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Influence of the completeness of chemical shift assignments on NMR structures obtained with automated NOE assignment

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Abstract

Reliable automated NOE assignment and structure calculation on the basis of a largely complete, assigned input chemical shift list and a list of unassigned NOESY cross peaks has recently become feasible for routine NMR protein structure calculation and has been shown to yield results that are equivalent to those of the conventional, manual approach. However, these algorithms rely on the availability of a virtually complete list of the chemical shifts. This paper investigates the influence of incomplete chemical shift assignments on the reliability of NMR structures obtained with automated NOESY cross peak assignment. The program CYANA was used for combined automated NOESY assignment with the CANDID algorithm and structure calculations with torsion angle dynamics at various degrees of completeness of the chemical shift assignment which was simulated by random omission of entries in the experimental ^{1}H chemical shift lists that had been used for the earlier, conventional structure determinations of two proteins. Sets of structure calculations were performed choosing the omitted chemical shifts randomly among all assigned hydrogen atoms, or among aromatic hydrogen atoms. For comparison, automated NOESY assignment and structure calculations were performed with the complete experimental chemical shift but under random omission of NOESY cross peaks. When heteronuclear-resolved three-dimensional NOESY spectra are available the current CANDID algorithm yields in the absence of up to about 10% of the experimental ^{1}H chemical shifts reliable NOE assignments and three-dimensional structures that deviate by less than 2 Å from the reference structure obtained using all experimental chemical shift assignments. In contrast, the algorithm can accommodate the omission of up to 50% of the cross peaks in heteronuclear- resolved NOESY spectra without producing structures with a RMSD of more than 2 Å to the reference structure. When only homonuclear NOESY spectra are available, the algorithm is slightly more susceptible to missing data and can tolerate the absence of up to about 7% of the experimental ^{1}H chemical shifts or of up to 30% of the NOESY peaks.

Abbreviations: BmPBP^{A} – Bombyx mori pheromone binding protein form A; CYANA – combined assignment and dynamics algorithm for NMR applications; NMR – nuclear magnetic resonance; NOE – nuclear Overhauser effect; NOESY – NOE spectroscopy; RMSD – root-mean-square deviation; WmKT – *Williopsis mrakii* killer toxin

Introduction

Improving the efficiency of NMR protein structure determination through partial or full automation of the assignment process has recently attracted much attention [1], especially because of its importance for NMR-based structural genomics [2–3]. Among the different assignment analysis steps that lead from NMR spectra to the three-dimensional protein structure, the NOESY cross peak assignment has proved to be most accessible to automation. Several fully automated approaches for combined automated NOESY

assignment and structure calculations have been developed [4–10]. Automated NOESY assignment replaces the most time-consuming part of the interactive spectral analysis by a fast computational method and has thus significantly enhanced the overall efficiency of NMR structure determination. Nevertheless, a limiting factor for the application of these automated NOE assignment procedures is that they rely on the availability of an essentially complete list of chemical shifts from the preceding sequence-specific resonance assignment. At present, chemical shift assignment remains largely the domain of interactive or semi-automated methods, despite of many promising attempts towards automation [1]. Experience shows that in general most of the chemical shifts can be assigned readily whereas others pose difficulties that may require a disproportionate amount of the spectroscopist's time. Hence, NMR structure determination would be speeded up significantly if NOE assignment and structure calculation could be based on incomplete lists of assigned chemical shifts, provided that doing so does not compromise the reliability and robustness of the NMR method for protein structure determination. This paper investigates the sensitivity of the CANDID NOE assignment algorithm [9] to missing chemical shifts. For comparison, also the effects of incomplete NOESY peak picking have been studied.

Methods

Structure calculations were performed with the program CYANA, version 1.0, using combined torsion angle dynamics [11] and automated NOESY assignment with the CANDID algorithm [9]. The standard protocol [9] was used with seven cycles of combined automated NOE assignment and structure calculation of 100 conformers in each cycle, of which the 20 with lowest target function value were retained for analysis. For each conformer, the standard simulated annealing schedule [11] with 10,000 torsion angle dynamics steps was applied, starting from initial structures with random values of the torsion angles. All structure calculations were performed using in parallel between 8 and 32 processors of a 64-processor Silicon Graphics computer.

The test calculations were performed using the experimental NMR data sets for the *Bombyx mori* pheromone binding protein form A ($BmPBP^A$; BioMagResBank ID 4849, Protein Data Bank ID 1GM0) [12] and for the *Williopsis mrakii* killer toxin (WmKT; BioMagResBank ID 5255, Protein Data Bank ID 1WKT) [13] that have previously been collected for the determination of the three-dimensional structure of these proteins in solution. The data include assignments for 97.1% and 97.0% of the non-labile and backbone amide ^1H chemical shifts of $BmPBP^A$ and WmKT, respectively, and lists of NOESY peak positions and volumes. For $BmPBP^A$, an α-helical protein of 142 amino acid residues, there are a total of 781 assigned ^1H chemical shifts, of which 643 (82.3%) are for H^α and side-chain protons, and 47 (6.0%) for aromatic protons. The three peak lists from three three-dimensional NOESY spectra of the doubly labeled $BmPBP^A$ protein contain a total of 5614 peak entries [12]. For WmKT, a β-sheet protein of 88 amino acid residues, there are a total of 455 assigned ^1H chemical shifts, of which 370 (81.3%) concern H^α and side-chain protons and 37 (8.1%) are for aromatic protons. The NOESY peak list for WmKT is based on a single homonuclear [^1H,^1H]-NOESY spectrum and contains 1998 peak entries [13].

Four groups of structure calculations were performed with differently prepared input data but following exactly the same computational procedure:

(a) Random omission of chemical shifts: From the complete experimental chemical shift list, a given percentage P of randomly selected, assigned ^1H chemical shifts was removed. The experimental peak list(s) were used without modification. The omission ratio P was varied between 0 and 30% of all assigned ^1H chemical shifts. The experimental NOESY peak lists were used without modification.

(b) Random omission of H^α and side-chain chemical shifts: The same as (a), but only randomly selected chemical shifts of H^α and side-chain protons were removed. The backbone amide ^1H chemical shifts were used without modification.

(c) Random omission of aromatic chemical shifts: The same as (a), but only randomly selected aromatic ^1H chemical shifts were removed. All other chemical shifts were used without modification.

(d) Random omission of NOESY peaks: A given percentage P of randomly selected entries were removed from the NOESY peak list(s). The experimental chemical shift list was used without modification.

Within each of the four groups (a)-(d), five independent runs of combined automated NOESY assignment and structure calculation were performed for each

value P of the percentage of omitted chemical shifts or peaks with different random selection of the omitted entries and different random initial structures. In addition, five runs with different random initial structures were performed with the complete experimental chemical shift and NOESY peak lists, and the final structure obtained from the first of these runs was used as a reference for the analysis of the calculations with reduced completeness of the chemical shift assignment or NOESY peak lists.

In the case of the protein BmPBP^A for which heteronuclear-resolved 3D NOESY data is available, the complete set of experimentally determined ^{13}C and ^{15}N chemical shifts was included in all calculations. Since the correct assignment of a NOESY cross peak in a heteronuclear-resolved 3D NOESY spectrum requires the simultaneous presence of the chemical shifts of the two corresponding ^{1}H protons and the associated heteronucleus, identical results would be obtained whether only a given ^{1}H chemical shift is omitted or also its associated ^{13}C or ^{15}N chemical shift.

Each run of the program CYANA was analyzed with respect to the following parameters:

(i) RMSD bias [14]: The RMSD between the average reference structure and the average final structure of the run. This parameter measures the overall accuracy of the final structure obtained from CYANA.

(ii) RMSD in cycle 1: The average RMSD value of the 20 conformers of CANDID cycle 1 with respect to their mean coordinates. This parameter measures the precision of the first structure obtained without use of any previous information on the three-dimensional structure.

(iii) RMSD drift: The RMSD between the average structures of the first and last (seventh) CANDID cycle of the run.

(iv) RMSD in cycle 7: The same as (ii) but calculated for the structure of the final CANDID cycle. This parameter measures the precision of the final structure.

(v) Target function: The average final target function value of the 20 conformers of cycle 7 is a measure of the size of violations of the constraints that are retained by CANDID for the final structure calculation.

(vi) Unassigned NOEs: The percentage of unassigned NOESY cross peaks in cycle 7.

(vii) Discarded long-range NOEs: The percentage of NOEs discarded by the CANDID algorithm among all NOEs between atoms separated by 4 or more residues along the polypeptide sequence. The previous and this parameter indicate the percentage of all NOEs and of all long-range NOEs that are not used by CANDID to generate distance constraints for the final structure calculation, and thus give information about the completeness with which the picked NOE cross peaks can be explained by the resulting structure

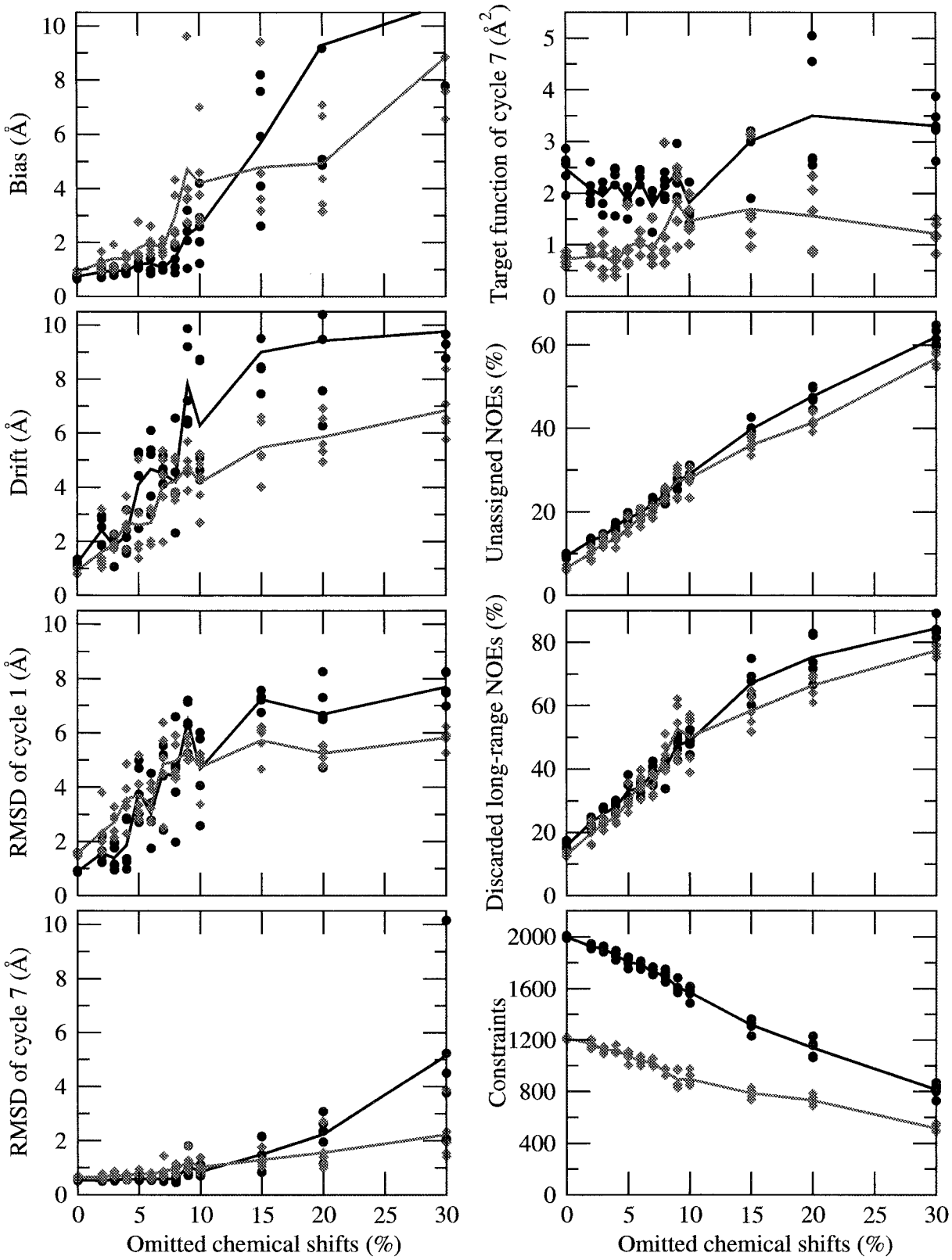
(viii) Number of distance constraints: The number of meaningful [15] upper distance limit constraints from NOEs.

Average structures were obtained by superimposing a bundle of conformers on its first member for minimal RMSD, and subsequently taking the mean value of the Cartesian coordinates. RMSD values were calculated for the backbone atoms N, C$^\alpha$ and C' of the well-defined regions of the protein structure, i.e. residues 8–142 of BmPBP^A [12] and residues 4–39 and 47–87 of WmKT [13], using the programs CYANA and MOLMOL [16].

Results

Random omission of ^{1}H chemical shift assignments

For both proteins the calculations using datasets with randomly omitted chemical shift assignments among all or only among the H$^\alpha$ and side-chain protons yielded equivalent results. The following analysis of the results therefore concentrates on the representative case of random omission among all ^{1}H chemical shift assigments (Figure 1). The deviation of the structures obtained with incomplete chemical shift assignment from the reference structure as measured by the RMSD bias increased only slowly with increasing omission ratio P up to about $P = 10\%$ for BmPBP^A or 7% for WmKT, from where onwards the RMSD bias rose abruptly, reflecting that severely distorted structures had been obtained. It is noteworthy that higher omission ratios did not only result in high mean values of the RMSD bias but also in pronounced variations among the five individual runs at a given P value. The patterns of the RMSD in cycle 1 and the RMSD drift were similar. This can be rationalized by the fact that the drift, i.e. the deviation between the structures of the first and last cycle, is dominated by the "loose" structure of cycle 1 rather than by the generally well-defined final structure. There was no remarkable variation among the final


Bias (Å)
Drift (Å)
RMSD of cycle 1 (Å)
RMSD of cycle 7 (Å)
Target function of cycle 7 (Å^2)
Unassigned NOEs (%)
Discarded long-range NOEs (%)
Constraints
Omitted chemical shifts (%)
Omitted chemical shifts (%)

target function values that were almost always below 5 $Å^2$ regardless of the omission ratio. The percentages of unassigned NOEs and discarded long-range NOEs as well as the number of distance constraints varied almost linearly with the omission rate and the dispersion among these quantities at a given rate was smaller than for the RMSD bias. Comparing the results between $BmPBP^A$ and WmKT indicates a higher tolerance against missing chemical shifts for $BmPBP^A$ than for WmKT. This can be explained by the availability of ^{13}C and ^{15}N chemical shifts for $BmPBP^A$ that allowed resolving many of ^1H chemical shifts degeneracies, resulting in a lower probability of accidental erroneous NOE assignments than for the homonuclear data of WmKT.

Random or complete omission of aromatic ^1H chemical shift assignments

The omission of aromatic ^1H chemical shift assignments in general causes more severe problems than the omission of the same number of chemical shifts chosen randomly among all assigned ^1H chemical shifts (Figure 2). In the case of $BmPBP^A$ the omission of all assigned aromatic chemical shifts, corresponding to 6.0% of all assigned protons, led to 2 Å RMSD bias already. In the case of WmKT (Figure 2) significant deviations from the reference structure were in some cases observed already when 20% of the aromatic chemical shifts were omitted, which corresponds to an overall omission ratio of merely 1.6% of all assigned ^1H chemical shifts. Despite the sometimes larger deviation from the reference structure, the values of the RMSD in cycle 7, the final target function, the total number of unassigned cross peaks and the number of constraints behaved similarly to those of $BmPBP^A$. However, the fluctuations in the RMSD bias were clearly visible in the corresponding values of the discarded long-range NOEs and the RMSD in cycle 1.

Random omission of NOESY peaks

In contrast to the effects seen under the omission of chemical shift assignments, the random omission of NOESY peaks does not cause severe problems in both proteins (Figure 3). Even when 50% of the NOESY peaks were omitted from the experimental input peak lists for $BmPBP^A$, most of the RMSD bias values remained in the region of 2 Å. At an NOE peak omission ratio of 50%, the mean number of final, meaningful NOE distance constraints dropped to 1197, which is 60% of the initial number, or 8.4 constraints per residues. An outlier with RMSD bias close to 4 Å shows that for $BmPBP^A$ the algorithm starts to loose its stability at 50% NOE omission ratio. The data of WmKT showed similar patterns, albeit with a somewhat stronger dependence on the omission rate and RMSD bias values exceeding 2 Å already in some runs with 30% NOESY peak omission ratio.

Discussion

Particular attention should be paid to those runs that exhibit a significant RMSD bias despite of having low values of the RMSD in cycle 7 that let them appear as well-defined structures. In a conventional structure calculation based on manual NOESY assignment, incomplete or inconsistent input data will be manifested by large RMSD and/or target function values of the final structure bundle, which will prompt the spectroscopist to discard the run and correct the input data for a new structure calculation. Our results show that for structure calculation with automated NOE assignment these two quantities, the RMSD in cycle 7 and the

Figure 1. Results of structure calculations with the program CYANA for the proteins $BmPBP^A$ [12] and WmKT [13] using combined automated NOESY assignment by the CANDID algorithm [9] at different levels of completeness of the input chemical shift assignment. The horizontal axis indicates the percentage of chemical shift assignments that were randomly omitted from the complete experimental chemical shift list. Each filled black circle and grey triangle represents the result of one complete run of seven cycles of NOE assignment and structure calculation for $BmPBP^A$ and WmKT, respectively. Five independent runs were performed at each percentage value of randomly omitted chemical shifts. The black and grey lines connect the average values over the five runs for $BmPBP^A$ and WmKT, respectively. The quantities on the vertical axis are: Bias, the RMSD between the average reference structure obtained by CYANA from the complete experimental input data set and the average final structure of the run. Drift, the RMSD between the average structures of the first and last (seventh) CANDID cycle. RMSD of cycle 1, the average RMSD value between the 20 conformers of CANDID cycle 1 and their mean coordinates. RMSD of cycle 7, the average RMSD value between the 20 conformers of the final cycle 7 and their mean coordinates. Target function of cycle 7, the average final target function value of the 20 conformers of cycle 7; Unassigned NOEs, the percentage of unassigned NOESY cross peaks in cycle7. Discarded long-range NOEs, the percentage of NOEs discarded by the CANDID algorithm among all NOEs between atoms separated by 4 or more residues along the polypeptide sequence. Constraints, the number of meaningful upper distance limit constraints from NOEs. The backbone atoms of residues 8–142 in $BmPBP^A$ and 4–39 and 47–87 in WmKT were used for RMSD calculation and comparison.

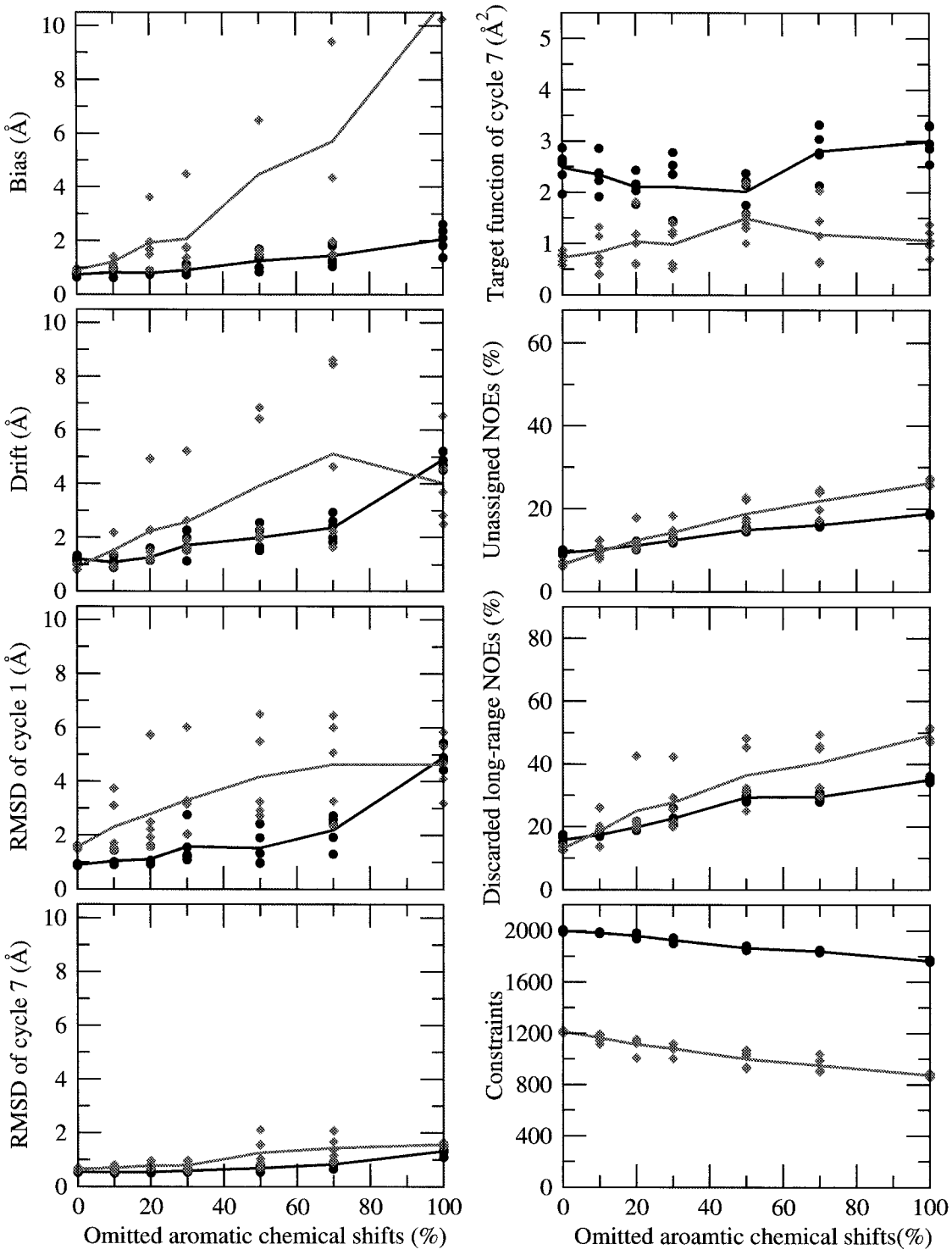


Figure 2. Results of structure calculations with the program CYANA for the protein BmPBP^A and WmKT at different levels of completeness of the input chemical shift assignments for aromatic ring protons of Tyr, Phe, His and Trp residues. For all other protons the complete set of experimental chemical shift assignments was used. All quantities are defined as in Fig. 1 except that the horizontal axis indicates the percentage of aromatic ^{1}H chemical shift values that were randomly omitted from the input experimental chemical shift list.

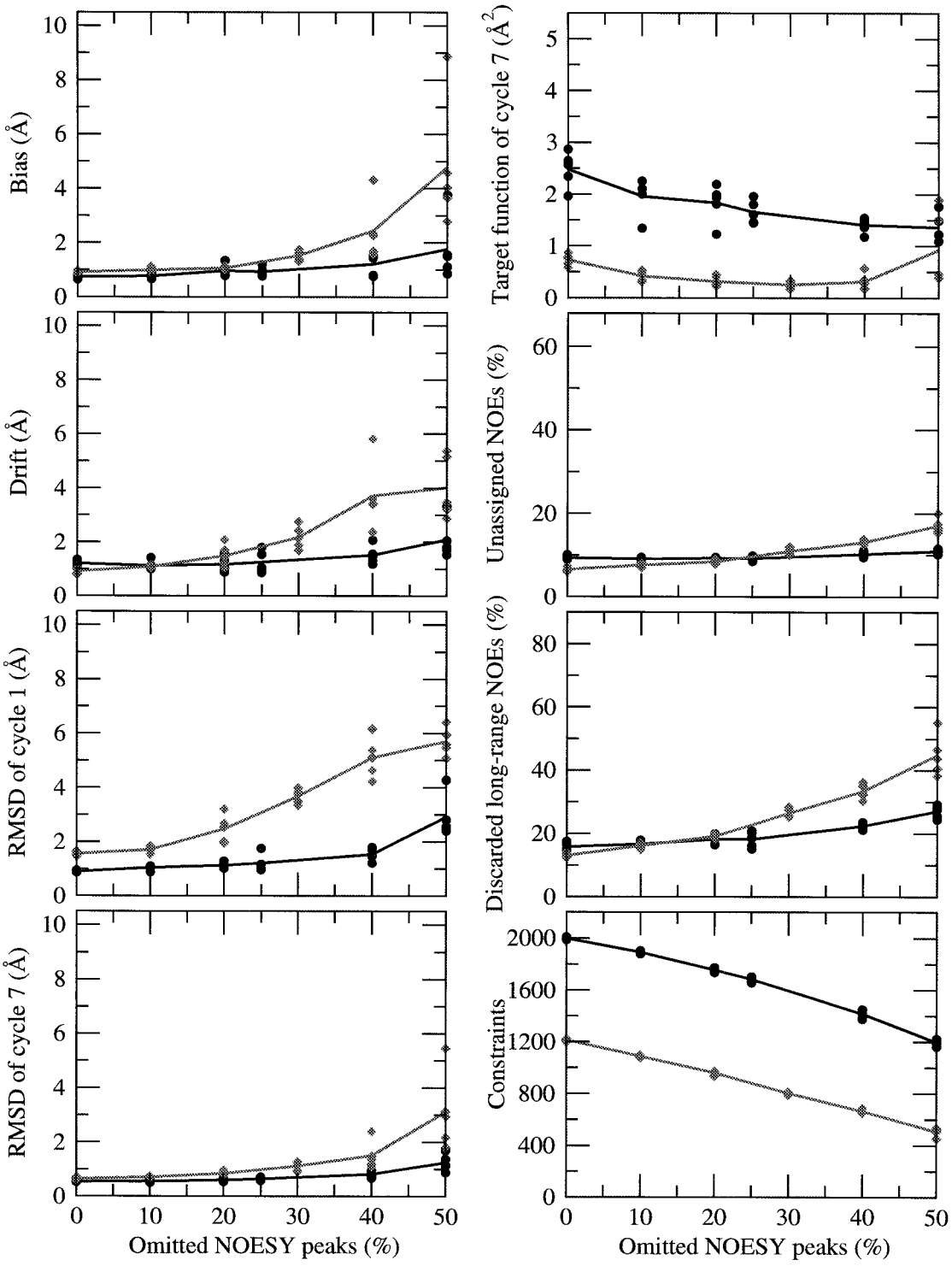


Figure 3. Results of structure calculations with the program CYANA for the protein BmPBP^A and WmKT at different levels of completeness of the input NOESY peak lists. All quantities are defined as in Fig. 1 except that the horizontal axis indicates the percentage of NOESY peaks that were omitted randomly from the input peak lists.

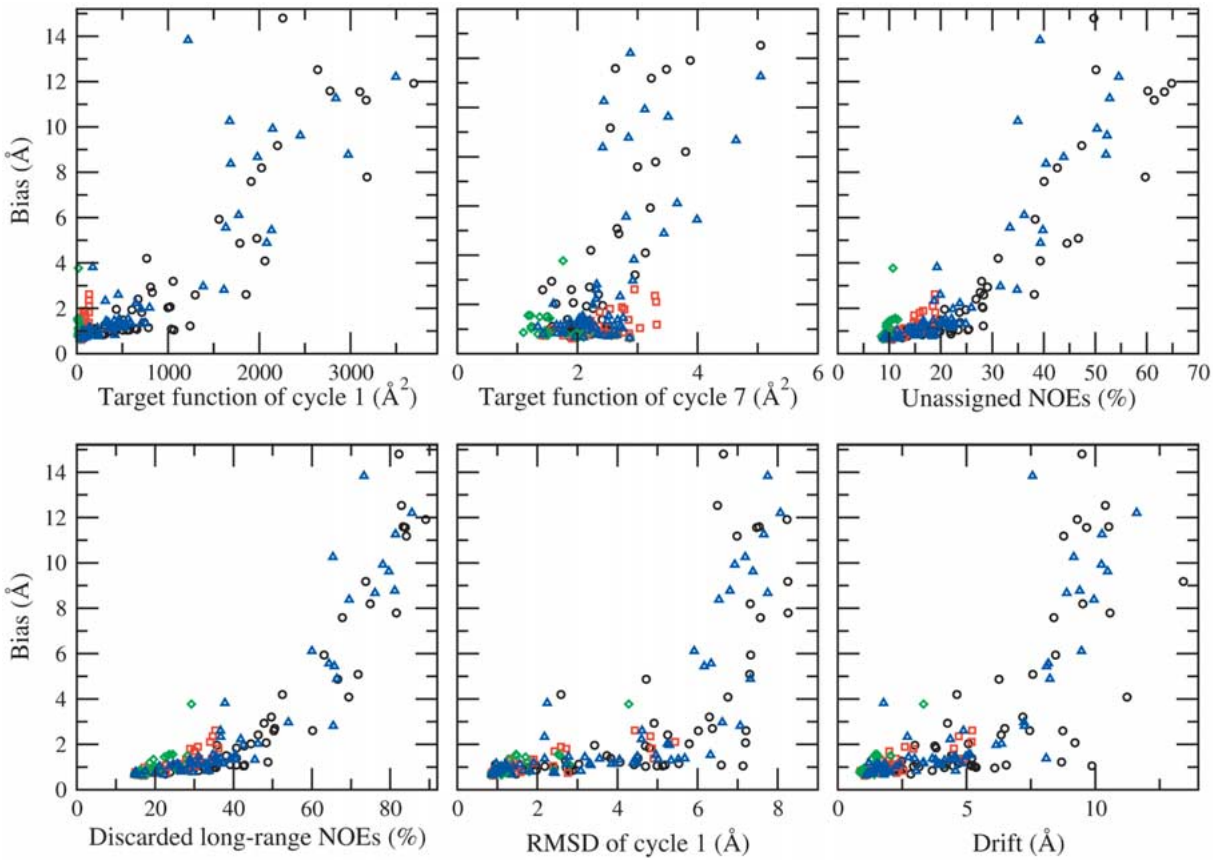


Figure 4. Correlation between various quantities defined in Fig. 1 and the accuracy of structures for the protein BmPBP^A obtained with the program CYANA from incomplete input chemical shift lists or incomplete input NOESY peak lists. Black circles, blue triangles, red squares and green diamonds, respectively, are for CYANA runs with random omission of proton chemical shifts as in Fig. 1, random omission of H^α and side-chain proton chemical shifts, random omission of aromatic ring proton chemical shifts as in Fig. 2, and random omission of NOESY peaks as in Fig. 3.

final target function value, are not suited to distinguish correct from biased results, and other criteria are needed to evaluate the outcome. In a previous paper [9], guidelines for successful structure calculations with NOE assignment by the automated CANDID algorithm were proposed. In the notation of the present paper, these guidelines comprise six criteria: (1) average target function value of cycle 1 below 250 Å^2, (2) average final target function value below 10 Å^2, (3) less than 20% unassigned NOEs, (4) less than 20% discarded long-range NOEs, (5) RMSD value in cycle 1 below 3 Å, and (6) RMSD drift below 3 Å. None of those structure calculations of this paper that exhibited an RMSD bias above 2 Å that was taken, somewhat arbitrarily, to indicate a significantly distorted structure fulfilled all six criteria, thereby confirming the validity of these guidelines [9] as sufficient conditions for successful CANDID runs. On the other hand, in many cases a structure with RMSD bias below 2 Å was obtained even if one or several of the six criteria were not fulfilled.

To investigate the general relationship between the accuracy of the structure and the parameters of Figures 1–3, the RMSD bias values from all runs performed with either chemical shift or NOE omission were plotted against these quantities in Figures 4 and 5. Since the omission ratio is not a variable in these plots, the results from all four groups of runs (see Methods) can be represented and juxtaposed simultaneously in order to reveal general correlations between these parameters and the RMSD bias that hold independent of the detailed nature of the input data. Among the six parameters in the guidelines for CANDID [9], the strongest correlation to the RMSD

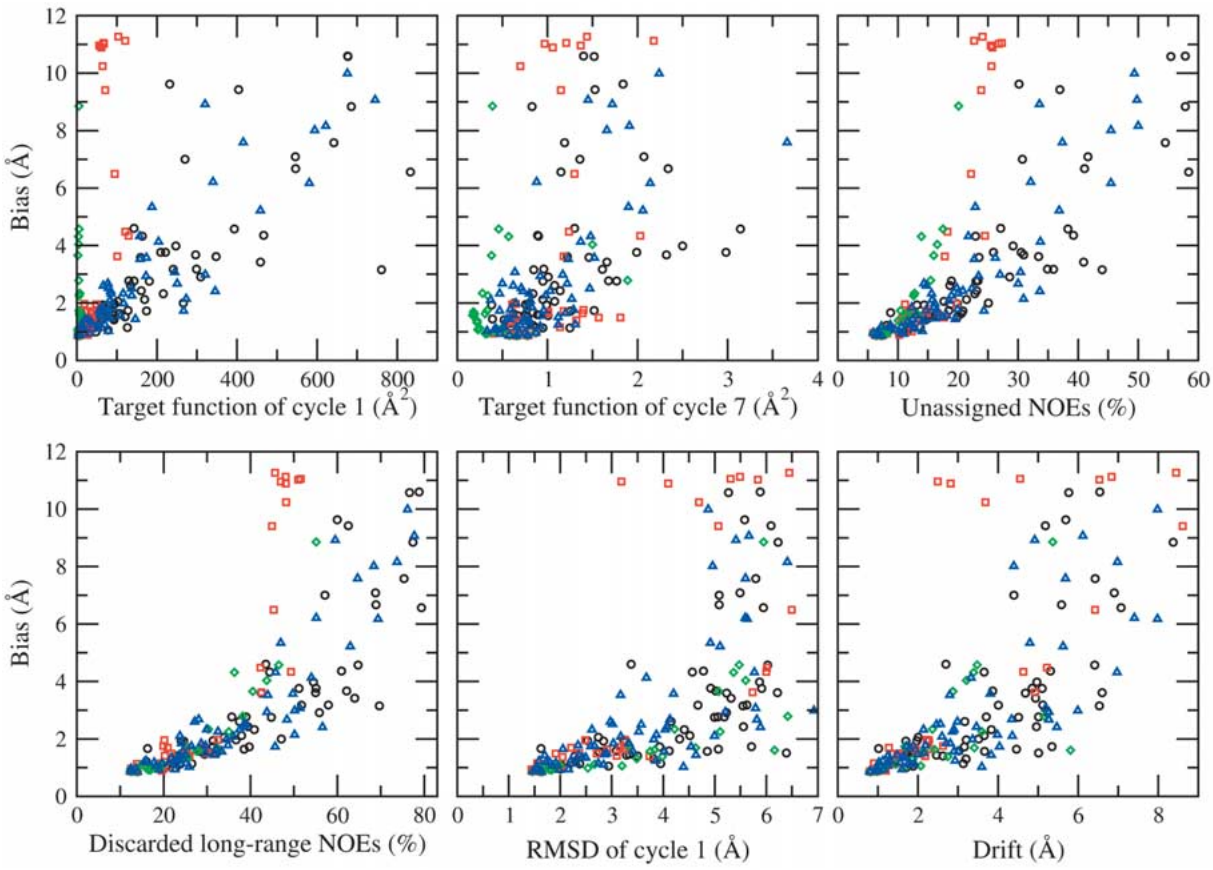


Figure 5. Correlation between various quantities defined in Fig. 1 and the accuracy of structures for the protein WmKT obtained with the program CYANA from incomplete input chemical shift lists or incomplete input NOESY peak lists. All quantities are defined as in Fig. 4.

bias is found for the percentage of discarded long-range NOEs. Good correlation is observed also with the percentage of unassigned NOEs, and, to a somewhat lesser degree, for the RMSD in cycle 1, and similarly for the RMSD drift, whereas the correlation is weak for the target function value in cycle 1 and virtually absent for the target function value of cycle 7 (Figures 4 and 5). In the light of these findings, the guidelines [9] for successful combined NOESY assignment and structure calculation with CYANA may be simplified and refined. First of all, both criteria based on the target functions values may be dropped. This does not mean, however, that achieving a low final target function value, i.e. small remaining violations of the conformational constraints, is no longer an aim of the structure calculation. Among the two criteria that measure the amount of unused NOEs, the percentage of discarded long-range NOEs is a slightly more sensitive indicator of the accuracy of the final structure than the overall percentage of unused cross peaks that includes also cross peaks with short-range assignment or with no assignment possibility at all. Since these two quantities are strongly correlated with each other, it is sufficient to consider one of them, preferably the discarded long-range NOEs. It is, however, not straightforward to calculate the percentage of discarded long-range NOEs outside the CYANA program, because it requires knowledge of the possible assignments also for the discarded NOESY cross peaks, whereas it is straightforward to obtain the overall percentage of unused NOESY cross peaks from the final assigned peak lists, in which unused cross peaks remain unassigned. To evaluate the outcome of a structure calculation outside the CYANA program, the percentage of unused cross peaks can therefore be used as an alternative to the percentage of discarded long-range NOEs. However, for data sets with a low density of NOEs, as evidenced by the cal-

culations with NOE omission, the discriminatory power of the unassigned NOEs criterion is significantly weakened. The results of this paper further suggest that the tolerable percentage of discarded long-range NOEs can be slightly higher than proposed originally [9], namely 25%, or, with only a single exception at high NOE omission rate, 30%. Of course, these criteria are only valid if the input peak lists provide a faithful representation of the underlying NOESY spectra, i.e. if the peak lists do not deliberately misinterpret the spectrum (to which the CANDID algorithm has no direct access).

The ability of the program to find a well-defined structure in the initial cycle of NOE assignment and structure calculation, as measured by the RMSD in cycle 1, is another important factor that strongly influences the accuracy of the final structure, as measured by the RMSD bias. This can be understood by considering the iterative nature of the CANDID algorithm, by which each cycle except cycle 1 is dependent on the structure obtained in the preceding cycle [9]. Using network-anchoring and constraint-combination, the algorithm tries to obtain a well-defined structure already in the first cycle [9]. A low precision of the structure from cycle 1 may hinder convergence to a well-defined final structure, or, more dangerously, opens the possibility of a structural drift in later cycles towards a precise but inaccurate final structure. In certain cases, such as the "outlying" calculation with around 4 Å RMSD bias at 50% NOE omission for $BmPBP^A$ (Figure 4), the RMSD in cycle 1 provides a better discrimination between successful and unsuccessful runs than the percentage of discarded long-range NOEs.

It is therefore safer to apply both criteria, even though for the calculations of this paper the percentage of discarded long-range NOEs alone would have been sufficient to detect all runs that resulted in a structure with more than 2 Å RMSD bias. The discriminatory power of these two criteria is maybe best illustrated by the calculations with omission or aromatic chemical shifts for WmKT (Figure 2). These calculations show a large dispersion in the accuracy of the final structure even among the runs with equal omission ratio, which is reflected reliably by the percentage of discarded long-range NOEs and the RMSD in cycle 1 but could not readily be discerned from the values of the target function after cycle 1 or 7, the RMSD at cycle 7, or the percentage of unassigned NOEs.

Conclusions

The calculations in this paper show that for reliable automated NOESY assignment with the CANDID algorithm around 90% completeness of the chemical shift assignment is necessary, whereas the algorithm is remarkably tolerant with respect to incomplete NOESY peak picking. The calculations with omission of aromatic proton chemical shifts show that in certain cases even the lack of a small number of "essential" chemical shifts can lead to a significant deviation of the structure. On the other hand the algorithm might be expected to tolerate a slightly higher degree of incompleteness in the chemical shift assignments than the simulations of this paper suggest provided that mostly assignments of "unimportant" chemical shifts that are involved in only few NOEs are missing. In practice this is usually the case because the chemical shifts of protons that are involved in many NOEs, and, if absent, prevent the program from correctly assigning any of these NOEs, are intrinsically easier to assign than those exhibiting only few NOEs. This effect is not reflected by the random selection of omitted chemical shifts in the simulations of this paper but indirectly confirmed by the finding that in general the lack of aromatic chemical shifts is more harmful to the outcome of the structure calculation than that of a similar number of other protons because aromatic protons tend to be located in the hydrophobic core of the protein where they give rise to a higher-than-average number of NOEs.

The results of this paper further revealed a certain redundancy in the original criteria [9] for successful CANDID runs. Provided that the input peak lists represent faithfully the underlying NOESY spectra, the different criteria in [9] can be condensed into the two conditions that less than 25% of the long-range NOEs have been discarded by the automated NOESY assignment algorithm for the final structure calculation, and that the backbone RMSD to the mean for the structure bundle of cycle 1 is below 3 Å.

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