Relaxation Matrix Refinement of the Solution Structure of Squash Trypsin Inhibitor

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The structure of the small squash trypsin inhibitor CMTI-I is refined by directly minimizing the difference between the observed two-dimensional nuclear Overhauser enhancement (NOE) intensities and those calculated by the full relaxation matrix approach. To achieve this, a term proportional to this difference was added to the potential energy function of the molecular dynamics program X-PLOR. Derivatives with respect to atomic co-ordinates are calculated analytically. Spin diffusion effects are thus accounted for fully during the refinement. Initial structures for the refinement were those determined recently by solution nuclear magnetic resonance using the isolated two-spin approximation to derive distance range estimates. The fits to the nuclear magnetic resonance data improve significantly with only small shifts in the refined structures during a few cycles of conjugate gradient minimization. However, larger changes (≥ 1 Å) in the conformation occur during simulated annealing, which is accompanied by a further reduction of the difference between experimental and calculated two-dimensional NOE intensities. The refined structures are closer to the X-ray structure of the inhibitor complexed with trypsin than the initial structures. The root-mean-square difference for backbone atoms between the initial structures and the X-ray structure is 0.96 Å, and that between the refined structures and the X-ray structure 0.61 Å.

Keywords: n.m.r. structures; NOE relaxation matrix refinement

1. Introduction

The ¹H nuclear Overhauser enhancement spectroscopy (NOESY) relies on the proton-proton dipolar interactions of a spin system. The intensity of the observed effects is a function of all interproton distances, thus providing information on the geometry of the system. For a rigid spin system and short mixing times in a first approximation (isolated two-spin approximation) the NOE intensity between a pair of protons is directly proportional to the inverse of the sixth power of the distance separating the protons (Macura & Ernst, 1980). In the case of large molecules the relationship between the crosspeak intensity and the distance of two protons is more complicated, since indirect magnetization transfer via other protons ("spin diffusion") contributes as well (Kalk & Berendsen, 1976). This, together with uncertainties in the motional behavior of a biomolecule, allows the determination of interproton distances derived with the isolated two-spin approximation only within approximate ranges. Until very recently, structure determination with n.m.r. data has relied on these approximate distance ranges.

Techniques to calculate a NOESY spectrum from atomic co-ordinates, either via the complete relaxation matrix (Keepers & James, 1984), or by numerical integration of the coupled differential equations which determine the relaxation (Marion et al., 1987; Lefèvre et al., 1987), allow a more direct comparison between observed crosspeak intensities and those calculated from a model. Spin diffusion is accounted for properly, so that weak peaks can be interpreted and the accessible distance range can be extended. Furthermore, there is no need to obtain spectra at very short mixing times, which leads to a gain in
accuracy in the measured crosspeak volumes. The back calculated 2D NOE intensities are very sensitive to small variations in the distance between protons, and to differences in the surrounding configurations (Madrid & Jardetzky, 1988). Together with the constraints imposed by the geometry of the molecule, even pure spin diffusion peaks may help to define the conformation of a molecule. A number of methods have been proposed to make use of calculated 2D NOE intensities in the refinement of n.m.r. structures. The target distances can be iteratively modified manually (Banks et al., 1989; Summers et al., 1990) or automatically (Guenet et al., 1990) to match observed and calculated 2D NOE intensities. Another approach is based on the back-transformation of the matrix of intensities to obtain the relaxation matrix and, hence, the cross-relaxation rates (Olejniczak et al., 1986). This requires a complete NOE matrix. All 2D NOE intensities have to be known and, for example, no spectral overlaps exist. Therefore, a "hybrid" NOE matrix is constructed where unknown experimental intensities are replaced by calculated ones (Boelens et al., 1988, 1989; Nikonowicz et al., 1990).

Ideally, though, a penalty function consisting of the differences between the calculated and observed 2D NOE intensities should be used directly as the driving force to change the conformation during refinement. Refinements using gradient-free optimization have been reported (Borgias & James, 1988; Borgias et al., 1990). Powerful minimization techniques, such as conjugate gradient minimization or molecular dynamics, require the gradient of the penalty function and thus of the calculated 2D NOE intensities. The gradient can be computed by a finite difference approximation (Baleja et al., 1990a, b) or analytically (Yip & Case, 1989; Koch et al., 1990). An extended version of the refinement program X-PLOR (Brünger, 1990c), which incorporates the difference between observed and calculated 2D NOE intensities as an effective energy term, and the analytical derivatives from Yip & Case (1989), we have refined the structure of the squash trypsin inhibitor CMTI-I, whose solution structure has been determined by n.m.r. and preliminary refined with distances derived from the isolated two-spin approximation (Holak et al., 1987).

2. Materials and Methods

(a) Acquisition of spectra and integration

Specifically for the purpose of the relaxation refinement NOESY spectra at various mixing times were acquired. The samples for n.m.r. contained approx. 3 mM CMTI-I in 2 mM sodium acetate (pH 4.3) in 90% H2O/10% D2O or in 100% D2O. The 2D n.m.r. spectra were recorded at 25°C in the pure-phase absorption mode on a Bruker AM-500 spectrometer. The 2D NOE intensities in H2O were recorded interleaved with mixing times of 50, 100, 150, 200 and 250 ms. The NOESY spectrum in H2O was recorded for a mixing time of 150 ms. The NOESY mixing times were randomly varied to eliminate coherent transfers (Macura et al., 1981). The water resonance was suppressed by presaturation. 512 T1 increments were collected, each with 1000 real data points, over a spectral width of 7 kHz in both dimensions. Base plane distortions were corrected by a polynomial baseline flattening of rows of the 2D matrices. The volumes of the crosspeaks at different mixing times were estimated with the relation

\[ V = \frac{aL_1L_2h}{2} \]

where \( L_1 \) and \( L_2 \) are the half line widths at the half-heights along the F1 and F2 axes, respectively, and \( h \) is the height of the peak (Holak et al., 1987). The heights were determined from the columns of the 2D matrix at the appropriate \( F_2 \) frequencies, in the majority of cases at the NH amide resonances. The intensities measured for the single 2H2O spectra were combined with the H2O spectra after appropriate scaling. For 150 ms and longer mixing times the errors in the intensities in most cases were very small (5 to 20%); errors refer to standard deviations in the integrations performed for different spectral limits as discussed by Holak et al., 1987); larger errors were associated with spectral overlap. At the shortest mixing time, the volume estimation was less precise (50 to 100%) because of the low intensity of the resonance peaks. The buildups of NOE intensities covering all mixing times were measured for 235 crosspeaks. In addition, 190 NOE intensities were measured only at the 150 ms and longer mixing times. These intensities were also used in the refinement. A complete table of the intensities and error estimates is included in the supplementary material.

Absence of peaks is an important part of the information in an n.m.r. spectrum. However, the computational cost for including a term for every possible crosspeak would be prohibitive. We have included an intensity with value zero only for crosspeaks whose absence had proven to be structurally important in the calculation of the initial structures (Holak et al., 1989), that is one for every "repulsive" distance constraint used there. There were 108 such constraints used in the refinement.

(b) Geometric energy function \( E_{\text{empirical}} \)

The total energy that is minimized in the refinement is constructed by combining experimental and empirical information in a way that has become important in refinement techniques (for a review see Brünger, 1990b): \[ E_{\text{total}} = E_{\text{empirical}} + E_{\text{experimental}} \] (1)

The form of the geometric energy function, \( E_{\text{empirical}} \), used here (Nilges et al., 1988) reflects the fact that our aim is not to obtain dynamical information from a molecular dynamics calculation but to maintain geometric ideality of the system, that is bond lengths, bond angles, planarity and repulsion. The energy constants used are the same as those used in the refinement of the initial structures using NOE-derived distance constraints (Holak et al., 1989).

Switching to a more conventional molecular dynamics force field (Brooks et al., 1983), including electrostatic interactions and a Lennard–Jones potential, at this point would have made it difficult to differentiate between the effects of the empirical and experimental parts of the target function, and to assess structural differences due to the relaxation matrix term alone.

The energy constants of the geometric energy function are in some cases substantially higher than in conventional molecular dynamics force fields. A case in point is

\[ \dagger \] The supplementary material has been deposited with the Brookhaven Protein Data Bank, accession number 3CTI.
the energy constant for the peptide plane dihedral angle, \( \omega \), which is 10 kcal mol\(^{-1}\) rad\(^{2}\) in the CHARMM PARAM19 force field (Brooks et al., 1983), while it is 200 kcal mol\(^{-1}\) rad\(^{2}\) in our calculations (1 cal = 4.184 J). The planarity of the peptide group is easily distorted during refinement with an unmodified molecular dynamics force field unless very low weights for the experimental terms are used. For this reason, the energy constant for the peptide plane angle had been increased for both n.m.r. and crystallographic refinement (e.g. see Brünger, 1988).

(c) Experimental energy function \( E_{\text{relaxation}} \)

The basis for the refinement is the calculation of the volume of a crosspeak between spins \( i \) and \( j \), \( I_{ij} \), from the atomic co-ordinates by means of the relaxation matrix \( R \) (Macura & Ernst, 1980; Ernst et al., 1987; Keepers & James, 1984):

\[
I_{ij} \propto \exp(-R_{ij}),
\]

where \( \tau_{ij} \) is the mixing time. The relaxation matrix \( R \) is a function of the transition rates \( \Omega^{ij} \):

\[
R_{ij} = \begin{cases} 
\Omega_{ij}^{ij} - \Omega_{ij}^{0} & \text{if } i \neq j \\
\sum_{j \neq i} \Omega_{ij}^{ij} + \Omega_{ij}^{0} & \text{if } i = j.
\end{cases}
\]

which are determined by spectral densities and dipolar coupling strengths (Solomon, 1955):

\[
\Omega_{ij}^{ij} = d_{ij} J(0)
\]

\[
\Omega_{ij}^{ij} = \frac{3}{2}d_{ij} J(\omega)
\]

\[
\Omega_{ij}^{ij} = 6d_{ij} J(2\omega)
\]

and

\[
d_{ij} = \gamma_{n} h_{0}^{2}/10\nu_{0}^{2},
\]

\( \gamma \) is the gyromagnetic ratio of the proton, and \( \tau_{n} \) is the distance between spins \( i \) and \( j \). For the calculations reported in this paper, we use a simple model, where a single isotropic correlation time \( \tau_{c} \) is assumed to be sufficient to describe the spectral densities \( J(\omega) \) (Solomon, 1955):

\[
J(\omega) = \frac{\tau_{c}}{1 + \omega^{2}\tau_{c}^{2}}.
\]

As the matrix \( R \) is symmetric, it can be diagonalized:

\[
R = L^{\dagger} A L.
\]

where \( A \) is diagonal and contains the Eigenvalues of \( R \). The derivative of \( I_{ij}^{\mu} \) with respect to a co-ordinate \( \mu \) (eqn (12) of Yip & Case, 1989) can be written as:

\[
V_{\mu} I_{ij}^{\mu} = V_{\mu} \exp(-R_{ij}) I_{ij}^{0} + \text{Trace}[V_{\mu} R L F^{(0)} L^{\dagger}],
\]

where \( F^{(0)} \) is defined as:

\[
F_{\alpha \beta}^{(0)} = \begin{cases} 
- L_{\alpha} L_{\beta} \exp(-\lambda \tau) - \exp(-\lambda \tau) / (\tau \lambda) & \text{if } \alpha \neq \beta \\
L_{\alpha} L_{\alpha} \exp(-\lambda \tau) & \text{else}
\end{cases}
\]

\( \lambda \) is the rth Eigenvalue of the relaxation matrix. This definition is similar to that of the matrix \( M^{(0)} \) in eqn (13) of Yip & Case (1989). \( E_{\text{relaxation}} \) can now be expressed as a function of the difference between (functions of) observed and calculated intensities, and analytic derivatives with respect to atomic co-ordinates can be readily obtained by using the chain rule:

\[
E_{\text{relaxation}} = K_{E} \sum_{\text{Spectra}} \sum_{i=1}^{N_{s}} w_{i} \text{well}(I_{ij}^{\mu}, k_{E} I_{ij}^{\mu}, \Delta_{E}, n)_{m},
\]

where \( K_{E} \) is the energy constant for the relaxation term, \( I_{ij}^{\mu} \) and \( I_{ij}^{0} \) are the calculated and observed intensities, respectively, \( \Delta_{E} \) is an error estimate for \( I_{ij}^{\mu} \), \( w_{i} \) is a weight factor, \( k_{E} \) is the calibration factor for each spectrum, and \( N_{s} \) is the number of crosspeaks in each spectrum. The function well\((a, b, \Delta, n)\) is defined as the absolute value of the difference between the \( n \)th powers of \( a \) and \( b \), where \( b \) has an error estimate \( \Delta \):

\[
\text{well}(a, b, \Delta, n) = \begin{cases} 
(b - \Delta)^{n} - a^{n} & \text{if } a^{n} \leq (b - \Delta)^{n} \\
0 & \text{if } (b - \Delta)^{n} < a^{n} < (b + \Delta)^{n} \\
(a^{n} - (b + \Delta)^{n}) & \text{if } a^{n} \geq (b + \Delta)^{n}.
\end{cases}
\]

The individual error estimates \( \Delta \) reflect the integration error due to noise and spectral overlap. Only \( n = 1 \) and \( m = 2 \) were used (eqns (10) and (11)) for the calculations in this paper, but other values are being tested. As a measure of the fit of the refined structure to the NOE data we use a generalized \( R \)-factor:

\[
R_{E} = \left[ \sum_{\text{Spectra}} \sum_{i=1}^{N_{s}} w_{i} \text{well}(I_{ij}^{\mu}, k_{E} I_{ij}^{\mu}, \Delta_{E}, n) \right]^{1/2}.
\]

For \( n = 1 \), \( \Delta = 0 \) and \( w_{i} = 1 \) this is the \( R \)-factor used in crystallography (Stout & Jensen, 1989), but we also report values with \( n = 1/6 \) as suggested by James et al. (1990). The value of the calibration factor \( k_{E} \) is evaluated simply as:

\[
k_{E} = \left( \frac{\sum_{\text{Spectra}} \sum_{i=1}^{N_{s}} w_{i} I_{ij}^{\mu}}{\sum_{\text{Spectra}} \sum_{i=1}^{N_{s}} w_{i} (k_{E} I_{ij}^{\mu})^{n}} \right)^{1/n}
\]

where the sum runs over all \( N_{s} \) peaks which are well determined; for a peak to be included in the calculation of the calibration factor, it has to be at least 3 times as big as the error estimate.

For the calculations in this paper, the scale was determined independently for each mixing time. The determined values for the scale for the different mixing times do not differ greatly, and the difference to using only one overall calibration factor is small. The advantage in using different calibration factors for different mixing times is simply that the experimental spectra do not need to be scaled accurately relative to each other. Also, non-NOE relaxation effects (termed overall Z-leakage by Summers et al., 1990) are dealt with in a simple way. The calibration factor is updated several times during the refinement (see section (e), below), in addition to the individual weights \( w_{i} \) which are applied to each term in the sum of eqns (10) and (12), in order to increase the relative weight of the small intensities. We have used \( w_{i} = (1/I_{ij}^{0})^{n-2} \), which is the weighting used in crystallography if experimental \( \sigma \) values are unavailable. It should be noted, however, that in the n.m.r. case there is no theoretical justification for this weighting scheme (in crystallography, the statistical error \( \sigma \) of an intensity measurement \( I_{ij}^{0} \) is \( \sqrt{I_{ij}^{0}} \)). The weights are scaled such that \( \text{min}(w_{i}) = 1 \). For absent crosspeaks, the weighting scheme would result in dividing by zero. In order to avoid this, the maximum weight used is determined by the smallest peak which is well determined, where we use the same criterion as in the determination of the calibration factor, that is a 2D NOE intensity has to be at least 3 times as large as its error estimate.
Methyl groups were treated similarly to the method used in COORMA, version 1.5 (Keepers & James, 1984): each methyl group is represented by one spin whose intensity is scaled by a factor of 3, and the distance to a methyl group is calculated as the \((r^{-3})^{-1/2}\) average over the 3 methyl protons (eqn (5)). The stereospecific assignment of diastereotopic proton or methyl pairs, determined by Holak et al. (1989) and Habazettl et al. (1990) was used. For ambiguous peaks and not stereospecifically assigned methylene protons, the sum of the calculated intensities was restrained to the sum of the observed ones.

The relaxation matrix contribution of the target function was supplemented by distance constraints for the clearly identified hydrogen bonds and experimental torsion angle constraints (Holak et al., 1989).

(d) Increasing the efficiency

The major drawback of the use of the relaxation matrix in n.m.r. refinement is the high computational cost. The diagonalization necessary to calculate intensities from a conformation is \(O(N x^3)\), where \(N x^3\) is the number of spins; the calculation of the gradient by eqn (8) is \(O(N x^3)\) for every crosspeak. As noted by Yip & Case (1989), the contribution of the relaxation matrix to the gradient may not have to be calculated every step during a dynamics/simulated annealing refinement. In our case, we found it is sufficient to recalculate the relaxation matrix whenever a hydrogen atom has moved by more than 0.075 Å (1 Å = 0.1 nm). Increasing this number (the tolerance) beyond 0.075 Å resulted in heating the system and large temperature fluctuations. Experience with the refinement of other molecules indicates that the optimal value for the tolerance depends on the system and, generally, a lower value than 0.075 Å is probably advisable. For a tolerance of 0.02 Å, the relaxation matrix term is recalculated approximately every 2 fs at an annealing temperature of 300 K; for a tolerance of 0.075 Å, every 2 to 4 fs.

The largest reduction in computation time was achieved by the introduction of a distance cutoff for the relaxation matrix and gradient calculations. To a good approximation, the size of a 2D NOE crosspeak between spins \(i\) and \(j\) is affected only by the relaxation pathways \(i\) via spins close to \(i\) or \(j\). Thus, individual distance cutoff spheres were used around \(i\) and \(j\) for the calculation of \(H_{ij}\) and the contribution to the gradient due to this crosspeak. For every pair of spins, a relaxation matrix is generated and diagonalized separately.

The size of the cutoff, that is the number of cross-relaxation pathways that need to be included in the calculation for a given crosspeak, depends on the longest mixing time, and the rotational correlation time of the refined molecule (200 ns and 2.5 ns respectively, in the present study). Calculations with different cutoff values showed that the central processor unit time starts rising steeply for a cutoff larger than 4 Å, while \(E_{\text{relaxation}}\) and the gradient are not greatly affected by increasing the cutoff beyond 3.5 Å (3% for increasing the cutoff from 3.5 to 4 Å, and 1% for increasing it from 4.0 to 4.5 Å). A cutoff of 4.0 Å seemed therefore a good compromise. For molecules with a higher mass and therefore larger isotropic correlation time, or longer mixing times, this may have to be increased. Details of this method and further test calculations will be published elsewhere (M. Nilges & A. T. Brünger, unpublished results).

(e) Refinement protocol

The following refinement protocol was used:

1. (1) 25 steps minimization. \(K_R = 100\);
2. (2) simulated annealing stage: 0.5 ps dynamics (300K). \(K_R = 100\), timestep 0.5 fs, tolerance 0.075 Å. 0.5 ps dynamics (300K). \(K_R = 200\), timestep 0.5 fs, tolerance 0.075 Å.
3. (3) 30 steps minimization. \(K_R = 400\).

Tolerance denotes the maximum distance a hydrogen atom is allowed to move before the relaxation matrix and gradient are recalculated (see section (d), above). With the value used, the relaxation term was recalculated during dynamics every 2 to 4 fs. The calibration factors \(k_p\) were recalculated after the initial minimization, and every 0.5 ps during the simulated annealing stage. Increasing the weight on the relaxation matrix term beyond \(K_R = 400\) did not improve the structures (see Results). Results from additional calculations with longer simulation times and smaller values for the tolerance were not significantly different from the reported ones. With a cutoff of 4.0 Å a refinement of CMMT-1 with data comprising a total of 1301 crosspeaks with this protocol took approximately 6 to 8 h central processor unit time on a Convex (210 computer, and 8 to 10 h on an IRIS 4D/220 GTX workstation.

3. Results

Independent refinements were performed from eight different initial conformations: six from n.m.r. structures refined with NOE derived distances, which are a subset of the structures from Holak et al. (1989), one from the X-ray structure (Bode et al., 1989), and one from a random structure. The random structure was generated by assigning random values to \(\phi\) and \(\psi\), 180° to all \(\chi_i\), and removing non-bonded contacts in the resulting structures before and after refinement.

<table>
<thead>
<tr>
<th>Structure</th>
<th>X-ray</th>
<th>Unweighted</th>
<th>Weighted</th>
<th>(r^{1.5})</th>
</tr>
</thead>
<tbody>
<tr>
<td>n.m.r., initial</td>
<td>0.31 ± 0.02</td>
<td>0.46 ± 0.02</td>
<td>0.64 ± 0.05</td>
<td>0.05 ± 0.002</td>
</tr>
<tr>
<td>n.m.r., refined</td>
<td>0.29 ± 0.01</td>
<td>0.35 ± 0.02</td>
<td>0.52 ± 0.05</td>
<td>0.075 ± 0.002</td>
</tr>
<tr>
<td>X-ray, refined</td>
<td>0.26 ± 0.01</td>
<td>0.22 ± 0.01</td>
<td>0.39 ± 0.02</td>
<td>0.060 ± 0.004</td>
</tr>
<tr>
<td>Random, unrefined</td>
<td>0.34</td>
<td>0.49</td>
<td>0.70</td>
<td>0.05</td>
</tr>
<tr>
<td>Random, minimized</td>
<td>0.44</td>
<td>0.39</td>
<td>0.57</td>
<td>0.087</td>
</tr>
<tr>
<td>X-ray, refined</td>
<td>0.52</td>
<td>0.28</td>
<td>0.44</td>
<td>0.089</td>
</tr>
<tr>
<td>Random, unrefined</td>
<td>1.12</td>
<td>1.06</td>
<td>3.00</td>
<td>0.32</td>
</tr>
<tr>
<td>Random, minimized</td>
<td>0.91</td>
<td>0.87</td>
<td>1.59</td>
<td>0.30</td>
</tr>
<tr>
<td>Random, refined</td>
<td>0.63</td>
<td>0.58</td>
<td>0.91</td>
<td>0.26</td>
</tr>
</tbody>
</table>
structure by minimization. The aim of this additional calculation was to obtain an estimate for the refined R-factor of an incorrect structure. A uniform isotropic correlation time of 2.3 nanoseconds was used in all calculations. The R-factor of the initial n.m.r. structures remained virtually unchanged over a large range of correlation times (1 to 10 ns; data not shown). Thus, no attempt was made to optimize the correlation time.

(a) Agreement with n.m.r. data

Figure 1 illustrates the improvement of the agreement with the n.m.r. data during the refinement. The Figure shows the value of the R-factor every time the relaxation matrix term is recalculated. The effect of the initial minimization is shown in the "negative time" part of the plot, the simulated annealing stage starts at the grey vertical line. R-factors for initial and final structures are compiled in Table 1. The values were calculated in four different ways (see eqn (12)): Weighted corresponds to the value calculated with the same individual weights \( w_i \) as used in the target function (see eqns (10) and (11)), with \( n \) set to 1 in equation (12); Unweighted is the unweighted R-factor (\( w_i = 1 \)); X-ray is the unweighted R-factor with all error estimates \( \Delta_i = 0 \), \( n = 1 \), and \( w_i = 1 \); and \( r^{1/6} \) an R-factor calculated with \( n \) set to \( \frac{1}{6} \), as suggested by James et al. (1990). The R-factors for the random structure given in Table 1 are lower estimates only, as only a few absent peaks were included in the calculation, namely only those that were of structural significance in the correct conformation (see Materials and Methods). Note that all refinements were carried out with the weighted target function, with \( n = 1 \) and \( m = 2 \) in equation (10).

Figure 2(a) to (c) shows the effect of minimization and simulated annealing stage on all deviations between \( P^0 \) and \( P^i \) for one of the structures. The size of the deviation is plotted against the observed value of the 2D NOE intensity.

(b) Structural changes during refinement

Figure 3 shows a superposition of one of the six initial and the corresponding refined n.m.r. structures. In Figure 4, the movement of the structures is monitored during the refinement. At each time point, the atomic r.m.s differences to the "previous" structure is plotted. Thus, the value at 500 femtoseconds to the r.m.s. difference between 500 and 0 femtoseconds, and so on. Figure 5 shows how the interproton distances changed. The distances between all proton pairs of each structure were calculated and the average number of the proton distances before and after refinement was plotted against the corresponding distance range.

Deviations from ideal values for bonds lengths, bond angles and planarity are compiled in Table 2. Atomic r.m.s. differences of initial and final structures are compiled in Table 3.

Figures 6 and 7 are stereoplots of the six initial and refined n.m.r. structures. Figure 8 shows the average pairwise r.m.s. difference among the six initial and refined n.m.r. structures. Figure 9 shows
4. Discussion

(a) Agreement with n.m.r. data

Almost half of the reduction in the R-factor is achieved in the initial minimization (see Fig. 1 and Table 1). This improvement of the agreement with the observed spectrum is achieved by surprisingly small shifts in the structure (~0.1 Å, see Fig. 4). From Figure 2(a) and (b) it becomes clear that the large reduction in R-factor during minimization is mainly due to a reduction of the largest deviations.

The R-factor slowly decreases further during the simulated annealing stage. As Figure 4 shows, this further reduction in the R-factor is accompanied by larger changes in the conformation. The “steps” that are visible in the time-course of the R-factor are a consequence of the doubling of $K_R$ every 500 femtoseconds (see Materials and Methods). Minimization of the structures after 500, 1000, 1500 and 2000 femtoseconds of simulated annealing had virtually no effect on the R-factor (data not shown).

Figures 1 and 4 indicate that the refinement has “converged” after the third cycle of the simulated annealing stage, with a final weight on the relaxation matrix term of 400. First, the r.m.s. difference between the conformations after 1.5 and 2.0 picoseconds (already that between those at 1.0 and 1.5 ps) is small (~0.1 Å). Second, a further reduction of the R-factor is only achieved at the cost of a rise in the deviations from ideality in bond angles and planarity (data not shown).

All four reported R-factors in Table 1 are much higher for the random structure than for the n.m.r. structures or the X-ray structure, both before and after the refinement. The $r^{16}$ R-factor decreases less during the refinement of the random structure. This, together with the fact that the difference in values between refined “correct” and “incorrect”
Figure 6. Stereo view of all non-hydrogen atoms of the 6 initial n.m.r. structures. The least-squares fit (Kabsch, 1976) was carried out for residues 2 to 29 as residue 1 is undetermined by the n.m.r. data.

Figure 7. Stereo view of all non-hydrogen atoms of the 6 refined n.m.r. structures. The least-squares fit (Kabsch, 1976) was carried out for residues 2 to 29 as residue 1 is undetermined by the n.m.r. data.

The improvement of the agreement with the observed n.m.r. spectra is also evident in Figure 2. The width of the distribution of the deviations is generally reduced. We note, however, that a few large deviations remain, and are virtually unaffected by the refinement. The two large deviations labeled 1 and 2 in Figure 2 are present in all seven final structures. Deviation 1 corresponds to a cross-peak from one of the β protons of Tyr27 to its own δ protons, and may be attributed to several factors.

Table 2
Average deviations from ideality

<table>
<thead>
<tr>
<th>Structure</th>
<th>Bonds (Å)</th>
<th>Angles (deg.)</th>
<th>Planarity (deg.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>0.012 ± 0.001</td>
<td>2.13 ± 0.03</td>
<td>0.36 ± 0.04</td>
</tr>
<tr>
<td>Refined</td>
<td>0.008 ± 0.001</td>
<td>2.25 ± 0.02</td>
<td>1.61 ± 0.13</td>
</tr>
</tbody>
</table>

Table 3
Atomic r.m.s. differences (Å) between starting, refined and X-ray structures of CMT1-I

<table>
<thead>
<tr>
<th>Structure</th>
<th>Heavy backbone atoms</th>
<th>All heavy atoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starting versus starting</td>
<td>0.46 ± 0.09</td>
<td>1.23 ± 0.19</td>
</tr>
<tr>
<td>Refined versus refined</td>
<td>0.38 ± 0.12</td>
<td>1.21 ± 0.13</td>
</tr>
<tr>
<td>Starting versus refined</td>
<td>0.87 ± 0.07</td>
<td>1.54 ± 0.17</td>
</tr>
<tr>
<td>Starting versus X-ray</td>
<td>0.96 ± 0.10</td>
<td>1.79 ± 0.08</td>
</tr>
<tr>
<td>Refined versus X-ray</td>
<td>0.61 ± 0.08</td>
<td>1.57 ± 0.14</td>
</tr>
<tr>
<td>Pairwise refined versus starting</td>
<td>0.86 ± 0.07</td>
<td>1.24 ± 0.05</td>
</tr>
<tr>
<td>Refined X-ray versus refined n.m.r.</td>
<td>0.42 ± 0.11</td>
<td>1.29 ± 0.14</td>
</tr>
<tr>
<td>Refined X-ray versus X-ray</td>
<td>0.54</td>
<td>1.18</td>
</tr>
</tbody>
</table>
The degeneracy of the δ proton chemical shifts indicates the possibility of rotation of the aromatic ring, which would increase the peak intensity. In addition, the intensity is in a region of the spectrum with overlap, and the error estimates may simply have been set too small in this case. The cause for the large deviation is unclear. Experimental work to clarify the reason for this large deviation, including three-dimensional n.m.r. spectroscopy to exclude the possibility of spectral overlap, is in progress.

Figure 8. Average pairwise atomic r.m.s. difference among the initial structures (open squares), and the refined structures (filled circles, line). The bars represent the standard deviations (only shown for the refined structures). The residue numbers are given for the 6 Cys residues.

The shifts in the structures during refinement are more than twice as large as the pairwise r.m.s. difference between different n.m.r. structures (≈0.9 Å versus ≈0.4 Å; see Table 3). Part of this shift is due to an expansion of interproton distances, which is evident in Figure 5. In all but one distance range the average number of interproton distances is smaller after the refinement than before. This is probably due to the influence of spin diffusion in the original distance estimates. Especially in the ranges from 3.6 to 4.0 Å and 4.1 to 4.4 Å the effect of spin diffusion is seen, approximately 25 proton distances in each range had too close proton contacts before refinement.

Plots of the atomic r.m.s. difference between the refined n.m.r. structures and the X-ray structure are shown in Figure 10, the r.m.s. difference between the initial n.m.r. structures and the X-ray structure is indicated as squares. Figure 10 shows that compared to the initial structures, the backbone as well as the side-chains of the refined structures are closer to the X-ray structure. In Figure 9 the backbone conformation of the binding loop (residues 2 to 10) is identical in the two structures. The side-chain conformations are less well determined by the n.m.r. data than that of the backbone, resulting in larger differences between the n.m.r. structures and the X-ray structure. It is interesting to note that, with the exception of Glu24 and His25, the largest r.m.s. differences between the n.m.r. and X-ray side-chain conformations occur for a residue for which no stereospecific assignment of the side-chain protons was achieved (Lys5; see Fig. 10 and Holak et al., 1989). Figure 9 shows that the positions of many side-chains of the refined structure are close to those of the X-ray structure at least up to the Cβ atoms.

(c) Refinement of the X-ray structure with the n.m.r. data

Although the refined n.m.r. structures are very close to the X-ray structure (Bode et al., 1989), some differences remain. In order to study if these differ-
enences are due to the experimental data or to a local minimum problem, we performed a test by carrying out a refinement starting from the X-ray structure.

The results of these calculations are compiled in Figures 11 and 12, and Tables 3 and 1. The $R$-factor improves during refinement by approximately the same amount as for the n.m.r. structures. The $R$-factor of the X-ray structure is only a little larger than the average over the $R$-factors of the initial n.m.r. structures. After refinement, it remains somewhat larger than the average over the $R$-factors of the refined n.m.r. structures.

During n.m.r. refinement, the structure moves away from the X-ray structure (the starting structure), and approaches the refined n.m.r. structures (cf. continuous lines in Figs 11 and 12). The final atomic r.m.s. difference between the n.m.r. refined X-ray structure and the six refined n.m.r. structures is similar to that among the six refined n.m.r. structures (see Fig. 12).

5. Conclusions

The n.m.r. $R$-factor is very sensitive to small differences in the atomic co-ordinates. A large part of the reduction in the $R$-factor was achieved by

Figure 10. Average atomic r.m.s. difference between the 6 refined n.m.r. structures and the X-ray structure (filled circles, line), and the initial n.m.r. structures and the X-ray structure (open squares). The bars represent the standard deviations shown for the refined versus X-ray only. The residue numbers are given for the 6 Cys residues.
Figure 11. Atomic r.m.s. difference between the n.m.r. refined X-ray structure and the X-ray structure (filled circles, line). For comparison, the atomic r.m.s. difference between the 6 refined n.m.r. structures and the X-ray structure is also shown (open squares). The residue numbers are given for the 6 Cys residues.

small atomic shifts during conjugate gradient minimization. Also during simulated annealing, no drastic changes occurred in the structure. This is in marked contrast to the situation in crystallography, where larger conformational changes may be necessary to achieve large reductions in the R-factor. This is probably due to the fact that the initial structures for n.m.r. refinement are already

Figure 12. Average atomic r.m.s. difference between the n.m.r. refined X-ray structure and the other 6 n.m.r. structures (filled circles, line). For comparison, the pairwise atomic r.m.s. difference among the refined structures is also shown (open squares). The residue numbers are given for the 6 Cys residues.
close to a local minimum of $E_{\text{total}}$ (1). The isolated two-spin approximation used to obtain the initial structures defines the atomic positions fairly well.

The significant shifts observed during the relaxation matrix refinement of CMTI-I are of a more subtle nature. For all refined structures, the atomic r.m.s. from the X-ray structure decreased during the refinement, while the size of the ensemble, which is sometimes used as an ad hoc measure for precision, decreased only slightly. This, together with the fact that the structures expanded slightly during the refinement, implies that the distance constraints derived from the isolated two-spin approximation were systematically slightly underestimated, although a relatively short mixing time was used. This stresses the importance of taking spin diffusion into account during n.m.r. refinement. But our results also show that the bias due to neglect of spin diffusion can be removed in a relatively short relaxation matrix refinement. Thus, the isolated two-spin approximation seems "safe" for initial relaxation matrix refinement. Thus, the isolated structure calculations even if the mixing times are not short enough, provided that the initial calculations are followed by a refinement which takes spin diffusion into account.

Although the $R$-factor could be significantly reduced during the refinement, its final value is far from zero, and a few large discrepancies between calculated and observed 2D NOE intensities did not disappear. Considering the approximations that were used in the refinement, this is not surprising. Especially the effects of motions and multiple substates were neglected in the present calculations. We are currently studying if a combination of methods such as suggested by Kim & Prestegard (1989) or Torda et al. (1989) with the relaxation matrix is a useful approach to treat multiple substates. Different motional behaviour may be taken into account by going beyond the approximation used in equation (6) (Lipari & Szabo, 1982).

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References


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