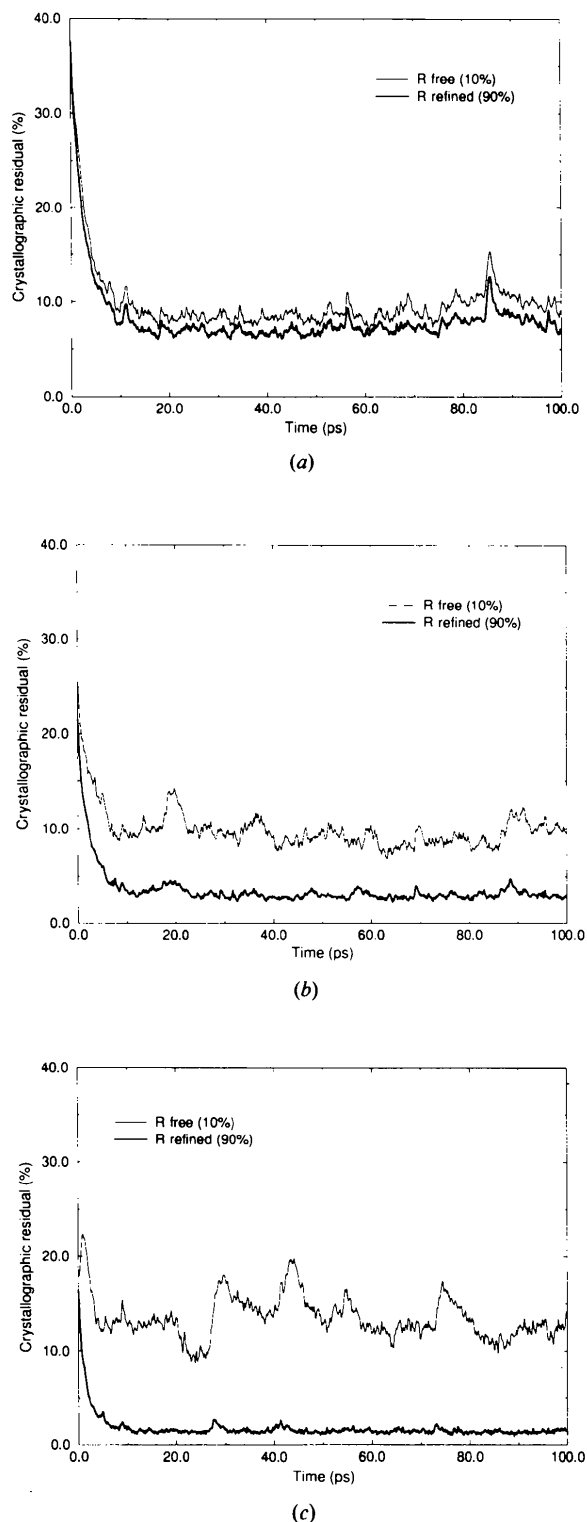
(a)

(b)

(c)

Fig. 1. The refined (solid line) and free R factor (dashed line) calculated using (3) for a 100 ps time-averaging refinement trajectory with a relaxation time τ_x of 5 ps, a temperature factor of 1 Å^2 and using data to a resolution of (*a*) 1.0, (*b*) 2.0 and (*c*) 3.0 Å.

graphic R factor from 44.7 to 15.9 to 6.87 by increasing the relaxation time from 0 to 0.5 to 2 ps at 1.0 Å resolution (Table 1). Similar behaviour is observed for the time-averaging refinements at 2.0 Å resolution, for which the refined and free R factors are plotted *versus* relaxation time in Fig. 2. As seen for the 1.0 Å refinement the R factor decreases quickly even at short relaxation times. As the relaxation time continues to be increased the refined R factor decreases but levels off. The R factor decreases only by 0.24% with a doubling of the relaxation time from 10 to 20 ps. This is not surprising considering the fact that the 'observed' data were generated over a 10 ps period. Thus, the optimal relaxation time for 2.0 Å resolution data representing local, short time (10 ps) motions of α-cyclodextrin is approximately 5 ps.

Overall temperature factor

Time-averaging refinement refines an ensemble of structures to the crystallographic data. In traditional single-structure refinement mobility in the structure is represented by the use of atomic temperature factors. In this section the use of an overall temperature factor to possibly increase the efficiency of time averaging is investigated. In Table 3 three pairs of simulations are compared. Enlarging the temperature factor of an atom effectively increases the radius of the electron density of that atom, thus by having a higher temperature factor the atom can cover more of space in the same amount of time. However, at high resolution, 1.0 Å, and 5 ps relaxation time increasing the temperature factor actually hinders the refinement. Since the data was generated using atoms with a temperature factor of 1.0 Å^2, the electron-
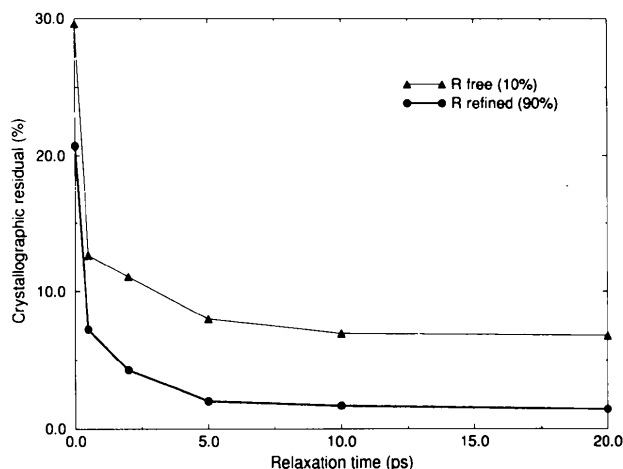

Fig. 2. The final refined and free R factor (taken from Table 1) resulting from a series of time-averaging refinements as a function of relaxation time τ_x at 2.0 Å resolution.

Table 3. *Effect of changing the temperature factor*

R factors were calculated from averaged structure factors using (4) over the last 50 ps of 100 ps trajectories.

Temperature factor (Å²)	Relaxation time τ_x (ps)	Resolution (Å)	Overall R factor	Refined R factor	Free R factor
1.0	5	1.0	5.23	5.04	6.86
2.0	5	1.0	5.85	5.73	6.85
1.0	0.5	1.0	16.1	15.9	18.4
5.0	0.5	1.0	15.7	15.6	16.8
1.0	2	2.0	5.01	4.27	11.1
2.0	2	2.0	3.87	3.12	10.1

density map possesses detailed features. These cannot be reproduced with temperature factors of 2 Å², resulting in a poorer R factor than when 1 Å² temperature factors were used.

The second pair of refinement simulations illustrates the effect of using a high temperature factor at a very short relaxation time. At 1.0 Å resolution and a 0.5 ps relaxation time, increasing the temperature factor from 1.0 to 5.0 Å² did not significantly improve the accuracy of the refinement. This is not surprising since an electron-density map with fine detailed features cannot be described well with relatively large atoms.

In the final pair of simulations the effect of changing the temperature factor at intermediate resolution, 2.0 Å, and medium-length relaxation time, 2 ps, is investigated. Here increasing the overall temperature factor does aid the refinement by decreasing the R factor by nearly one fourth. At this resolution this increase in accuracy is of the same order of magnitude as obtained by a doubling of the relaxation time. The difference at 2.0 Å is that the electron density does not have as sharp peaks as at 1.0 Å and, thus, increasing the effective radii of the atoms is advantageous to the refinement.

Comparison with anisotropic refinement

As a comparative test to the time-averaging crystallographic refinement a refinement with anisotropic temperature factors was performed with the generated data to 1.0 Å using the *SHELXL93* program (Sheldrick, 1990). Refinement of anisotropic temperature factors reflects that atoms do not vibrate in a spherically symmetric way and thus an ellipsoid is defined to represent the anisotropic motion of the atoms. The refinement of anisotropic temperature factors adds six more unknowns to refine per atom. The anisotropic temperature factor is defined as,

$$B = \exp[-2\pi^2(U_{11}h^2\mathbf{a}^{*2} + U_{22}k^2\mathbf{b}^{*2} + U_{33}l^2\mathbf{c}^{*2}$$
$$+ 2U_{12}hk\mathbf{a}^*\mathbf{b}^*\cos\gamma^* + 2U_{13}hl\mathbf{a}^*\mathbf{c}^*\cos\beta^*$$
$$+ 2U_{23}kl\mathbf{b}^*\mathbf{c}^*\cos\alpha^*)], \qquad (6)$$

where U_{ij} (six additional degrees of freedom per

atom) are the thermal parameters expressed in terms of mean-square amplitudes of vibration, h, k and l are the Miller indices, and $\mathbf{a}^*$, $\mathbf{b}^*$, $\mathbf{c}^*$, α^*, β^*, γ^*, are the reciprocal lattice unit vectors and angles. Thus, refinement of anisotropic temperature factors should be able to account for some of the motion that is accounted for with time-averaging crystallographic refinement.

The starting conformation was the same structure as was used at the start of the various time-averaged refinements. Initially the coordinates were given full occupancy and individual temperature factors of 5.0 Å² which yielded an R factor of 44.2%. After 20 steps of least-squares refinement and refinement of individual isotropic temperatures factors the R factor was 21.1%. Another such round of refinement brought the R factor to 16.2%. At this point a further 15 cycles of least-squares refinement was performed and a round of anisotropic refinement. Several atoms were flagged as potentially having two positions. These atoms, along with the water molecules, were given isotropic temperature factors and another ten steps of refinement were performed. The R factor was then 12.7%. Once again anisotropic refinement was tried on all atoms, but seven were again flagged as having two positions. These atoms were then assigned two positions each with 50% occupancy, ten steps of least-squares refinement were performed and the occupancies were also refined. The R factor dropped to 9.47%. The occupancies which showed two atoms in predominantly one position, were re-refined using a single isotropic position again for ten steps. The R factor became 9.41%. Then anisotropic refinement was tried on the three atoms which were no longer considered in split positions and a final 20 cycles of least-squares positional refinement were performed. The final resulting R factor was 8.74%, with four atoms placed in two distinct positions, the six water molecules refined isotropically and the remaining 62 atoms refined anisotropically. All the atoms that were split were O atoms, two were in the O6 position in the sugar ring, one was in the O2 position and one was in the O5 position, three of the atoms were part of the same sugar ring.

To compare the results of the *SHELXL93* refinement with the time-averaging refinement, the electron density of the ring with the three atoms with multiple positions was examined. In Fig. 3(a) the electron density from the *SHELXL93* model is shown, in Fig. 3(b) the density of the 1.0 Å, $\tau_x = 5$ ps, time-averaging refinement is shown, and in Fig. 3(c) the exact 'observed' electron density. The electron-density map obtained by time-averaging refinement almost perfectly matches the exact 'observed' one, although a small amount of noise is seen in a straight difference map. One of the factors
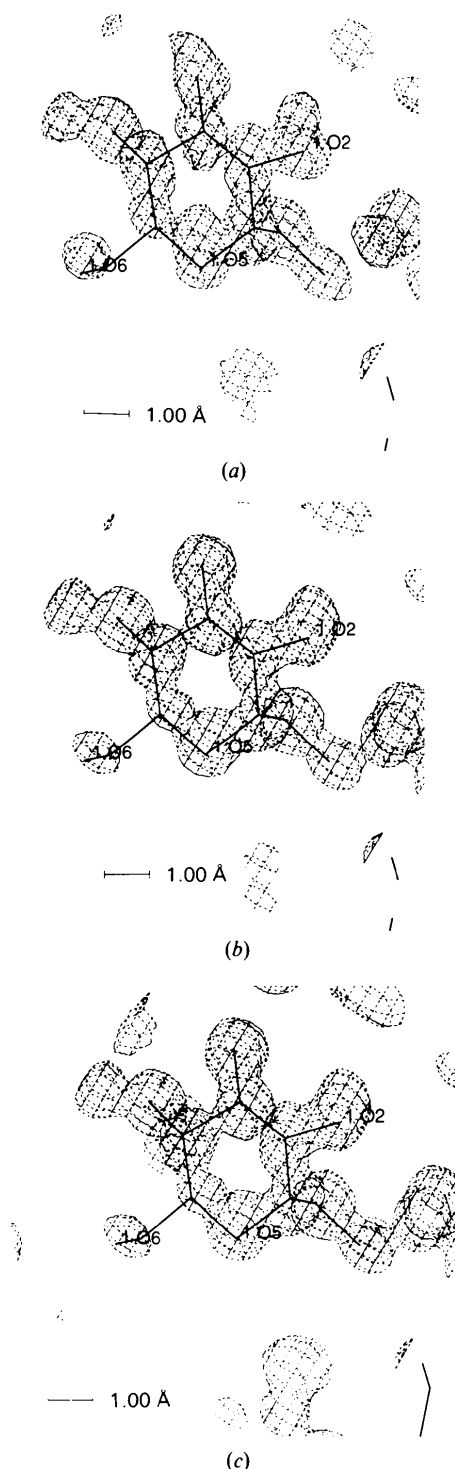
— 1.00 Å

(a)

— 1.00 Å

(b)

— 1.00 Å

(c)

Fig. 3. $2F_{obs} - F_{calc}$ electron-density map of one ring in the α-cyclodextrin contoured at 1σ using data to 1.0 Å resolution, (a) resulting from the SHELXL93 program, (b) resulting from the time-averaging simulation with a 5 ps relaxation time, (c) exact 'observed' density resulting from the structure-factor generating simulation. [The electron-density maps were produced with X-PLOR (Brünger, 1992b) and displayed with CHAIN (Sack, 1988).]

contributing to this perfect match is the use of the same force field in generating the 'observed' data as in the refinement. The electron density from the time-averaging refinement is much smoother and continuous around the ring with the positions of O2 and O5 in a single slightly broader, peak each than is observed in anisotropic temperature-factor refinement. The SHELXL93 electron density is less continuous, with very sharp edges around many of the atoms. In all three electron-density maps there is very little density around the C6 and O6 positions, indicating that the positions of these atoms are not well defined.

Conclusions and implications to macromolecular crystallography

In this study the range and modes of applicability of time-averaging crystallographic refinement were examined by simulations in which the resolution, relaxation time and temperature factor of the system are varied. Having sufficient resolution, and thus redundancy in the experimental data is the most critical parameter as to whether time averaging will work. Increasing the relaxation time of the system increases the size of the ensemble used for the refinement and thus better describes the system, which is reflected by decreasing (free) R factors. Choosing the relaxation time by a factor of two longer than the time period over which the 'observed' structure factors were collected ($\tau_x = 20$ versus 10 ps at 2.0 Å resolution) does not lead to a deteriorated model, although no improvement is obtained either; the free R factor slightly decreases, whereas the number of degrees of freedom effectively increases. At high resolution, 1.0 Å, increasing the temperature factor of the system does not aid in the refinement, whereas at 2.0 Å it has an effect similar to doubling the relaxation time. Thus, the appropriate parameters for time-averaging refinement are dependent on the experimental data of system of interest, the amount of computer time available and the intrinsic mobility and disorder of the molecular crystal.

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