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Automated resonance assignment of the 21 kDa stereo-array isotope labeled thioldisulfide oxidoreductase DsbA

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ABSTRACT

The automated chemical shift assignment algorithm FLYA has been extended for use with stereo-array isotope labeled (SAIL) proteins to determine the sequence-specific resonance assignments of large proteins. Here we present the assignment of the backbone and sidechain chemical shifts of the 21 kDa thioldisulfide oxidoreductase DsbA from *Escherichia coli* that were determined with the SAIL-FLYA algorithm in conjunction with automated peak picking. No manual corrections of peak lists or assignments were applied. The assignments agreed with manually determined reference assignments in 95.4% of the cases if 16 input spectra were used, 94.1% if only 3D $^{13}C/^{15}N$-resolved NOESY, CBCA(CO)NH, and 2D [$^{13}C/^{15}N$,^{1}H]-HSQC were used, and 86.8% if exclusively 3D $^{13}C/^{15}N$-resolved NOESY spectra were used. Considering only the assignments that are classified as reliable by the SAIL-FLYA algorithm, the degrees of agreement increased to 97.5%, 96.5%, and 94.2%, respectively. With our approach it is thus possible to automatically obtain almost complete and correct assignments of proteins larger than 20 kDa.

1. Introduction

The stereo-array isotope labeling (SAIL) technique [1,2] was developed with the aim of improving NMR spectra in order to enable the analysis of large proteins. Reduced relaxation during magnetization transfer steps and reduced long-range couplings, which result from SAIL labeling, lead to enhanced signal strength and sharper lines. The reduction of the number of detectable atoms in the protein leads to a decreased number of signals without loss of important information.

Signal overlap in overcrowded spectra, noise, artifacts, and missing peaks are the main challenges for automated chemical shift assignment of NMR spectra [3–5]. Hence, SAIL labeling improves the conditions for automated chemical shift assignment and provides a basis for the automated assignment of large proteins. The advantages of SAIL for challenging automated resonance assignment tasks became evident with the first assignment of a protein based exclusively on NOESY data, which was achieved for SAIL ubiquitin [6] using the program Garant [7,8], a predecessor of the FLYA automated assignment algorithm.

Recently, we introduced the FLYA algorithm [9] which is able to assign the chemical shifts measured for a protein to the respective atoms. The flexible network approach implemented in FLYA allows the use of virtually all known multidimensional NMR spectra for the assignment calculation and can in principle be applied to all types of molecules including arbitrarily isotope labeled proteins. FLYA has been used to assign several protein targets with data from solution and solid-state NMR [9,10]. Furthermore, the applicability of FLYA to NMR data of RNAs [11,12] and to "NOESY-only" data of proteins [13] has been shown.

In this paper the automated chemical shift assignment of a large SAIL labeled protein, i.e. the *Escherichia coli* thioldisulfide oxidoreductase DsbA, is presented. DsbA is a monomeric 21 kDa protein that consists of 189 amino acid residues and has two domains. The catalytic domain, spanning residues 1–62 and 139–189, exhibits a thioredoxin fold [14]. DsbA is required for the disulfide bond formation during protein folding in the periplasmic space by transferring a disulfide bond to a target protein. Structures of DsbA exist from solution NMR [14], X-ray crystallography (e.g. [15,16]), and

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joint X-ray and solid-state NMR data [17] but were not used as input for the assignment calculations of this paper.

2. Materials and methods

2.1. Protein and experimental data

NMR measurements were performed with reduced SAIL labeled DsbA from *E. coli*. The DsbA sample was produced by *E. coli* cell-free protein synthesis that was optimized for the preparation of labeled NMR samples [18,19]. All amino acids were labeled according to the SAIL standard labeling pattern [1] with the following modifications. In Tyr and Phe ^{13}C–^{1}H groups were located at the δ-positions and ^{12}C–^{2}H groups were located at the ε-positions in the ring. In Pro $H^{\delta 2}$ is a ^{1}H nucleus, and $H^{\delta 3}$ is labeled with ^{2}H. Example spectra of SAIL DsbA are shown in Fig. 1.

NMR spectra were obtained at 25 °C and pH 3.7 from a single 0.36 mM sample of reduced DsbA with all SAIL amino acids and 20 mM sodium phosphate in H_2O. The measurements were performed on a Bruker AV950 spectrometer for ^{15}N-resolved NOESY, ^{13}C-resolved NOESY of the aliphatic region, ^{13}C-resolved NOESY of the aromatic region, [^{15}N,^{1}H]-HSQC, [^{13}C,^{1}H]-HSQC of the aliphatic region, [^{13}C,^{1}H]-HSQC of the aromatic region, with a Bruker AV900 spectrometer for CBCANH, with a Bruker AV700 spectrometer for CBCA(CO)NH, with a Bruker AV600 spectrometer for C(CCO)NH and a Bruker AV500 spectrometer for HNCO, HN(CA)CO, HBHA(CO)NH, H(CCCO)NH, (H)CCH-TOCSY, HCCH-TOCSY, and HCCH-COSY. NOESY spectra were processed with quantitative maximum entropy (QME) [20], all other spectra were processed with Azara (W. Boucher, University of Cambridge, UK, http://www2.ccpn.ac.uk/azara/). Peak lists for the two 2D HSQC spectra were obtained by automatic peak picking followed by visual confirmation, in particular for avoiding water signals in the [^{13}C,^{1}H]-HSQC spectra. Peak lists for the 3D spectra were collected by automatic peak picking based on the two HSQC peak lists with the program CcpNmr Analysis [21] without manual corrections or modifications. Statistics on the contents and quality of the peak lists are given in Table 1.

2.2. FLYA automated chemical shift assignment algorithm

The FLYA algorithm, which has been described in detail recently [9], generates an assignment by mapping a network of expected peaks to the measured peaks in the peak lists obtained from NMR spectra. The network of expected peaks is built up using the sequence of the protein and a list of NMR experiment specifications, which can be defined and modified by the user. Experiment specifications comprise magnetization transfer rules for

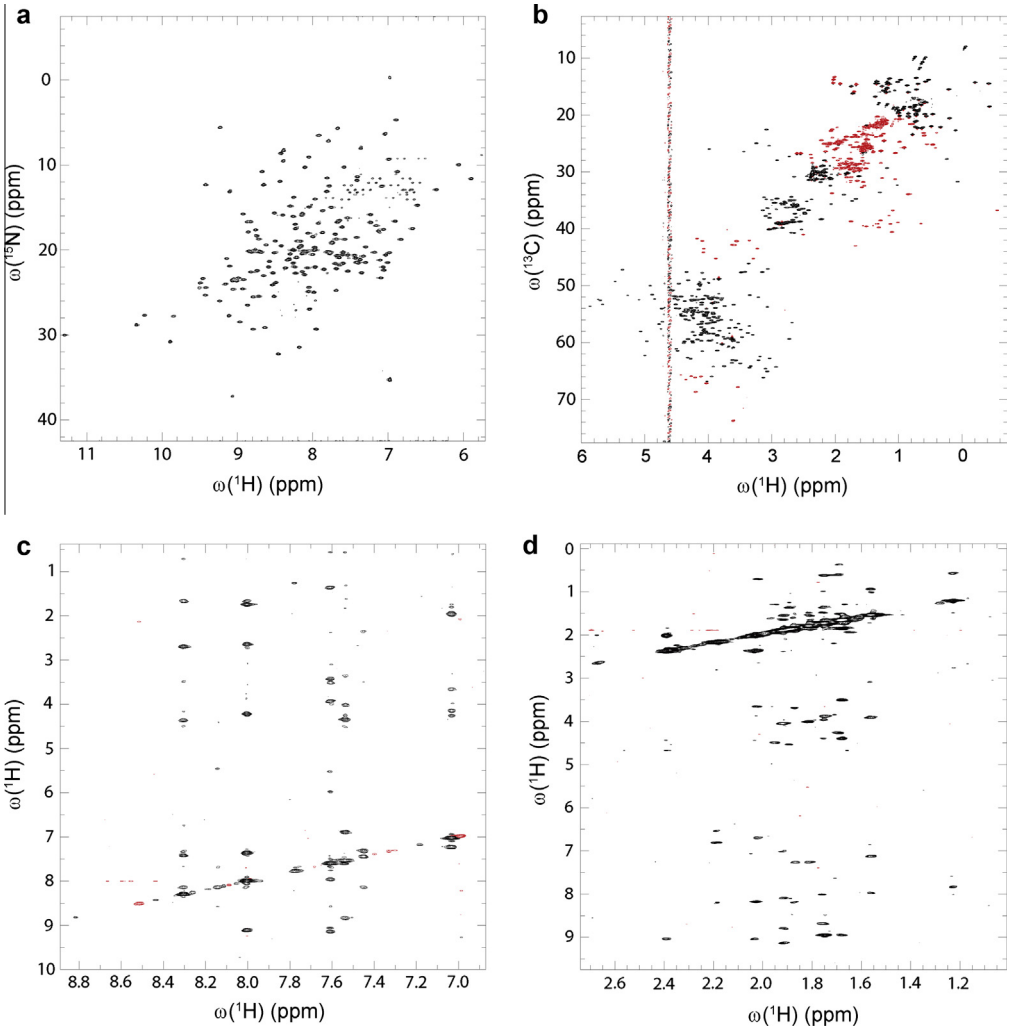

Fig. 1. NMR spectra of SAIL DsbA. (a) 2D [^{15}N, ^{1}H]-HSQC. (b) 2D [^{13}C,^{1}H]-HSQC. (c) 3D ^{15}N-resolved NOESY. ^{1}H–^{1}H plane at $\omega(^{15}N)$ = 116.48 ppm. (d) 3D ^{13}C-resolved NOESY. ^{1}H–^{1}H plane at $\omega(^{13}C)$ = 52.87 ppm.

Table 1
Experimental peak lists.[a]

Peak list	Expected peaks	Measured peaks	Complete (%)	Assigned (%)
^{15}N-resolved NOESY	5607	9933	53.6	26.9
^{13}C-resolved NOESY (aliphatic)	13548	12685	36.5	33.7
^{13}C-resolved NOESY (aromatic)	677	1469	36.2	14.8
[^{15}N,^{1}H]-HSQC	232	257	86.2	76.3
[^{13}C,^{1}H]-HSQC (aliphatic)	543	988	92.8	49.1
[^{13}C,^{1}H]-HSQC (aromatic)	31	86	74.2	25.6
HNCO	181	188	98.3	94.7
HN(CA)CO	363	199	57.9	97.0
CBCANH	712	558	73.3	89.6
CBCA(CO)NH	363	597	80.4	48.7
HBHA(CO)NH	349	985	93.1	32.8
H(CCCO)NH	623	640	45.3	43.0
C(CCO)NH	623	1687	71.8	26.1
(H)CCH-TOCSY	1843	3565	22.3	10.3
HCCH-TOCSY	1767	6373	65.0	16.5
HCCH-COSY	1270	3277	76.9	26.6

[a] *Expected peaks:* Number of expected peaks by FLYA using a DsbA X-ray structure (PDB 1FVK) [16] for the generation of distance-dependent expected peaks. The structure was *not* used for the assignment calculations. *Measured peaks:* Number of measured peaks. *Complete:* Percentage of expected peaks that can be mapped to a measured peak based on the reference chemical shift assignments. The theoretical maximum of 100% corresponds to the situation that the spectra "explain" all expected peaks. Each expected peak can be mapped to at most one measured peak. Remaining expected peaks correspond to missing peaks in the measured peak list. *Assigned:* Percentage of measured peaks that can be assigned within a tolerance of 0.025 ppm for ^{1}H and 0.3 ppm for ^{13}C and ^{15}N, based on the reference chemical shift assignments. The theoretical maximum of 100% corresponds to having all measured peaks assigned. Note that several expected peaks can be mapped to the same measured peak, i.e. the assignments of measured peaks can be unambiguous or ambiguous. Remaining unassigned measured peaks are likely to be artifacts.

through-bond as well as through-space magnetization transfer. To implement SAIL labeling, the generation of expected peaks in SAIL-FLYA is restricted to the subset of all atoms that are labeled with NMR-observable nuclei according to the SAIL isotope labeling patterns [1,2]. With this approach, SAIL-FLYA can be applied to completely (all amino acids) or partially (selected amino acids) SAIL labeled proteins. The subset of NMR-observable nuclei can either be specified by setting the corresponding atom types in the CYANA residue library, for instance to H_ALI for an aliphatic ^{1}H and D_ALI for aliphatic ^{2}H, or by specifying the NMR-observable nuclei in a CYANA atom selection command. Whereas the former method applies simultaneously to all input spectra, the latter approach has the advantage that it can be applied to specific input spectra, e.g. if multiple samples with different isotope labeling patterns are used.

An assignment solution is determined by a combination of an evolutionary algorithm and a local optimization maximizing a scoring function that takes into account the distribution of chemical shift values with respect to general chemical shift statistics, the alignment of peaks assigned to the same atom, the completeness of the assignment, and a penalty for chemical shift degeneracy. The SAIL-FLYA algorithm for automated resonance assignment and structure calculation is part of the CYANA software package [3].

2.3. Chemical shift assignment calculations

The tolerance for chemical shift matching in the FLYA algorithm was 0.025 ppm for ^{1}H and 0.3 ppm for ^{13}C and ^{15}N for all calculations. The same tolerances were used for the determination of the assignments and their evaluation by comparison with the manually determined reference assignments. Manually determined chemical shift assignments were used only as reference to evaluate the quality of the automated assignment.

Expected peaks in through-bond spectra were generated according to the magnetization transfer rules of the CYANA library. Expected peaks for the NOESY spectra were generated on the basis of 20 conformers, calculated with CYANA, that fulfill the steric restraints but are otherwise random. Expected NOESY peaks with probabilities 0.9, 0.8, 0.7, 0.6, and 0.5 were generated for ^{1}H–^{1}H distances shorter than 4.0, 4.5, 5.0, 5.5, and 6.0 Å, respectively, in all 20 random conformers. The computation time for generating the random conformers (8 s) was less than 1% of that for the subsequent assignment calculation (about 1100 s with all peak lists). Alternatively, FLYA provides the possibility to use a general set of rules, similar to those used for example for TOCSY spectra, for the generation of the expected short-range NOESY peaks (not used for the calculations of this paper).

The population size for the evolutionary algorithm was 50 in the calculation with the full set of available spectra, 200 for the calculation with NOESY, HSQC, and CBCA(CO)NH, and 300 for the calculation in which solely NOESY peak lists were used (see below). Hydroxyl protons and the side-chain terminal amide groups of Lys and Arg were excluded from the calculations.

Chemical shift assignments were consolidated from 20 independent runs. The assignment of an atom was classified as 'strong' if 80% or more of the 20 chemical shift values from these runs differed by less than the matching tolerance from the consensus value, and 'weak' otherwise. Earlier experience [9–13] has shown that strong assignments are more reliable than weak ones.

3. Results and discussion

Automatic assignment calculations were performed for the 189-residue protein DsbA using three different sets of peak lists. The first set consisted of all 16 available peak lists, including 3D ^{13}C/^{15}N-resolved NOESY, 2D [^{13}C/^{15}N,^{1}H]-HSQC, and several through-bond backbone and sidechain assignment experiments. The second set comprised seven peak lists including all NOESY and HSQC experiments and one experiment for backbone assignment, i.e. CBCA(CO)NH. The third calculation was done using only the three peak lists obtained from the NOESY experiments as input.

In order to obtain the correctness of the automated procedure, the resonance assignment of DsbA was also determined manually using all available spectra. These assignments were used as reference chemical shifts. Automatically obtained atom assignments were considered correct if they agreed with the reference within a tolerance of 0.025 ppm for ^{1}H atoms and 0.3 ppm for ^{13}C or ^{15}N atoms, respectively. The manually and automatically determined

Table 2
Chemical shift assignment statistics for SAIL-DsbA.

Atom selection	All peak lists[a]	Selected peak lists[b]	NOESY peak lists[c]
All (strong and weak)[d] assignments:			
All atoms reference[e]	1684	1497	1497
All atoms correct[f]	1607 (95.4%)	1408 (94.1%)	1300 (86.8%)
Backbone reference[e]	906	719	719
Backbone correct[f]	883 (97.5%)	691 (96.1%)	628 (87.3%)
Side chain reference[e]	778	778	778
Side chain correct[f]	724 (93.1%)	717 (92.2%)	672 (86.4%)
Strong[d] assignments:			
All atoms reference[e]	1613	1396	1223
All atoms correct[f]	1573 (97.5%)	1347 (96.5%)	1152 (94.2%)
Backbone reference[e]	883	685	602
Backbone correct[f]	874 (99.0%)	672 (98.1%)	572 (95.0%)
Sidechain reference[e]	730	711	621
Sidechain correct[f]	699 (95.8%)	675 (94.9%)	580 (93.4%)

[a] Calculations performed with all experimental peak lists given in Table 1.
[b] Calculations performed with ^{15}N-resolved NOESY, ^{13}C-resolved NOESY, [^{15}N, ^{1}H]-HSQC, [^{13}C, ^{1}H]-HSQC, and CBCA(CO)NH peak lists.
[c] Calculations performed with ^{15}N-resolved NOESY and ^{13}C-resolved NOESY peak lists.
[d] 'Strong' includes the assignments that are self-consistent within at least 16 of 20 runs of the algorithm. Other assignments are classified as 'weak'. Note that these categories only refer to the subset of atoms that could be assigned automatically.
[e] Number of atoms in the category that have been assigned manually. Note that C′ nuclei cannot be assigned by the algorithm if using only selected peak lists or exclusively NOESY peak lists. 'Backbone' refers to the atoms that can be assigned using standard triple resonance experiments for backbone assignment, i.e. backbone N and H, C′, C$^{\alpha}$, and C$^{\beta}$. 'Side chain' refers to all other ^{1}H, ^{13}C, and ^{15}N nuclei, including H$^{\alpha}$ and H$^{\beta}$. The category 'All atoms' includes both groups.
[f] Number and percentage of chemical shifts that are, within the chemical shift tolerance of 0.025 ppm for ^{1}H and 0.3 ppm for ^{13}C and ^{15}N, in agreement with the manually determined assignment. The percentage is relative to the corresponding number in the preceding row.

assignments have been deposited in the BMRB database [22] with accession number 25117.

Results of the automated assignment are summarized in Table 2. An assignment correctness of 100% means that for all nuclei assigned by automatic and manual methods all automatically obtained assignments agreed with the reference assignments. Nuclei that could not be assigned manually were not considered in the percentage of correct assignments because their correctness cannot be established. The total number of manual assignments was 1684. The FLYA calculation with all peak lists yielded 1803 assignments (1683 for atoms that could also be assigned manually, and 120 for atoms that could not be assigned manually). The two FLYA calculations with fewer peak lists yielded only 1614 assignments because C′ atoms cannot be assigned in the absence of the HNCO and HN(CA)CO spectra. It should be noted that FLYA yields a chemical shift value for every atom that occurs in at least one assigned peak, although not all of these assignments are reliable (see 'strong' and 'weak' assignments below), whereas the manual assignment includes only reliably assigned atoms. Using all available peak lists a correctness of 95.4% was obtained with FLYA. This corresponds to 1607 correct chemical shift assignments out of 1684 manually obtained reference assignments. For backbone atoms the percentage of correct assignments was 2.1 percentage points higher, for side chains the percentage was 2.3 percentage points lower than for all atoms.

Atom chemical shift assignments are classified into 'strong' and 'weak' assignments. Strong assignments are consistent over at least 80% of several runs with different random seed numbers [9], and therefore considered to be more reliable than others. If only the 1666 strong assignments were considered in the present calculation, the percentage of correct assignments increased by 2.1 percentage points to 97.5%. In case of the assignment calculation

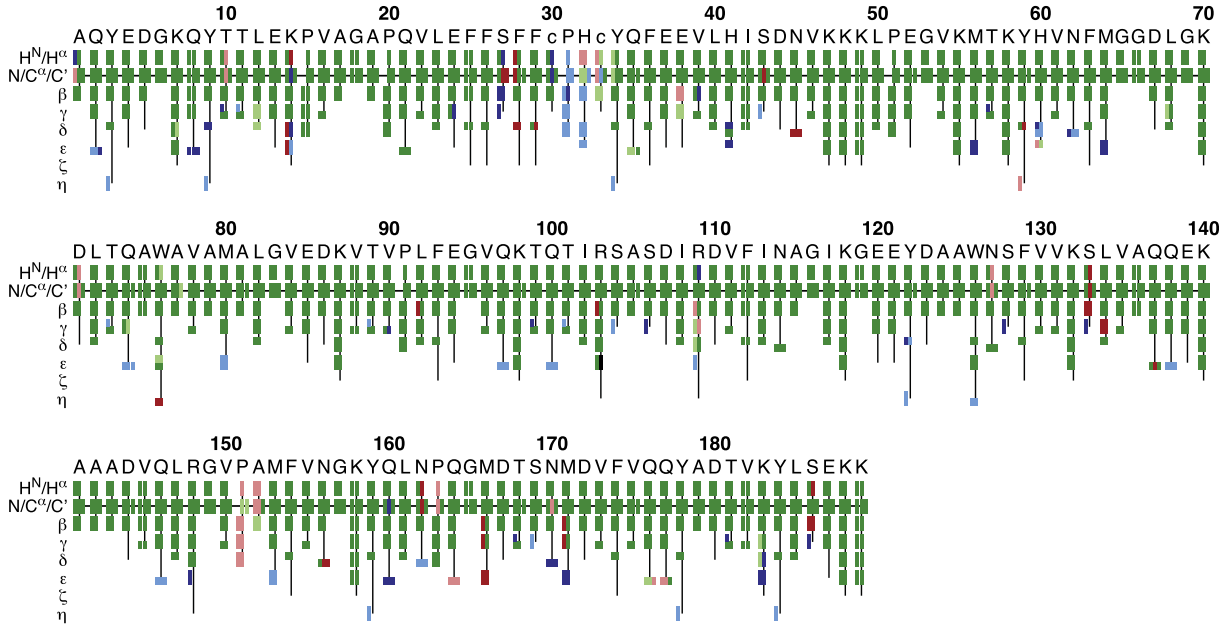

Fig. 2. Extent, correctness, and reliability of individual assignments obtained with the FLYA automated resonance assignment algorithm using the full sets of automatically prepared peak lists. Each assignment for an atom is represented by a colored rectangle: green, assignment by FLYA agrees with the manually determined reference chemical shifts within a tolerance of 0.025 ppm for ^{1}H ppm and 0.3 ppm for ^{13}C and ^{15}N; red, assignment differs from reference; blue, assigned by FLYA but no reference available; black, with reference assignment but not assigned by FLYA. Respective light colors indicate assignments classified as 'weak' by the chemical shift consolidation. The row labeled H^{N}/H$^{\alpha}$ shows for each residue H^{N} on the left and H$^{\alpha}$ in the center. The N/C$^{\alpha}$/C′ row shows for each residue the N, C$^{\alpha}$, and C′ assignments from left to right. The rows β–η show the side-chain assignments for the heavy atoms in the center and hydrogen atoms to the left and right. In the case of branched side chains, the corresponding row is split into an upper part for one branch and a lower part for the other branch. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

using the CBCA(CO)NH peak list combined with NOESY and HSQC peak lists, 1408 correct assignments could be obtained corresponding to 94.1% of the reference assignments. The absolute number of assignments decreased because C′ atoms are not observable in these spectra. The percentage of correct assignments increased to 96.5% for the subset of strong assignments. If only NOESY peak lists were used for the automated assignment calculation, 86.8% of the assignments were correct, and the percentage of correct assignments increased to 94.2% for strong assignments.

Hence, in the present case a set of 7 peak lists led to results that are comparable to those obtained using the set of 16 peak lists. Even if the overall number of correct assignments drops using only NOESY spectra, a correctness of 94.2% could still be obtained for the subset of strong assignments on the dispense of 11% of the correct assignments. The extent of correct assignments with 16 and 7 peak lists fulfills the requirement of 90% correct chemical shift assignments for automated NOE assignment and structure calculations that were established in systematic test calculations [23,24]. The results obtained using exclusively NOESY peak lists are slightly below but still close to this threshold.

The results for individual atoms obtained with the three different sets of peak lists are given in Figs. 2–4. Wrong assignments were located in all different residue types. If 16 or 7 peak lists were used (Figs. 2 or 3), single residues or residue pairs containing wrong assignments were distributed over the sequence, but did not accumulate in contiguous clusters. In these two calculations most of the correct assignments were classified as strong.

Due to missing signals the manual assignment was difficult to obtain in the region around residue 30 and some manual assignments are still missing in residues 27 and 30–33. Hence, it must be assumed that automatically obtained assignments in this region are wrong. Ideally, atoms without reference assignment should not be assigned or the assignments should be classified as weak. In all calculations most of these assignments and neighboring wrong

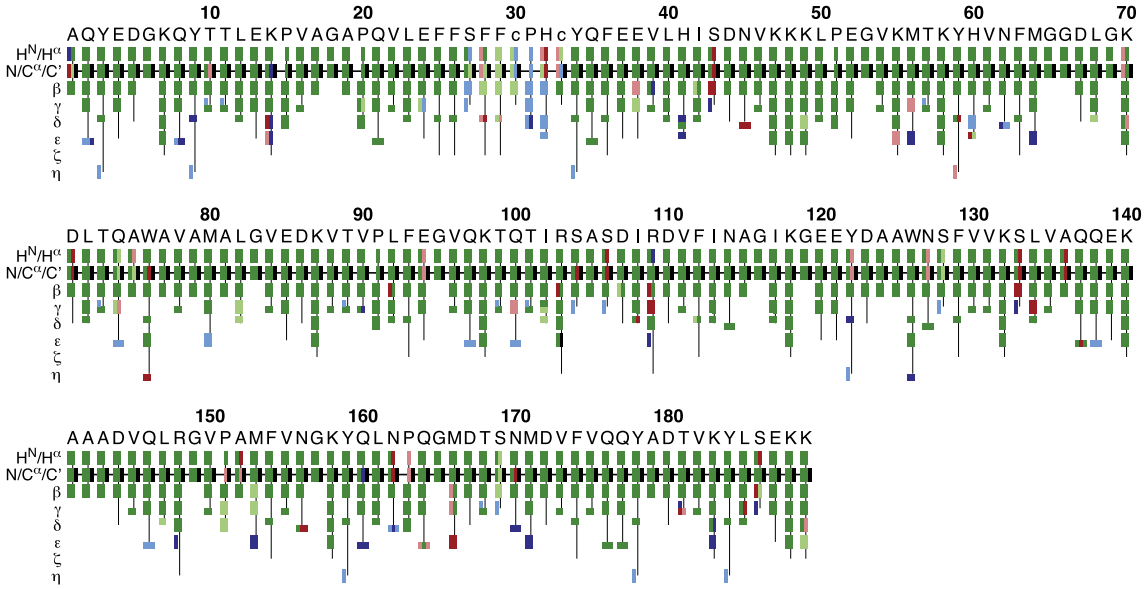

Fig. 3. Extent, correctness, and reliability of individual assignments obtained with the FLYA automated resonance assignment algorithm using ^{13}C-resolved NOESY, ^{15}N-resolved NOESY, [^{15}N, ^{1}H]-HSQC, and [^{13}C, ^{1}H]-HSQC and CBCA(CO)NH. For details see Fig. 2.

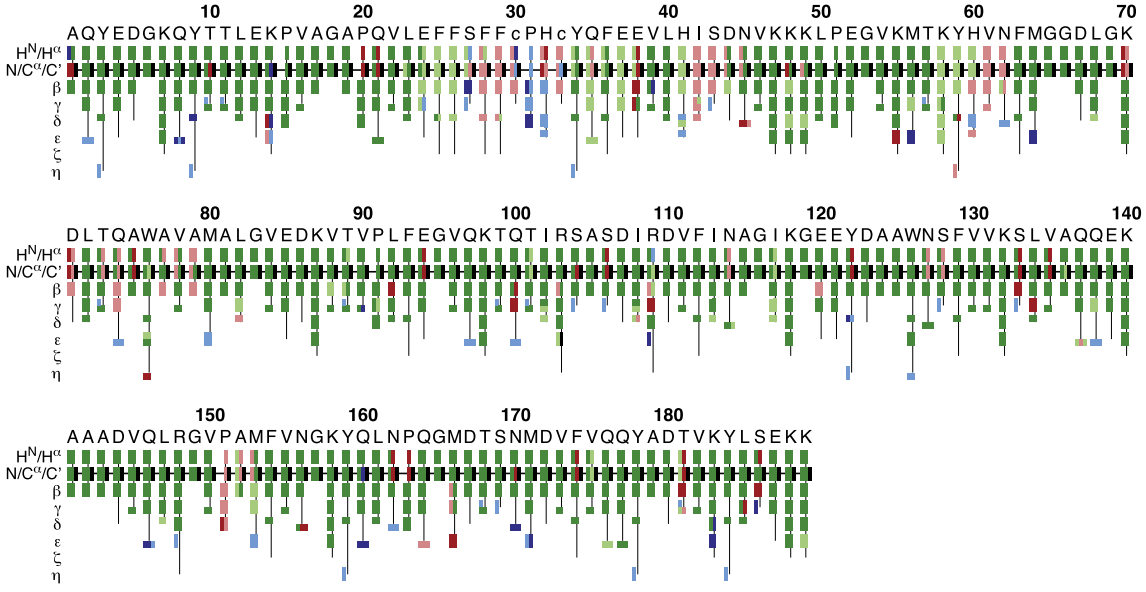

Fig. 4. Extent, correctness, and reliability of individual assignments obtained with the FLYA automated resonance assignment algorithm using ^{13}C-resolved NOESY and ^{15}N-resolved NOESY. For details see Fig. 2.

assignments were correctly classified as weak. In addition, some neighboring correct assignments were also classified as weak.

The calculation for which only NOESY spectra were used (Fig. 4) differed from the other calculations in that several clusters of wrong and weak assignments spanning several residues were located in the first part of the sequence. These clusters include atoms in residues 41–45, 58–62, 70–79, and residues 24–38, the latter containing the region in which many signals are missing in the spectra.

The region of residues 105–110 provides an illustrative example of the quality of the assignments obtained with the three input peak list sets. With all 16 peak lists all strong assignments are correct (2 have no reference shift). The only inconsistencies with the manual assignment occur for the weak assignments of $H^{\beta 3}$ and C^{γ} of Arg 109. FLYA assigned the former to the same shift as $H^{\gamma 3}$ in the same residue (a local mistake that is expected to have only minimal effect in a structure calculation), and the latter to a shift value with no local correspondence in the manual assignment. It should be noted that H^{α} of Arg 109 could not be assigned manually. Using 7 peak lists, FLYA made 6 incorrect assignments, i.e. C^{α}/H^{α} of Ser 106, $C^{\delta 1}$ of Ile 108, and $H^{\beta 3}$, $H^{\gamma 3}/C^{\gamma}$ of Arg 109. Using only NOESY spectra, the same 6 incorrect assignments occurred. In the calculation based on 7 peak lists these incorrect assignments were all classified as strong by FLYA, whereas two of them were classified as weak if using only NOESY spectra. The incorrect chemical shift values of these atoms were (within tolerance) the same in both cases, suggesting that their assignment is based mostly on the NOESY data. In contrast, using all 16 peak lists the information from the additional through-bond spectra allows to avoid 4 of these 6 incorrect assignments, and to classify the two remaining ones as weak.

4. Conclusions

In this paper we have demonstrated that the FLYA automated resonance assignment algorithm is able to reliably assign large proteins such as the 189-residue SAIL labeled DsbA. SAIL labeling facilitates the assignment process, since the number of signals is reduced by nearly a factor of 2 and lines are sharpened [1]. Due to the improved spectral quality, a smaller chemical shift tolerance compared to standard calculations with uniformly labeled proteins could be applied. The results for backbone atoms are comparable to those that have been obtained previously [9] using nearly the same set of spectra for smaller, uniformly $^{13}C/^{15}N$-labeled proteins with 114–140 residues. Results improved significantly, i.e. by more than 6 percentage points, for the side chain assignments. Remarkably, almost equally correct assignments were obtained for DsbA from only 3D NOESY spectra, 2D HSQC spectra, and a single, rapidly measurable, through-bond spectrum. The correctness decreased by merely 0.9–1.4% for the different categories in Table 1. Hence, it appears sufficient to record the smaller set of spectra, in particular since the additional through-bond spectra in the full set are in general not used for any other purpose than the resonance assignment.

The measurement of NOESY spectra is required for the structure determination of proteins by solution-state NMR, since they deliver the crucial $^{1}H–^{1}H$ distance information. In addition, a set of spectra is usually measured specifically for chemical shift assignment. The calculations of this paper show that with SAIL it is possible to assign 86.8% of the atoms in a 21 kDa protein correctly only using NOESY spectra.

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