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# Assignment Strategy for Proteins with Known Structure

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**In protein NMR the assignment of nuclear spin resonances is a prerequisite for all subsequent applications, such as studies of ligand binding, protein-DNA interactions, and dynamics. Resonance assignment is a time consuming step even when the 3D x-ray structure of the protein is available. A new strategy is presented to solve the "inverse" assignment problem, which is the determination of the NMR resonance assignment from a known 3D protein structure. The protocol employs NMR data in the form of residual dipolar couplings and chemical shifts, while it does not require any sequential NMR connectivity information. The assignment problem is mathematically formulated in terms of a weighted matching problem that can be computationally efficiently solved by a combinatorial optimization algorithm. The protocol is applied to ubiquitin using two or three residual dipolar couplings per amino acid measured in Pf1 phage medium together with chemical shift information. The algorithm yields for more than 90% of the protein backbone resonances the correct assignment.** © 2002 Elsevier Science (USA)

*Key Words*: **NMR resonance assignment; residual dipolar couplings; x-ray structure; amino-acid specific chemical shifts.**

## INTRODUCTION

A prerequisite for NMR studies of proteins is the assignment of the NMR spin resonances to specific atoms (*1*). Common assignment strategies (*1–3*) use sequential backbone connectivities but no 3D structural information as input. On the other hand, for many proteins the 3D structure is known from x-ray crystallography and further investigations of their biophysical and biomedical properties, such as interactions with DNA and RNA, other proteins, and small ligands as well as dynamics, can be subsequently carried out by the large arsenal of NMR techniques provided that complete or partial NMR assignment information is available.

A new assignment strategy is presented here for proteins with known structure. It primarily uses residual dipolar couplings (RDCs) (*4, 5*) measured, in a single alignment medium, and chemical shifts. RDCs of $^{15}N$–$^{1}H^{N}$, $^{13}C'$–$^{15}N$, and $^{13}C'$–$^{13}C^{\alpha}$ spin pairs belonging to the backbone peptide planes are employed that can be measured using a variety of different 2D and 3D NMR pulse-sequence schemes (*6–13*). To optimize the number of correct assignments, the RDC data are supplemented by $C^{\alpha}$ and $C^{\beta}$ chemical shifts which can be correlated to NH backbone resonances of the succeeding amino acid using 3D CBCA(CO)NH-type NMR experiments (*14*). Since $C^{\alpha}$ and $C^{\beta}$ chemical shifts depend on the amino-acid type (*15*) as well as on the backbone conformation (*16–19*), they are useful in combination with the knowledge of the amino-acid sequence and the 3D structure to reduce the number of possible assignments. Resonances belonging to highly mobile residues are identified on the basis of their heteronuclear $\{^{1}H\}$–$^{15}N$ nuclear Overhauser enhancements (NOEs) which tend towards small or negative values. Since these residues are likely to exhibit motionally averaged RDCs that may be inconsistent with RDCs calculated from a static x-ray structure, RDCs from such residues will be treated separately. In contrast to traditional assignment protocols (*1–3*), the method does not use sequential connectivity information between peptide units.

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## ASSIGNMENT ALGORITHM

The nomenclature that will be used in the following is first introduced. The polypeptide chain is considered as a set of disjunct *peptide units*, where unit $i$ contains atoms $C_{i-1}^{\alpha(\beta)}$, $C'_{i-1}$, $N_i$, and $H_i^N$ of amino acids $i-1$ and $i$. The NMR resonances belonging to a peptide unit are called a *resonance set*. A resonance set is correctly assigned if the corresponding peptide unit is correctly identified and vice versa.

For a given alignment, the geometric dependence of RDCs can be characterized by a traceless symmetric alignment tensor $\boldsymbol{D}$, which has eigenvalues $D_{xx}$, $D_{yy}$, $D_{zz}$. In the eigenframe of this tensor the dipolar coupling $D$ between two spins connected by an internuclear vector with orientation $\Omega = (\theta, \varphi)$, where $\theta$, $\varphi$ denote the polar angles in the eigenframe of $\boldsymbol{D}$, is given by

$$D = D_a\{3\cos^2\theta - 1 + (3/2)R\sin^2\theta\cos 2\varphi\}, \qquad [1]$$

where $D_a = D_{zz}/2$ is the axial component and $R = 2/3 \cdot (D_{xx} - D_{yy})/D_{zz}$ is the rhombicity of $\boldsymbol{D}$. In the general case, the alignment tensor $\boldsymbol{D}$ is specified by 5 independent parameters, for example, $D_a$, $R$, $\alpha$, $\beta$, $\gamma$ where $\alpha$, $\beta$, $\gamma$ represent the three Euler angles that relate a molecular frame to the alignment frame.

The problem of finding the most probable NMR assignment for a known 3D protein structure using residue-specific assignment information in the form of RDCs and chemical shifts



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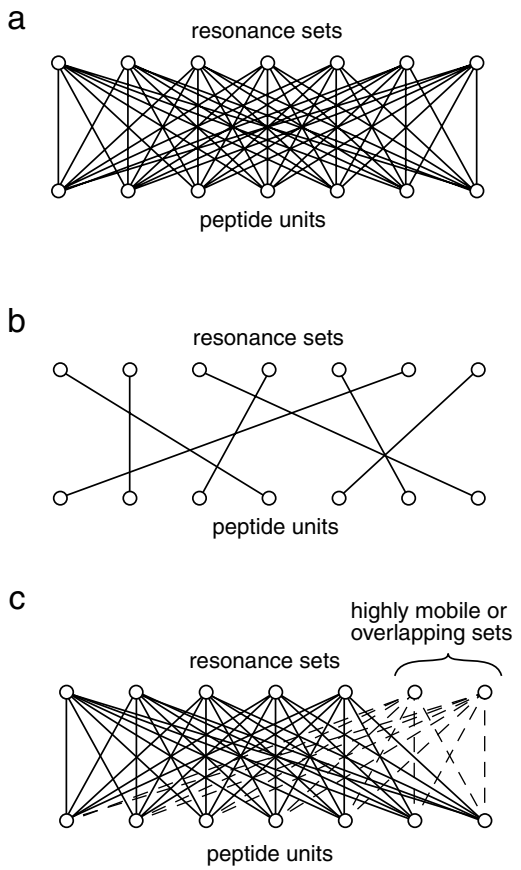


**FIG. 1.** (a) Schematic presentation of the assignment problem of NMR resonance sets to peptide units as a complete edge-weighted bipartite graph. Each edge carries a cost that corresponds to the agreement between experimental NMR parameters (RDCs and chemical shifts) and the ones back-calculated from the x-ray structure. (b) Solution that optimizes the agreement between experimental and back-calculated data. (c) Resonance sets that overlap or that belong to mobile residues are treated as extra nodes with equally weighted edges to all peptide units.

is expressed in terms of the general class of combinatorial optimization problems known as *assignment problems* or *weighted matching problems* (*20*). It can be formulated in form of a complete edge-weighted bipartite graph (see Fig. 1), which is a graph whose nodes can be partitioned into two subsets each containing $n$ nodes numbered $\{1, 2, \ldots, n\}$ (top and bottom row in Fig. 1a), with no edge joining nodes belonging to the same subset. Each node $i$ in one of the subsets is joined to each node $j$ in the other subset by an edge that has a specific weight or cost $C(i, j) \geq 0$. The optimal solution of the assignment problem is to find a permutation $\pi$ of $\{1, 2, \ldots, n\}$ that minimizes

$$\chi^2 = \sum_{i=1}^{n} C(i, \pi(i)). \qquad [2]$$

Deterministic algorithms exist that solve this problem in polynomial time $O(n^3)$ (*20, 21*). In the present context, the $n \times n$ cost matrix

$$C(i, j) = C_{rdc}(i, j) + C_{cs}(i, j) \qquad [3]$$

describes the discrepancy between experimental RDC ($C_{rdc}$) and chemical shift ($C_{cs}$) data of resonance set $i$ with the corresponding parameters of peptide unit $j$ derived from the x-ray structure. If $C(i, j)$ is small, the RDCs and chemical shifts of resonance set $i$ fit well to peptide unit $j$ for the assumed alignment tensor making them potential candidates for assignment (although in the global $\chi^2$ minimum resonance set $i$ is not necessarily assigned to peptide unit $j$).

The proposed procedure involves the following steps, which are explained in more detail below:

(1) Convert experimental $C^\alpha$ and $C^\beta$ chemical shifts into cost matrix $C_{cs}$; remove N-terminal amino acid and all proline residues from the cost matrix
(2) Optional step: identify dynamic resonance sets based on heteronuclear NOEs; remove corresponding RDCs from list and set cost matrix elements $C(i,j)$ involving these resonances to maximal value $C_{max}$, which is larger than any other element of $C$
(3) Estimate from remaining RDCs initial values for axial and rhombic components of alignment tensor $D_a$ and $R$
(4) WHILE convergence has not been reached:
  (a) Choose new Euler angles $\alpha$, $\beta$, $\gamma$
  (b) Calculate cost matrix $C$ based on x-ray structure and chemical-shift derived probabilities (see Eqs. [3]–[5])
  (c) Solve weighted matching problem
  (d) Define the subset of peptide planes that show good agreement between RDCs and prediction from x-ray structure
  (e) Refine Euler angles $\alpha$, $\beta$, $\gamma$ and $D_a$ and $R$ values on this subset with the assignment obtained in 4c
  (f) Calculate new cost matrix $C$
  (g) Solve weighted matching problem
  END WHILE
(5) Assignment of resonances belonging to mobile residues and residues with only chemical information (if any)

In step 1, the likelihood that chemical shifts of $C^\alpha$ and $C^\beta$ nuclei (except for glycines) of resonance set $i$ belong to amino-acid type $j$ is translated into the cost $C_{cs}(i, j)$ entering Eq. [3]

$$C_{cs}(i, j) = K \cdot \left\{ \frac{\left(\delta_\alpha(i) - \delta_\alpha^{av}(j)\right)^2}{\sigma_\alpha^2(j)} + \frac{\left(\delta_\beta(i) - \delta_\beta^{av}(j)\right)^2}{\sigma_\beta^2(j)} \right\}, \qquad [4]$$

where $\delta_{\alpha(\beta)}(i)$ is the $C^{\alpha(\beta)}$ chemical shift of resonance set $i$ and $\delta_{\alpha(\beta)}^{av}(j)$ is the $C^{\alpha(\beta)}$ chemical shift belonging to peptide unit $j$ obtained by averaging the $C^{\alpha(\beta)}$ shifts of amino acids of the same type over the proteins deposited in the BMRB database (*15*) and $\sigma_{\alpha(\beta)}^2$ is the corresponding variance. Alternatively, $\delta_{\alpha(\beta)}^{av}(j)$ values

can be used that are predicted from a database analysis with the backbone $\varphi, \psi$ dihedral angles of the known x-ray structure (*19*). Prefactor $K$ defines the relative weight of $C_{cs}$ with respect to $C_{rdc}$.

In steps 4b and 4f the $C_{rdc}(i, j)$ part of the total cost matrix $C(i, j)$ is given by

$$C_{rdc}(i, j) = \sum_k \frac{(D^{exp}(i, k) - D^x(j, k))^2}{\sigma^2_{rdc}(i, k)}, \qquad [5]$$

where $D^{exp}(i, k)$ is the experimental RDC of spin pair $k$ belonging to resonance set $i$ with experimental error $\sigma_{rdc}(i, k)$ and $D^x(j, k)$ is the RDC calculated from the x-ray structure and a given alignment tensor $\boldsymbol{D}$ for peptide unit $j$.

In step 2, which is optional, resonance sets with a significantly reduced or negative $\{^1H\}$–$^{15}N$ NOE are identified and their cost matrix elements are set to a high $C_{max}$ value. This guarantees that at this stage of the assignment process they cannot compete for assignment with "well-behaved" resonance sets.

In step 3, initial values for the axial and rhombic components $D_a$ and $R$ of the alignment tensor are estimated using a powder pattern analysis (*22–24*).

In step 4, a grid search is performed in the Euler angles $\alpha$, $\beta$, $\gamma$. At each grid point the cost matrix $\boldsymbol{C}$ is calculated and the assignment problem is solved using a weighted matching algorithm (*21*). The peptide units whose assigned resonance sets have the lowest $C_{rdc}$ costs are used to refine the alignment tensor.

In step 5, resonance sets belonging to mobile residues and residues with only chemical shift information are assigned to the remaining peptide planes that have not been assigned to rigid residues. Steps 4f and 4g are repeated with the original $C_{rdc}$ values of resonance sets belonging to mobile residues to identify their most probable assignments.

## RESULTS AND DISCUSSION

The algorithm was applied to the 76 amino acid protein ubiquitin for which a 1.8-Å resolution x-ray structure exists (PDB entry 1UBQ) (*25*). Dipolar couplings have been measured using Pf1 phage (ASLA Ltd., Riga, Latvia) as alignment medium (*26, 27*). The sample consists of a 500-$\mu$l solution of 1 mM $^{15}N$, $^{13}C$-labeled ubiquitin in the presence of 5.7 mg Pf1 phage dissolved in a 10-mM phosphate buffer at pH 6.55 with 50 mM NaCl and 0.02% $NaN_3$ in 95% $H_2O$ and 5% $D_2O$. As an isotropic reference, a second sample was prepared with 2 mM ubiquitin under identical buffer conditions without the phage alignment medium.

All NMR experiments were performed at 303 K on a 600-MHz spectrometer. Intra-peptide-plane RDCs of the $^{15}N$–$^1H^N$, $^{13}C'$–$^{15}N$, and $^{13}C'$–$^{13}C^\alpha$ spin pairs were measured as follows. For the $^{15}N$–$^1H^N$ coupling the inphase-antiphase (IPAP) method was used (*6, 7*). RDCs of $^{13}C'$–$^{15}N$ and $^{13}C'$–$^{13}C^\alpha$ spin pairs were measured using 2D versions of the HNCO-based pulse sequences of Permi *et al.* (*8*). For the $^{13}C'$–$^{15}N$ coupling the HNCO($\alpha/\beta$-NC′-J)-pulse sequence (see Fig. 2D of Ref. (*8*)) was used with $\lambda = 0$. For the $^{13}C'$–$^{13}C^\alpha$ coupling the HNCO($\alpha/\beta$-C′C$^\alpha$-J)-pulse sequence was used (see Fig. 2A of Ref. (*8*)). All RDCs were measured as changes of the positions of multiplet components along $\omega_1$. Resonance sets were established using CBCA(CO)NH and HNCO experiments (*3*). Assignments used as reference to test the new method were determined using standard procedures based on these experiments and a 3D HNCACB experiment (*28*) together with previously reported assignments of ubiquitin under different conditions (*29, 30*).

Some of the RDCs belonging to very weak signals ($^{13}C'$–$^{15}N$ RDCs of peptide units 9, 46, 75 and $^{13}C'$–$^{13}C^\alpha$ RDCs of units 9 and 46) and RDCs lying outside the fitted powder pattern distribution ($^{13}C'$–$^{15}N$ RDCs of peptide units 4 and 56 and $^{13}C'$–$^{13}C^\alpha$ RDCs of unit 59) were not included in subsequent analysis. For resonance sets belonging to peptide 53, neither RDC nor chemical shift data are available and no RDC data are available for unit 24. Four peptide units (belonging to the C-terminal peptide units 73–76) exhibit increased mobility as reflected in the heteronuclear $\{^1H\}$–$^{15}N$ NOE data (this is in agreement with previous findings of ubiquitin measured under different conditions (*31, 32*)). For these resonance sets the cost matrix elements were set to $C_{max}$ (see step 2 of the algorithm) in the case where rigid and mobile residues are treated differently.

The refined alignment tensor of the Pf1 phage sample has a rhombicity of $R = 0.36$ and its $D_a$ value corresponds to 21.6 Hz for the largest possible $^{15}N$–$^1H^N$ coupling. Since Met 1 and the three prolines in ubiquitin do not appear in the NMR spectra considered here, there is a total of 72 resonance sets that can be assigned.

The algorithm was first applied to the rigid residues using three RDCs per peptide unit for spin pairs $^{15}N$–$^1H^N$, $^{13}C'$–$^{15}N$, and $^{13}C'$–$^{13}C^\alpha$ without chemical shift information. The resulting assignments are correct for 37 of the 72 resonance sets (51.4%) (see Table 1). This result illustrates the potential of dipolar couplings for resonance assignment, but it also indicates that for a higher assignment score additional experimental information needs to be included. Such information is provided by amino-acid type dependent $C^\alpha$ and $C^\beta$ chemical shifts which are incorporated into the cost matrix in form of the $C_{cs}(i, j)$ term defined in Eq. [4].

For the following applications the prefactor $K$ of Eq. [4] which determines the relative weights of the chemical shifts relative to the RDCs was chosen such that the chemical shifts and RDCs contribute on average with about the same weight to the $\chi^2$ of Eq. [2]. Incorporation of the chemical shift information substantially improves the assignment results as can be seen in Table 1. If 3 RDCs per peptide unit are used and mobile residues identified by their $\{^1H\}$–$^{15}N$ NOE are treated as described in the algorithm, the correct assignment is found for 64 of the 72 resonance sets (88.9%). The wrong assignments are related to each other by swaps between the peptide-unit pairs $24 \leftrightarrow 31$, $41 \leftrightarrow 73$, and a cyclic permutation involving four peptide units $9 \rightarrow 74 \rightarrow 59 \rightarrow 53 \rightarrow 9$. If the $\{^1H\}$–$^{15}N$ NOEs are ignored, i.e., no distinction is made between rigid and mobile residues by skipping step 2 of the algorithm, the assignment score slightly improves leading to 91.7% correct assignments.

**TABLE 1**
**Results of Assignment of Ubiquitin Using RDCs and $C^{\alpha}$ and $C^{\beta}$ Chemical Shifts**

| Number of RDCs per peptide unit[a] | Chemical shift information[b] | Assignment[c] (%) | | |
|---|---|---|---|---|
| | | Rigid residues[d] | Mobile residues[e] | All residues[f] |
| 3 RDCs[a1] | BMRB | 91.2 | 50.0 | 88.9 |
| 3 RDCs[a2] | | | | 91.7 |
| 2 RDCs[a1] | BMRB | 86.8 | 50.0 | 84.7 |
| 2 RDCs[a2] | | | | 84.7 |
| 3 RDCs[a1] | $\phi$, $\psi$ based | 92.6 | 75.0 | 91.7 |
| 3 RDCs[a2] | | | | 88.9 |
| 2 RDCs[a1] | $\phi$, $\psi$ based | 91.2 | 75.0 | 90.3 |
| 2 RDCs[a2] | | | | 86.1 |
| 3 RDCs[a1] | None | 54.4 | 0.0 | 51.4 |
| 3 RDCs[a2] | | | | 44.4 |

[a] Three RDCs per peptide unit were used belonging to $^{15}N$–$^{1}H^{N}$, $^{13}C'$–$^{15}N$, $^{13}C$–$^{13}C^{\alpha}$ vectors or only two RDCs were used belonging to $^{15}N$–$^{1}H^{N}$ and $^{13}C'$–$^{15}N$ vectors. The upper row (a1) refers to the calculation for which the mobile residues identified by a low heteronuclear NOE are treated separately in a second stage. In the lower row (a2) all resonance sets are treated the same way.

[b] Chemical shift information was interpreted either via the BMRB database or using predictions applied to the x-ray structure using empirical parametrizations of $C^{\alpha}$ and $C^{\beta}$ chemical shifts.

[c] Percentage of correctly assigned resonance sets with respect to the total of 72 resonance sets.

[d] Residues 2 to 72 without prolines but including residue 53 for which no data were measured.

[e] Residues 73 to 76 of the mobile tail of ubiquitin which exhibit reduced $\{^{1}H\}$–$^{15}N$ NOEs.

[f] Residues belonging to all 72 resonance sets.

Errors correspond to the three swaps $24 \leftrightarrow 31$, $53 \leftrightarrow 59$, and $36 \leftrightarrow 76$. The assignment result is mapped in Fig. 2 onto the backbone structure of ubiquitin.

These assignment swaps are caused by a number of different factors: (i) Some or all RDC information is missing for these peptide units due to peak overlap or line broadening (units 24, 53, and 59), (ii) the side-chain types belonging to the swapped peptide units are identical leading to similar $C^{\alpha}$ and $C^{\beta}$ chemical shifts (isoleucine side chains for peptide units 24 and 31 and aspartic-acid side chains for peptide units 53 and 59), (iii) peptide units belonging to the same type of secondary structure have similar $\varphi$, $\psi$ dihedral angles that similarly affect $C^{\alpha}$ and $C^{\beta}$ chemical shifts (units 24 and 31), (iv) peptide units with similar or symmetry-related orientations exhibit similar RDCs, which makes them difficult to distinguish (units 24 and 31), and (v) units 73 and 76 are mobile residues whose experimental RDC data are not well explained by the static x-ray structure. Residues of other regions of ubiquitin with increased flexibility, such as the loop region of the N-terminal $\beta$-sheet [33], are correctly assigned.

Assignments that change upon inclusion of $\{^{1}H\}$–$^{15}N$ NOEs have a low level of confidence. The level of confidence for each assignment can be further assessed by inspection of the cost matrix. Generally for the erroneous assignments there exists a second assignment possibility with similar cost indicating a low level of confidence for these assignments.

The assignment protocol was applied using only 2 RDCs per peptide unit. The highest assignment scores are achieved when the RDCs belonging to the $^{15}N$–$^{1}H^{N}$ and $^{13}C'$–$^{15}N$ vectors are used. The results (see Table 1) are only slightly worse than when 3 RDCs per peptide unit are used. It reflects a significant degree of redundancy of assignment information contained in the 3 RDCs belonging to vectors lying in the same peptide plane.

The robustness of the algorithm was further tested by the addition of 10% random noise to the experimental RDCs which corresponds to a standard deviation of about 1 Hz for the $^{15}N$–$^{1}H^{N}$ RDCs. As a consequence, changes in the assignment swaps can occur, but the total assignment score remains unchanged. It demonstrates that the assignment results are rather insensitive to the experimental accuracy of the RDC data. The results are also insensitive with respect to the input structure. Essentially the same assignment scores are obtained for a different x-ray structure (*34*) and a NMR structure (*35*) of ubiquitin.

It is known that $C^{\alpha}$ and $C^{\beta}$ chemical shifts depend also on the local backbone $\varphi$, $\psi$ dihedral angles. Using a chemical shift database of proteins with known structure, an empirical relationship between $C^{\alpha}$, $C^{\beta}$ chemical shifts and $\varphi$, $\psi$ angles was parameterized by Iwadate *et al.* (*19*). However, the use of this relationship instead of the amino-acid type specific chemical shift averages does not lead to an improvement of the assignment score (see Table 1).

The total measurement time required for the determination of RDCs depends on numerous factors, such as the sample

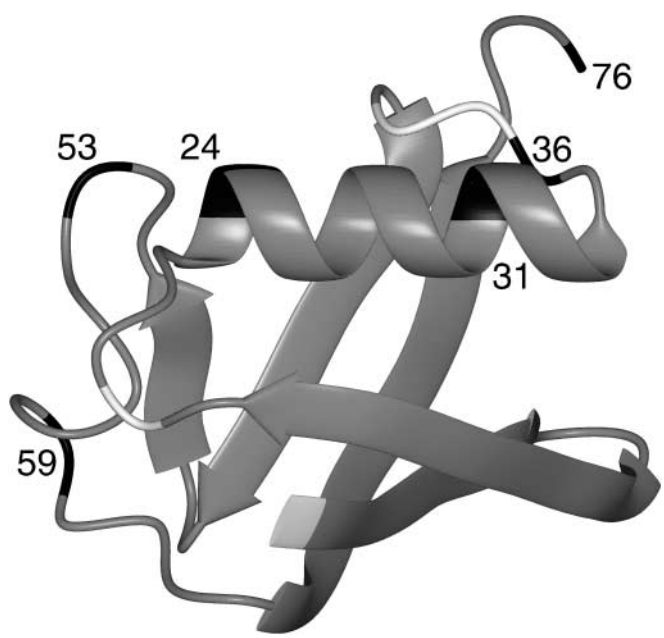


**FIG. 2.** Results of the assignment protocol for ubiquitin from the second row of Table 1 displayed using MolMol (*36*). The correctly assigned peptide units are colored in grey, the peptide units which do not give rise to NMR signals in the experiments considered here (Met 1, Pro 19, Pro 37, Pro 38) are displayed in white, and in black are the wrongly assigned peptide units $24 \leftrightarrow 31$, $53 \leftrightarrow 59$, and $36 \leftrightarrow 76$.

concentration, magnetic field strength, the availability of a cryoprobe, and the NMR pulse sequence. In the present study, no cryoprobe was used and each RDC set measured using 2D experiments (including the experiment in phage medium and the one in isotropic solution) required about one day of spectrometer time. Using a cryoprobe it should be possible to measure all RDCs in one day. Alternatively, NMR pulse sequences are available that allow the measurement of two or three RDCs in the same 3D experiment (*9–11*). In combination with the algorithm described here, this may allow shortening and simplification of the assignment. The identification of highly mobile residues on the basis of their NOE has in the case of ubiquitin no major effect on the assignment score. Since the NOE information is used purely qualitatively here, it can be collected within a few hours.

A hallmark of traditional NMR assignment protocols is the use of sequential NMR connectivity information along the polypeptide backbone regardless of 3D structural knowledge (*1–3*). The new strategy does not rely on sequential connectivity information. It is solely based on RDCs and chemical shifts within individual peptide units. The optimization algorithm scales only polynomially ($O(n^3)$) with the number of peptide units $n$, which makes it applicable also to larger protein systems that can be studied by NMR. The strategy should also be suitable for resonance assignments by solid-state NMR.

In conclusion, an efficient algorithm for the NMR assignment problem has been presented that uses intraresidual NMR information together with the 3D x-ray structure. While the percentage of correct assignments is high, it does not reach 100% in the present application. Because the method provides information about the confidence into each proposed assignment, a subset of assignments can be identified that is correct with high probability. If this subset includes residues or resonances that are of particular interest such incomplete assignment information may be useful in practice.

If complete assignment is mandatory, it is conceivable to incorporate additional intraresidual NMR information. This includes scalar $^3J$ couplings, cross-correlated relaxation parameters, chemical shifts of additional nuclei, pseudo-contact shifts, residual dipolar couplings of other interatomic vectors, and RDCs measured in a second alignment medium. Moreover, the incorporation of some sequential connectivity information that may be sparse and incomplete is feasible. Investigations of these possibilities are currently under way in our group. The presented strategy represents a first step towards the use of x-ray structural information for the NMR assignment task with the goal to make the fast growing number of proteins whose structures have been determined by x-ray crystallography amenable to detailed NMR investigations for studying dynamics, protein interactions, and for screening of inhibitors for drug discovery.

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