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Assignment strategies for large proteins by magic-angle spinning NMR: the 21-kDa disulfide bond forming enzyme DsbA

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Summary

We present strategies for chemical shift assignments of large proteins by magic-angle spinning (MAS) solid-state NMR (SSNMR), using the 21-kDa disulfide bond forming enzyme DsbA as a prototype. Previous studies have demonstrated that complete *de novo* assignments are possible for proteins up to ~17 kDa, and partial assignments have been performed for several larger proteins. Here we show that combinations of isotopic labeling strategies, high field correlation spectroscopy and 3D and 4D backbone correlation experiments yield highly confident assignments for more than 90% of the backbone resonances in DsbA. Samples were prepared as nanocrystalline precipitates by a dialysis procedure, resulting in heterogeneous linewidths under 0.2 ppm. Thus, high magnetic fields, selective decoupling pulse sequences, and sparse isotopic labeling all improved spectral resolution. Assignments by amino acid type were facilitated by particular combinations of pulse sequences and isotopic labeling; for example, TEDOR experiments enhanced sensitivity for Pro and Gly residues, 2-¹³C-glycerol labeling clarified Val, Ile and Leu assignments, IPAP correlation spectra enabled interpretation of otherwise crowded Glx/Asx sidechain regions, and 3D NCACX experiments on 2-¹³C-glycerol samples provided unique sets of aromatic (Phe, Tyr, Trp) correlations. Together with high sensitivity CANCOCA 4D and CANCOCX 3D experiments, unambiguous backbone walks could be performed throughout the majority of the sequence. At 189 residues, DsbA represents the largest monomeric unit for which essentially complete solid-state NMR assignments have so far been achieved. These results will facilitate studies of nanocrystalline DsbA structure and dynamics and enable analysis of its 41-kDa covalent complex with the membrane protein DsbB, for which we demonstrate a high-resolution 2D ¹³C-¹³C spectrum.

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Keywords

chemical shift assignment; correlation spectroscopy; dipolar recoupling; selective pulses; solid-state NMR

Introduction

DsbA, with its membrane-bound partner, DsbB, belong to the disulfide bond transfer pathway of *Escherichia coli*, and together serve as the sole source of *de novo* disulfide bonds in the periplasm.¹⁻³ From genome analysis, DsbA is predicted to catalyze the folding of more than 300 periplasmic proteins by donating a disulfide bond; over a dozen of these have been experimentally confirmed.⁴⁻⁷ DsbA is reoxidized by DsbB through the formation of a covalent complex that serves as a transient intermediate in the reaction pathway. Analogs of DsbA and DsbB are found widely among bacteria, and the target proteins of this pathway in pathogenic bacteria include a variety of virulence factors.⁷⁻⁹ DsbA is required for folding and activity of these proteins, and therefore has been identified as a novel potential target for drug development.

DsbA is a 21 kDa soluble protein with a thioredoxin fold that has an insertion of a second, helical cap domain.¹⁰ This domain is responsible for positioning the –CPHC— active site in a relatively hydrophobic patch that has been postulated to be particularly suitable for specific binding of DsbB. The weaker of unfolded polypeptides substrates may be accommodated by hydrogen-bonding to the amide backbone of the *cis*-proline loop adjacent to the active site,¹¹ in a similar fashion as observed with substrate binding to thioredoxin.¹²

Among members of the thioredoxin superfamily, the redox potential of DsbA (–120 mV) is one of the most oxidizing, and this stabilization of DsbA in its reduced state aids the transfer of disulfide bonds to its periplasmic substrates.¹³ Redox tuning of the DsbA and thioredoxin active-site cysteines has been shown to be chiefly a function of three amino acid residues: the two between these cysteines (–CXXC—),¹⁴⁻¹⁶ and the one that precedes the highly conserved *cis*-proline (V150 in *E. coli* DsbA).¹⁷ The conformational changes in the backbone of DsbA upon oxidation/reduction are minimal and confined to the vicinity of the active site; the hinge motion of the thioredoxin and cap domains relative to each other do not correlate with redox state.^{18,19} Solution NMR has likewise indicated changes in chemical shift for residues in the vicinity of the active site, as expected for an altered chemical environment.^{19,20}

The high redox potential of DsbA makes its reoxidation by DsbB challenging, and the mechanistic details of how this is accomplished remains a topic of intense interest.^{3,21} To date, X-ray crystal structures of DsbB include the DsbA C33S/DsbB C130S complex (3.7 Å)^{22,23}, DsbA C33A/DsbB wt complex (3.7 Å),²⁴ and DsbB C41S bound to a Fab fragment (3.4 Å).²³ In addition, a high-resolution structure of DsbB C44S, C104S in dodecylphosphocholine micelles has been determined by solution NMR.²⁵ Comparison of free and DsbA-bound DsbB indicates that the binding of DsbA stabilizes portions of the periplasmic loops of DsbB, and induces a conformational change in DsbB. This change has been postulated to facilitate the transfer of the disulfide bond to DsbA and prevent the back-reaction. A highly-resolved structure of the DsbA/DsbB interface has not been achieved, because of either crystalline disorder or loop dynamics, resulting in low crystallographic resolution or, in some loop regions, an absence of electron density in the X-ray data. Chemical shift perturbation mapping studies have been achieved by solution NMR,²⁵ but as yet a high-resolution structure of the complex by solution NMR remains to be solved.

In this context, MAS SSNMR offers the potential to bridge the gap between crystallography and solution NMR, providing site-specific insight into atomic-resolution changes in states that neither diffract to high resolution nor give sufficient quality solution NMR data sets. For example, SSNMR can examine intermediate states that may not be accessible to solution NMR due to the suboptimal timescales of motion at or above room temperature. In addition, samples prepared for SSNMR may mimic single crystal conditions, enabling the crystal structures to be refined in conjunction with chemical shift and other SSNMR data. Finally, comparison of spectra of the individual proteins to the complex will enable site-resolved chemical shift perturbation mapping to observe protein-protein interactions and rearrangements upon binding.^{25,26} Thus a combination of isotopic labeling strategies with SSNMR spectroscopy has the potential to yield a variety of structural, dynamic and mechanistic information on the DsbA/DsbB complex. We have previously reported the preparation of DsbB samples for SSNMR study²⁷ and utilized high-field 3D and 4D experiments to assign many of the helical residues.²⁸ Here we complement these results with a detailed investigation of the periplasmic partner of DsbB, and report essentially complete ^{13}C and ^{15}N assignments of solid DsbA.

At 21 kDa, DsbA is among the largest monomeric units so far studied at this level of detail by SSNMR. Proteins of similar size for which nearly complete (>90% of the backbone resonances) SSNMR assignments are available include matrix metalloproteinase (17.6 kDa)^{29,30} and superoxide dismutase (16 kDa).³¹ A number of other proteins ranging from ~15 to 30 kDa have been examined and partially assigned. For example, sensory rhodopsin II (27 kDa) was partially assigned using CC and NC 2Ds with dipolar and scalar transfer experiments with selectively labeled U[^{13}C , ^{15}N](V,L,F,Y)NpSR_{II} samples.³² Roughly half (residues 64-163) of the α B-crystallin protein (20 kDa) was assigned using selectively labeled samples and 3D heteronuclear spectroscopy,³³ and 3D and 4D methods were applied to proteorhodopsin (27 kDa) in combination with selective labeling to assign the ~43% of the residues.³⁴ In our previous study of DsbB, we were able to assign approximately a third of all amino acids, including the majority of the transmembrane helices.²⁸ Partial assignments also are available for much larger complexes, such as the light-harvesting complex 1 (160 kDa), which has been partially assigned (~15%) using NCOCA and NCACX 3D spectra and CC 2D spectra of selectively labeled samples.³⁵ These successes of SSNMR build upon early studies, which focused primarily on proteins of less than 15 kDa,³⁶⁻⁴³ resulting in high-resolution structures for proteins in this size range.⁴⁴⁻⁴⁹ With the continued development of SSNMR methods, and analysis protocols designed specifically to address larger proteins,⁵⁰ we anticipate that a similar degree of completeness and structural quality will be achieved for substantially larger proteins including DsbA, DsbB and the DsbA-DsbB complex.

Therefore the study here presents both responses to longstanding technical challenges and new opportunities for investigation into fundamental aspects of *E. coli* disulfide bond formation.

Results and Discussion

Evaluation of Nanocrystalline DsbA Samples by SSNMR

We prepared three types of DsbA samples for SSNMR studies (see Materials and Methods): (1) U- ^{13}C , ^{15}N -labeled DsbA (U-DsbA); (2) 2- ^{13}C -glycerol, ^{15}N DsbA (2-DsbA), and (3) 1,3- ^{13}C -glycerol, ^{15}N DsbA (1,3-DsbA). The U-DsbA samples were used initially to evaluate spectral resolution and sensitivity as a function of sample preparation protocol; principally the details of the preparation influence the quantity of protein that can be packed into the rotor, thereby influencing sensitivity (*vide infra*). However, the one-dimensional (1D) ^{13}C magic-angle spinning (CP-MAS) spectra (Figure 1) in all cases demonstrated the

high resolution exemplified here. Outlying resonances—including carboxylates (Asp, Glu), aromatics (Tyr), Thr CA, and Ile CD1—are prominently observed even in the U-DsbA sample (Figure 1a), and resolution is greatly enhanced in the 1,3-DsbA (Figure 1b) and 2-DsbA (Figure 1c) samples. As previously demonstrated in a number of studies,^{44,48,51,52} the sparse ^{13}C labeling with glycerol improves linewidths throughout the entire protein, with particular notable resolution enhancement among the aromatics, the C α region (45 to 70 ppm) of the 2-DsbA spectrum and the methyl region of the 1,3-DsbA spectrum. Intensity patterns are qualitatively consistent with the previously reported labeling percentages.^{44,53} Notably, Tyr CZ sites (~155-160 ppm) are not labeled to any significant extent in the 2-DsbA sample, revealing instead the unique Arg guanido CZ signals in this region of the spectrum. Conversely, the Asp and Glu sidechain carboxylate signals are very weak in the 1,3-DsbA but prominently observed in the 2-DsbA sample. Patterns of aromatic signal intensity (from ~110 to ~140 ppm) are also substantially different between these two samples. Overall the ^{13}C 1D spectra demonstrate the potential for performing complete assignments.

Likewise, the ^{15}N 1D spectrum (Figure 2) shows good chemical shift dispersion for the amide signals (~100-140 ppm), including several resolved signals in the downfield (~130-140 ppm) and upfield (~100-108 ppm) regions. Chemical shifts characteristic of His, Pro, Arg and Lys residue types are also prominent. For example, the strongly downfield shifted signal at ~156 ppm is presumably due to Pro amide site (and confirmed based on TEDOR spectra, *vide infra*), and further downfield a set of three signals at ~170-175 ppm arises from His imidazole ^{15}N sites. The characteristic Arg sidechain chemical shifts at ~85 and 70 ppm are observed for the NH and NH₂ groups, respectively. Finally, the sidechain NH₃⁺ groups of Lys residues range from ~30 to ~34 ppm, with several individually resolved signals among the 17 expected. Overall the chemical shift dispersion of the ^{15}N spectrum is more than adequate for performing backbone walk analysis with 3D data sets, and it is notable that such a variety of spectral signatures are evident even in simple 1D spectra.

Beyond demonstrating that the resolution in the 1D spectra for both ^{13}C and ^{15}N was promising for assignment studies, we utilized the absolute signal intensity of 1D spectra to assess sensitivity. We measured the signal intensity of the direct (Boltzmann) polarization (DP) with Bloch decay ^{13}C spectra utilizing a very long pulse delay (~10 s). We then compared this intensity to that observed from standard compounds of known quantity, and determined that in each of the three samples shown here, the total quantity of nanocrystalline protein was ~18 mg, corresponding to almost one micromole, in a volume of ~30 μL (~80% of the coil length) in limited speed 3.2 mm rotors (Varian, Palo Alto, CA). Cross polarization (CP) enhancements for the ^{13}C spectra, relative to DP, were approximately two (slightly higher for protonated sites, slightly less for carbonyls). Thus high quality 2D spectra could be obtained in a few hours and 3D spectra in a few days or less, at 500 and 750 MHz ^1H frequencies.

Experimental Optimizations for Amino Acid Type Assignment

Two-dimensional (2D) ^{13}C - ^{13}C correlation spectra were acquired to assess the achievable resolution and numbers of resolved spin systems. The CC 2D spectrum acquired with short mixing (25 ms of homonuclear ^{13}C - ^{13}C DARR54 mixing (Figure 3) allows for a few initial assignments of outlying resonances including Ala, Gly, Ile, Ser, Thr, and Val residues that yield unique and well-resolved chemical shift patterns. Within these specific amino acid types, the expected distribution of ^{13}C chemical shifts due to secondary structure is observed.⁵⁵⁻⁵⁷ For example, in the Ala CB-CA region of the CC 2D spectrum most of the signals resonate near (18, 55) ppm, but there are also four outlier peaks with CA shifts of 52 and 50 ppm and CB shifts of 25.5, 19 and 17.5 ppm. These intensities correlate well with previously published structures^{10,18,19} where, of the 17 total Ala residues in DsbA, three

reside in turns and one in a β -sheet (the outliers), and the other 12 reside in α -helices. However, even with the assignments by amino acid type, and a handful of site-specific assignments of outlier peaks, the majority of the sequence still remained unassigned after initial analysis. In order to complete site-specific assignments for a protein of this size, additional resolution *via* 3D experiments and/or sparse isotopic labeling are essential.

First we examine the use of sparse labeling. Preparing samples with 2- ^{13}C -glycerol as the sole ^{13}C source substantially enhances resolution for CA-CB correlations of Val residues, and CB-CG of Leu. Although the methyl regions for Val are generally well resolved even in the U-DsbA sample, overlap within the common frequency range of Val CA and Thr CA/CB leads to ambiguity regarding the amino acid type identification. We addressed this by collecting complementary data sets with the 2-DsbA sample, in which the Val CA-CB correlations are observed with very high sensitivity at moderate (~ 50 ms) mixing times (Figure 4). This enabled confident identification of the Val CA chemical shifts, which in turn were used to identify Val CA-CG1/2 correlations in the U-DsbA 2D ^{13}C - ^{13}C data set. We found also that Ile CA-CB signals appeared with significant intensity, because the Ile CB is labeled to nearly 100% and the CA site is $\sim 50\%$ labeled. However, no other amino acid CA-CB correlations are observed in the range $F_1=55\text{--}70$ ppm, $F_2=30\text{--}40$ ppm, and so we were able to identify the majority of Val and Ile CA-CB correlations with high reliability. In the same spectrum, the region $F_1=36\text{--}46$ ppm, $F_2=22\text{--}32$ ppm contains exclusively Leu CB-CG and Ile CB-CG1 correlations, which can be distinguished from one another based on the characteristic CB shifts of these two amino acid types. Thus, the 2D ^{13}C - ^{13}C spectrum of the 2-DsbA sample at moderate mixing times gives unambiguous identification of Val, Ile and Leu residues, and portions of the Pro spin systems.

Further confirmation of Pro signal patterns was achieved using TEDOR pulse sequences^{58,59} in combination with the 2-DsbA and 1,3-DsbA samples. Although in general we find that ^{15}N - ^{13}C correlations are weaker with TEDOR than SPECIFIC CP, Pro residues are an exception; i.e., the TEDOR spectra (Figure 5) yield stronger ^{15}N - ^{13}C cross peaks for Pro because the initial CP step is from directly bonded protons to ^{13}C , rather than a longer range ^1H to ^{15}N transfer. Likewise, TEDOR experiments can be beneficial in the context of intermediate timescale molecular motion, where rotating frame relaxation times are shortened; empirically, we observe that some Gly ^{15}N - ^{13}C correlations are stronger in the TEDOR spectra than with SPECIFIC CP, particularly when short (~ 1.4 ms) TEDOR mixing times are utilized (Figure 5a). Gly residues often occur in turns and loops where dynamics on the microsecond to millisecond timescale are anticipated, and preliminary ^1H - ^{15}N and ^1H - ^{13}C order parameter measurements (data not shown) are consistent with this trend. In addition, 2D TEDOR spectra were acquired on both samples at longer mixing time (5.76 ms, Figure 5b) to identify sequential correlations involving Pro and Gly residues. At 1.44 ms mixing time, the spectrum contains primarily directly bonded $^{15}\text{N}[i]\text{--}^{13}\text{C}[i\text{--}1]$ and $^{15}\text{N}[i]\text{--}^{13}\text{CA}[i]$ pairs, which have maximum intensity at this mixing time. In addition, at 5.76 ms mixing, two-bond correlations ($^{15}\text{N}[i]\text{--}^{13}\text{C}[i]$, $^{15}\text{N}[i]\text{--}^{13}\text{CB}[i]$, and $^{15}\text{N}[i]\text{--}^{13}\text{CA}[i\text{--}1]$) are observed with similar intensity (Figure 5b). These correlations enable partial sequential assignments of the Pro and Gly residues in particular, which tend to have well dispersed ^{15}N chemical shifts but relatively weak signals in the 3D spectra discussed below. Pairwise assignments of all Pro (7 total) and Gly residues (13 total) were made with TEDOR spectra acquired on the 2-DsbA sample, to complement the amino acid type identifications of Val, Ile, Leu from the CC 2D on 2-DsbA and Ala, Ile, Ser and Thr from U-DsbA.

A remaining challenge is to resolve the sidechain resonances of Asx and Glx residues, requiring the observation of one-bond correlations with minimal interference from two- or three-bond correlations from a variety of other amino acid types with CB or CG resonances

in the same chemical shift range. In this context, the resolution and sensitivity of 2D homonuclear correlation spectra was greatly increased by the CTUC-COSY experiment, 60,61 which utilizes scalar polarization transfers with selective decoupling, constant time evolution and fast MAS, in order to improve resolution and reduce the number of observed peaks compared to dipolar experiments for U-DsbA (Figure 6). Moreover, this transfer pathway is unaffected by motion within the protein, allowing for previously weak signals to be observed due to the increased sensitivity (by 2 or 3 times compared with dipolar experiments). Since the evolution periods are constant-time, the chemical shift evolution and scalar coherence transfer occur during the same time interval, so relaxation is minimized and scalar decoupling can be achieved of the passive spins. This results in overall better resolution and sensitivity than the analogous DARR experiment. Thus, the CTUC-COSY IPAP spectrum yielded ~189 peaks in the CACO region, of which ~155 (out of 189 expected) are C α -C' correlations and ~34 (out of 45 expected) are side-chain carbonyls of Asn, Asp, Gln, and Glu residues. These amino acids, containing carbonyl side chain signals, are especially difficult to assign using non-selective dipolar mixing methods. Yet the CTUC-COSY still resolved 80% of the CG resonances of the glutamic acid and glutamine residues and 70% of the CD resonances of the aspartic acid and asparagines residues, in contrast to the dipolar spectra in which only a few were resolved.

Another challenge of identifying amino acid types in DsbA is the degeneracy inherent to the aromatic regions. To address this, we used a combination of selective labeling to increase sensitivity and resolution in this region, along with experiments utilizing long mixing times to ensure polarization transfer throughout the aromatic ring. Some aromatic residues could be identified solely from CC 2Ds with medium to long mixing DARR times. However, this region is sometimes compromised by overlapping carbonyl and/or (weak) aliphatic sidebands, as well as relatively poor ^1H - ^{13}C CP efficiency for the aromatics, so the additional sensitivity from the glycerol samples proved especially valuable. Among the experiments performed, we found that the NCACX 3D on the 2-DsbA (with 225 ms mixing, Figure 7) and NCOCX 3D 1,3-DsbA (with 250 ms mixing) were most effective in making unique identifications of aromatic amino acid spin systems. The same data sets allowed us to identify sequential CA[i]-CA[$i\pm 1$] correlations, which in some cases provided unique pairwise assignments based on the unique sets of CA chemical shifts and knowledge of amino acid types. Using these 2D and 3D data (*vide infra*), we assigned 10 of 11 Phe, 1 of 3 His, 7 of 9 Tyr, and both Trp residues site-specifically.

Sequential Assignment

In addition to the aromatic assignments, a good fraction of sequential assignments in general could be performed directly from CC 2D spectra with long (~200-300 ms) DARR mixing times on the 2-DsbA and 1,3-DsbA samples. For example, the CA-region of the CC spectrum acquired on 2-DsbA with 250 ms DARR mixing (Figure 8) yields strong cross-peaks for neighboring CA sites, especially in cases where both amino acids are labeled at a high percentage, such as the Gly-Ala, Gly-Val, and Phe-Val pairs. However, there were a significant number of interresidue correlations that we expected to observe, but did not; e.g., Gly-Lys pairs are expected, since the Gly CA is 100% labeled, and the Lys CA should be approximately ~50% labeled. This could reflect variations from the anticipated labeling percentage within the Lys residues, dynamics of the sidechain, or the relatively large chemical shift difference between Gly and Lys, which would reduce the overlap of the zero quantum lineshapes and thereby reduce DARR transfer efficiency.^{54,62} In some cases, resolution and chemical shift patterns were sufficiently distinct that unique assignments could be made from the CC 2D spectrum alone. However, to confidently site-specifically identify backbone correlations required 3D data sets to complete a formal backbone walk.

We performed NCACX, NCOCX, and CAN(CO)CX 3D experiments to establish sequential correlations through the common amide ^{15}N and CA ^{13}C resonances (the latter only in the case of the CAN(CO)CX). The NCACX and NCOCX spectra were acquired with 25 and 35 ms of DARR mixing respectively, requiring approximately 50 hrs signal averaging time to observe the majority of sidechain ^{13}C signals (spectra were processed in 12.5 hr blocks for evaluation). The CAN(CO)CX 3D experiment with 45 ms DARR mixing required significantly longer measurement time (~120 hrs) due to the ~50% signal intensity loss of the additional (CA to N) polarization transfer event. Using these three experiments, site-specific *de novo* assignments were performed as a backbone walk (Figure 9) as utilized in previous studies.^{28,31,33-35,37,38,40-43,63} Approximately 70% of the backbone was assigned uniquely from these data sets. However, some sites in the protein have severely weakened signal intensities (presumably due to dynamics), complicating completion of the assignment of aromatic and Lys residues, of which DsbA has a large number (31 aromatic and 17 Lys). Moreover, these amino acid types display weaker sidechain crosspeak intensities than residues with fewer sidechain ^{13}C sites. Identifying these residues unambiguously in sequential backbone walks therefore required a similar set of 3D experiments with the 2-DsbA and 1,3-DsbA samples; with these experiments, the majority of the remaining sets of correlations could be identified. The CAN(CO)CX 3D experiment and the related CANCOCA 4D proved highly valuable for addressing the large number of residues with ^{15}N frequencies of ~120-121 ppm. Even in the well digitized 3D experiments, there is still significant overlap in the CX dimension for both the CA region, from ~55-60 ppm, and the methyl region, from ~15-25 ppm, in these spectra. As shown in the A77 and V78 CA regions of these strips, near the congested ^{15}N frequencies, the CA signals are not resolved from other CA signals in the same plane, complicating a precise assignment of chemical shift values to both the ^{15}N and the ^{13}C signals for these residues.

To address this severe overlap in the central region of the N-CA plane, we performed a 4D CANCOCA64 experiment with the U-DsbA sample. The uniform labeling is critical to this experiment, since the likelihood of all three ^{13}C sites being labeled in either glycerol sample would be <10%. Initially we performed the 4D experiment with broadband mixing prior to the ^{13}C detection, but concluded after ~100 hrs that this approach would not yield sufficient sensitivity in a feasible timeframe; so instead, we utilized rotational resonance tickling⁶⁵ for the final CO-CA transfer. This pulse sequence element resulted in nearly 50% efficiency of CO-CA transfer, compared to approximately 10-20% efficiency when using DARR mixing at moderate (~10 to 25 ms) mixing times, thereby at least doubling the sensitivity per unit time, and in some cases (such as aromatics, Lys, and other large residues) we found even greater benefit. The reduction in signal transfer to sidechain ^{13}C sites not only provided additional sensitivity, but simplified interpretation by eliminating the majority of CB resonances (with some exceptions such as Thr, which were already assigned) and correlations of the type CA[i]-N[i]-CO[i-1]-CA[i], which led to additional overlap among those sites observed in the broadband experiment. In addition to the R2T step, we utilized selective scalar decoupling in the indirect CA and CO evolution dimensions, using appropriately synchronized r-SNOB pulses.⁶⁶ Essentially every peak in the optimized CANCOCA 4D experiment was individually resolved, as illustrated by the 4D backbone walk from residue 76 to 84 in DsbA (Figure 10). This is the same stretch as shown above in the 3D backbone walk (Figure 9), and represents one of the most challenging stretches of the entire backbone to assign unambiguously. This helical region contains four CA chemical shifts over a 0.5 ppm range (54.4 to 54.9 ppm) and two amide ^{15}N signals that are with 0.2 ppm. However, the overlapping amide frequencies arise from residues with large differences in CA chemical shifts (A77 at 54.9 ppm and V78 at 67.5 ppm), and the overlapping CA shifts of A81 and L82 (at 54.4-54.5 ppm) are nicely separated by their amide frequencies of 123.6 ppm and 113.1 ppm, respectively. Similar site-specific chemical shift assignment confirmation is achieved for many of the residues in DsbA that were, from other data sets

described above, not possible to assign unambiguously. The 4D experiment also enabled unique identification of otherwise ambiguous strips in the 3D CAN(CO)CX experiment (Figure 9, in red), where the full ^{13}C side chain information is present. For example, this enabled the certain determination of methyl shifts for V78. Thus, the combination of the highly sensitive 4D experiment with the crosspeak-rich CAN(CO)CX 3D experiment permitted assignment of congested planes from the 3D experiments, allowing extensions of the backbone walks through the protein with minimal disruptions.

Although the majority of assignments were completed with the U-DsbA sample using 3D and 4D backbone walk strategies, additional assignments were confirmed with the higher sensitivity of samples prepared from 1,3- ^{13}C - and 2- ^{13}C -glycerol. We performed 3D NCOCX and NCACX spectra with mixing times sufficient to observe most interresidue correlations (~200-250 ms) (Figure 11). As with the CC 2D spectrum acquired on 2-DsbA with long mixing, not only can we assign resonances with the knowledge of backbone walk strategies for assigning neighboring sites, but the known patterns of labeling can also be used to assign new chemical shifts. The NCC spectra provide information also on medium-range correlations, observed at a single ^{15}N frequency in the NCOCX and NCACX spectra. These selectively labeled samples enable observation of longer distances due to the increased resolution and sensitivity.⁵⁰ New backbone assignments were identified and many side chain resonances confirmed in this manner. For example, at the F112 ^{15}N frequency (120.3 ppm), complementary correlations are observed in the two NCC spectra. The NCOCX (Figure 11, **red**) contains correlations from the F112N-V111C (F1-F2) to the V111 side chain resonances, as well as to its neighbors, F112CB and D110CA and CB. The carbonyl region in this spectrum reveals medium-range correlations (from V111C to N114C and CG). The NCACX (Figure 11, **blue**) provides the alternate labeling, revealing correlations that arise from F112N-F112CA followed by mixing not only to the F112 sidechain, but also to the V111CB. Differences in peak intensity among cross peaks arise due to the known labeling patterns, e.g., the V111CB is fully labeled and the F112CB is only partially labeled in the 2-DsbA sample. The labeling pattern also accounts for why no carbonyl or Val methyl resonances are observed in this NCACX plane, since the likelihood of carbonyl and methyl labeling is rare in the 2-DsbA sample.

Comparison with solution NMR chemical shifts

Concurrently with the solid-state NMR assignments, we confirmed solution NMR backbone assignments, utilizing the previously published results²⁰ in combination with HNCACB, HNCO, HN(CO)CACB, and an HSQC spectra acquired (at 750 MHz) on an exact replica sample of oxidized DsbA that we used for SSNMR studies. We found these experiments to be necessary in order to determine the extent to which sample-to-sample variations might impact the chemical shifts. The solution assignments generally agree very well with the backbone (^{15}N , ^{13}CA , ^{13}CB , and $^{13}\text{C}'$) solid-state NMR chemical shift assignments (Figure 12). Regression analysis of the entire set of chemical shifts of each type yields R^2 values ranging from 0.938 to 0.999, with the best agreement for CA and CB shifts, which are sensitive primarily to conformation, and slightly poorer agreement for N and C' , which depend more strongly upon hydrogen bonding and electrostatics. Similar trends have been observed in previous SSNMR studies, including our extensive investigations of GB1.^{42,67-70} Specifically, we have shown for GB1 that the solid-state chemical shifts are extremely sensitive to changes in environment of the preparation, including crystal contacts, nearby solvent molecules and protonation states (which may differ in the solid versus solution).^{42,69} Based on these investigations of a very well characterized protein, we would expect in general that some statistically significant variations between the solution and solid-state chemical shifts are likely to be present. Therefore it is not surprising that we observe outliers in DsbA; in the majority of cases, the outlying residues are in common with those

residues (such as E38, G65 and M64) that have been previously reported to show different chemical shifts between the oxidized and reduced forms of DsbA using solution NMR.²⁰ In addition, we expect that residues A1, Q2, Y3, G66 and V90 vary due to their proximity to crystal contacts. For example, G66 CA is 5 Å from the Y184 CA on the neighboring molecule, in the case of the crystallographic symmetry of PDB entry 1DSB. Additionally, intermolecular contacts can be observed between V90 CA and A1 CA (5.3 Å), as well as more distant crystal contacts between V90 and Q2 (6.7 Å). Overall the agreement between the solid-state NMR chemical shifts of DsbA, which we determined entirely *de novo*, and the solution chemical shifts shows the promise of combining solution and solid data sets, for example, to refine the resolution of crystal structures determined at moderate resolution.

The availability of complete ¹³C and ¹⁵N chemical shifts for DsbA in the nanocrystalline state will facilitate SSNMR dynamics studies and chemical shift perturbation mapping of the DsbA in complex with its membrane bound partner DsbB, for which both solution²⁵ and solid^{27,28} assignments are available. Mutation of the active site residue C33 in DsbA allows the formation of a covalent disulfide-bonded complex with wild type DsbB, which is believed to be representative of the structure of a transient intermediate in oxidation of DsbA by DsbB. Initial studies completed on the precipitated U,¹³C, ¹⁵N-DsbA C33S-n.a.DsbB complex have shown that the majority of backbone signals for DsbA do not shift from the crystalline form of oxidized DsbA to the complex containing the DsbA C31-DsbB C104 disulfide bond (Figure 13). Therefore most of the chemical shift assignments can be tentatively inferred from the chemical shifts of DsbA on its own. However, there are approximately 5% of chemical shifts that are observed in the spectra of the U,¹³C,¹⁵N-DsbA*-[n.a.]DsbB complex that have shifted greater than 0.5 ppm or appear to be new peaks altogether. Based upon the inferred assignments, it appears that most of the chemical shift changes are residues located in the interface of DsbA and DsbB or residues that experience changes in crystal contacts from one preparation to the other. Although the complete assignment of DsbA in the complex is beyond the scope of the current study, this initial 2D ¹³C-¹³C spectrum illustrates the potential of SSNMR-based structure determination methods to tackle large membrane protein complexes, where high-resolution data can be acquired despite the lack of single crystal preparations.

In summary, we used a wide array of techniques to obtain full chemical shift assignments of the 21 kDa enzyme DsbA and find excellent agreement with solution NMR values. We expect that these strategies for solid-state chemical shift assignment can be applied to other large proteins, especially those embedded in membranes, for which SSNMR is uniquely positioned to provide data to bridge gaps between crystallography and solution NMR. The methods discussed here for enhancing sensitivity while retaining high resolution will be key to assigning chemical shifts in large proteins by SSNMR.

Materials and Methods

Sample Preparation

Expression plasmids for wildtype DsbA, DsbAC33S, and His-tagged “wildtype” DsbB containing mutations of nonessential cysteines (C8A, C49V) were obtained from K. Inaba and K. Ito.^{71,72} For preparation of uniformly-labeled ¹³C, ¹⁵N DsbA, we used the following method, modified from Marley *et al.*⁷³ Freshly transformed *E. coli* C43 (DE3)/pREP4 cells were grown to A₆₀₀ of 0.8 in LB medium containing 60 µg/ml ampicillin, 25 µg/ml kanamycin and harvested in a sterile manner. The cells were then resuspended in 0.25 volume of uniform-labeling medium (50 mM Na₂HPO₄, 50 mM KH₂PO₄, 5 mM Na₂SO₄, 2 mM MgSO₄, 10 µg/ml thiamine, 10 µg/ml biotin, 0.3% ¹⁵N-NH₄Cl, 0.4% U-¹³C glucose, 10 ml/L ¹³C ¹⁵N BioExpress, and trace elements), with 60 µg/ml ampicillin. Trace elements consisted of 10 µM FeCl₃, 4 µM CaCl₂, 2 µM MnCl₂, 2 µM ZnSO₄, and 0.4 µM each of

CoCl₂, CuCl₂, NiCl₂, Na₂MoO₄, Na₂SeO₃, and H₃BO₃.74 Flasks (2L), with a culture volume of 250 ml, were shaken at 250 rpm for 1 hr at 37 °C. At this point, expression was induced with 0.8 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and growth continued under the same conditions for 16 hrs until harvest. DsbA was released from the cells using osmotic shock, and purified by QFF anion exchange chromatography (GE Healthcare, Piscataway, NJ) in 10 mM MOPS pH 7.75. The protein eluted at 30-50 mM NaCl. The yield of DsbA was over 100 mg protein/L of culture.

For preparation of partially ¹³C-, uniformly ¹⁵N-labeled DsbA, the uniform-labeling medium was used with the omission of glucose and Bioexpress, and the addition of 0.2% 2-¹³C glycerol and 9 mM Na¹³CO₃ (or 0.2% 1,3-¹³C glycerol and 9 mM Na¹²CO₃).⁵¹ The medium was inoculated directly with 2 ml/L of LB culture, grown to A₆₀₀= 0.8, induced with IPTG, and harvested after 5 hrs. Yield of DsbA was ~15 mg protein/L of culture. U-¹³C glucose, 2-¹³C-glycerol, 1,3-¹³C-glycerol, ¹⁵N-NH₄Cl, and U-¹³C ¹⁵N BioExpress were obtained from Cambridge Isotope Laboratories, Andover, MA.

To prepare nanocrystalline precipitant for packing into an NMR rotor, one volume of DsbA at 45 mg protein/ml in 25 mM MOPS, pH 7.0 was mixed with one volume of crystallization buffer (30% PEG 8,000, 50 mM cacodylate pH 6.5, and 1.5% 2-methyl-2,4-pentanediol, modified from Martin *et al.*⁷⁶ This solution was loaded into several 200 or 300 μl dialysis buttons, and covered with a 3,000 MWCO dialysis membrane. The buttons were immersed in ~15 ml of undiluted crystallization buffer, which was gently stirred overnight at 4°C. This treatment produced a shower of nanocrystals, which were harvested by centrifugation and packed into a NMR rotor.

To prepare U-¹³C ¹⁵N labeled DsbA in a covalent complex with natural abundance DsbB, DsbB was expressed in *E. coli* C43 (DE3) in Luria-Bertani medium containing 2 mM MgSO₄ and 90 μg/ml ampicillin. Induction of expression, membrane isolation, protein solubilization and purification were performed as described previously for isotopically labeled DsbB.^{27,28} The concentration of DsbB was determined by titration with DsbA C33S, with complex formation monitored at 500 nm.⁷² The sample for SSNMR was then prepared with a substoichiometric amount of labeled DsbA C33S, so that all would be covalently bound. The sample was concentrated, dialyzed for 2 days against 25 mM Tris, pH 8, and centrifuged for 1 h at 100,000 × g to remove large aggregates of contaminating protein and lipid. The supernatant was then centrifuged for 20 h at 100,000 × g to pellet the dark red DsbA/DsbB complex, which was packed into a NMR rotor.

SSNMR Spectroscopy

Experiments were performed on an 11.7 Tesla (500 MHz ¹H frequency) Varian Infinity Plus spectrometer and a 17.6 Tesla (750 MHz ¹H frequency) Varian Unity Inova spectrometer equipped with Varian Balun^{TM} ¹H-¹³C-¹⁵N 3.2-mm probes. Nanocrystals of oxidized DsbA were packed into 3.2-mm rotors with ~6-9 mg protein in standard wall rotors and ~18-20 mg in thin wall rotors. All data was acquired at 0±3 °C actual sample temperature (determined by ethylene glycol calibration).⁷⁷ All experiments utilized tangent ramped cross polarization⁷⁸ with TPPM⁷⁹ decoupling of the protons applied during acquisition and evolution periods on average at ~80 kHz. For 3D ¹⁵N-¹³C-¹³C and ¹³C-¹⁵N-¹³C correlation experiments, band-selective SPECIFIC CP80 was used for polarization transfer between ¹⁵N and ¹³C. The details of the 4D experiments closely followed Franks *et al.*,⁶⁴ with the exception of the r-SNOB pulses, which followed the recipe of Li *et al.*⁶⁶ Other experiments were performed according to published procedures as cited in the main text. Hard π/2 pulse widths were typically 3.0 μs for ¹H and ¹³C, and 4.5 ms for ¹⁵N.

Spectra were processed with nmrPipe,⁸¹ employing zero filling and Lorentzian-to-Gaussian line broadening for each dimension before Fourier transformation. Back linear prediction and polynomial baseline correction were applied to the frequency domain in the direct dimension. Chemical shifts were referenced externally with adamantane (assuming the downfield peak to resonate at 40.48 ppm).⁸² Additional experimental details are listed in the figure captions. Peak picking and assignments were performed with Sparky.⁸³

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

CP-MAS	cross polarization magic-angle spinning
DARR	dipolar assisted rotational resonance
MAS	magic-angle spinning
SSNMR	solid-state nuclear magnetic resonance
TEDOR	Transferred Echo Double Resonance

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Figure 1.

^{13}C CP-MAS spectra (1,024 scans each, -10°C , 12.500 kHz MAS, 750 MHz ^1H frequency) of nanocrystalline, oxidized DsbA, isotopically labeled with (a) uniform- ^{13}C , ^{15}N (b) 1,3- ^{13}C -glycerol, and (c) 2- ^{13}C -glycerol. Spectra were zero filled by a factor of four and Fourier transformed without apodization.

Figure 2.
 ^{15}N CP-MAS spectrum of U- ^{13}C , ^{15}N -DsbA (4096 scans, -10°C , 11.111 kHz MAS, 500 MHz ^1H frequency). The spectrum was zero filled and Fourier transformed without apodization.

Figure 3. $U\text{-}^{13}\text{C},^{15}\text{N}$ -DsbA 2D ^{13}C - ^{13}C correlation spectrum, 25 ms DARR mixing. The spectrum was acquired for 30 hrs (-10°C , 12.500 kHz MAS, 750 MHz), zero filled and apodized with Lorentzian-to-Gaussian functions, resulting in net broadening of 15 Hz and 35 Hz in the direct (F2) and indirect (F1) dimensions, respectively.

Figure 4. Selected regions of the 2D ^{13}C - ^{13}C spectrum of 2-DsbA, illustrating the (a) Val and Ile CACB and (b) Leu CB-CG and Ile CB-CG1 signals (50 ms DARR mixing, 5 hrs, -10°C , 12.5 kHz MAS, 750 MHz). The spectrum was zero filled and apodized with Lorentzian-to-Gaussian apodization with net broadening of 20 Hz and 25 Hz in the direct (F2) and indirect (F1) dimensions respectively.

Figure 5.

2D ZF-TEDOR spectrum of 2-DsbA with (a) 1.44 ms of mixing (4.5 hrs measurement time) and (b) 5.76 ms mixing (7 hrs measurement time) acquired at -10°C with 11.111 kHz MAS rate at 500 MHz ^1H frequency. TEDOR spectra yield strong Gly and Pro intrasidue correlations at short mixing time and interresidue N-CA correlations at ~ 6 ms mixing time.

Figure 6.

Comparison of DARR and IPAP CTUC COSY experiments for carbonyl region correlations. (a) 2D ^{13}C - ^{13}C spectrum with 25 ms DARR mixing (750 MHz, 12.5 kHz MAS) and (b) CA-CO optimized CTUC COSY IPAP spectrum (500 MHz, 22.222 kHz MAS, 125 kHz TPPM decoupling).

Figure 7.

CA-CX planes from an NCACX 3D spectrum with 225 ms DARR mixing acquired on 2-DsbA for 128 hrs on a 500 MHz (^1H frequency) Infinity Plus spectrometer at -10°C with 11.111 kHz MAS rate. The spectrum was processed with 75, 75, and 60 Hz Lorentzian-to-Gaussian apodization in F3, F2, and F1 respectively.

Figure 8.

CA-CA region of the 2D ^{13}C - ^{13}C DsbA spectrum with 250 ms DARR mixing acquired for 12 hrs at -10°C (12.5 kHz MAS rate, 750 MHz). The spectrum was zero filled to 16384 and 8192 and processed with 45 Hz and 45 Hz of Lorentzian-to-Gaussian apodization in the direct and indirect dimensions respectively.

Figure 9.

Strip plot of NCACX in blue (20 hrs, 25 ms mixing), NCOCX in red (14 hrs, 35 ms mixing), and CAN(CO)CX in orange (120 hrs, 45 ms mixing) acquired at -10°C with 11.111 kHz MAS rate on an Infinity Plus 500 MHz (^1H frequency) spectrometer on U-Dsba.

Figure 10. Strip plot of CANCOCA 4D (where R2T was employed for the CO-CA transfer) acquired for 115 hrs at -10°C with 9.090 kHz MAS rate on an Infinity Plus 500 MHz (^1H frequency) spectrometer on U-DsbA.

Figure 11.

Strip plot of the F112 ^{15}N frequency in a NCOCX (red, 250 ms, 112 hrs) 3D spectrum acquired on 1,3-DsbA and a NCACX 3D (blue, 225ms, 128 hrs) spectrum acquired on 2-DsbA on a 500 MHz (^1H frequency) Infinity Plus spectrometer at -10°C with 11.111 kHz MAS rate. The NCOCX and NCACX spectra were processed with 25, 25, and 15, and 40, 50, and 20 Hz Lorentzian-to-Gaussian apodization in F3, F2, and F1 respectively.

Figure 12. (a) $^{13}\text{C}\alpha$, (b) ^{15}N , (c) $^{13}\text{C}\beta$, and (d) $^{13}\text{C}'$ solid-state versus solution state chemical shift assignments of wild type oxidized DsbA. Labeled residues are for those that differ by greater than 1.5 ppm.

Figure 13.
2D ^{13}C - ^{13}C spectrum of U- ^{13}C , ^{15}N -DsbA/(n.a.)DsbB, acquired with 25 ms DARR mixing for 126 hrs at -20°C (12.5 kHz MAS rate, 750 MHz). The spectrum was zero filled to 16384 and 8192 and processed with 20 Hz and 20 Hz of Lorentzian-to-Gaussian apodization in the direct and indirect dimensions respectively.