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Biomolecular Solid-State NMR

Solid-State NMR Spectroscopy of a Paramagnetic Protein: Assignment and Study of Human Dimeric Oxidized Cu^{II}–Zn^{II} Superoxide Dismutase (SOD)**

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Solution NMR studies of paramagnetic systems are well-developed.[1] Paramagnetic centers originate from unpaired electrons that are intrinsic features of many transition-metal ions. A paramagnetic center changes the appearance of the NMR spectrum in several ways, most obviously by altering chemical shifts and increasing relaxation rates. On one side, these changes constitute a unique, direct probe of the electronic structure of the metals in these systems.[2] On the other hand, as the paramagnetic effects depend in a well-defined manner on the structure of the molecule, they provide a variety of structural restraints in the determination of the molecular geometry.[3] Paramagnetic phenomena thus provide information on electronic structure, protein–protein or protein–nucleic acid docking, ligand binding, solvent mapping, and the flexibility of multidomain proteins. However, NMR studies of paramagnetic molecules are often hindered by the very same large hyperfine effects, and mostly by the paramagnetically enhanced nuclear relaxation,[1] which undermines the acquisition of the NMR experiments.

Magic angle spinning (MAS) ^{13}C NMR spectroscopy in the solid state has recently made significant advances, thereby paving the way to a variety of assignment strategies and 3D structure determinations of macromolecules.[4] Nevertheless, the higher complexity of the paramagnetic interactions in crystals has so far limited the NMR study of paramagnetic molecules,[5] and paramagnetic proteins have barely been studied by solid-state NMR.[6] Herein, we present the first detailed investigation of a uniformly labeled protein containing a paramagnetic metal center. We have recorded NMR correlation spectra and obtained the complete assignment in the solid state. Contrary to the solution case, we notably find that the observation of correlations is minimally affected by paramagnetic relaxation, and we observe most of the contributions from nuclei that are close to the metal center.

As a benchmark, we studied the oxidized form of human copper–zinc superoxide dismutase (SOD). SOD is a dimeric enzyme of about 32 kDa containing two identical and symmetric units of 153 residues.[7] SOD has been thoroughly studied in the past, with several X-ray[8] and NMR solution structures[9] available of the enzyme and of several mutants.

Owing to the long electronic T_1 relaxation times of the so-called type II copper center, however, the oxidized form is virtually inaccessible to traditional solution NMR studies, with ^1H NMR resonances of protons that are closer than 12 Å from the metal ion broadened beyond detection.[10] Since the paramagnetic broadening depends on the square of the gyromagnetic ratio of the observed nucleus, ^{13}C or ^{15}N spins that are close to the metal ion are easier to detect by NMR than ^1H spins.[11] For example, in the case of a monomeric analogue of SOD, ^{13}C spins that are as close as 5 Å to the metal ion have been detected and assigned by solution-state NMR experiments by using a new instrumental and experimental setup tailored for ^{13}C direct acquisition.[12] In particular, as longitudinal relaxation is less influenced by the paramagnetic center, sequences based on longitudinal magnetization transfers (for example, ^{13}C–^{13}C NOESY) have proven particularly effective in studying resonances of atoms that are close to metal centers.[10,11]

Solid-state NMR is particularly suited to these latter approaches, since carbon-13 acquisition is routine, and transfer schemes based on dipolar interactions are available that allow directed, highly efficient homonuclear (^{13}C–^{13}C) and heteronuclear (^1H–^{13}C, ^1H–^{15}N, ^{13}C–^{15}N) transfers over short delays. Additionally, transfers such as proton-driven spin diffusion (PDSD)[13] or RFDR[14] can be used, during which coherence lifetimes are subject to processes that are dominated by T_1 relaxation, which are less prone than T_2 relaxation to paramagnetic enhancements.[15] In particular, in solids, ^1H–^{13}C polarization transfer is still feasible close to the metal, in

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contrast to solution. Indeed, cross-polarization transfer is efficient over contact times typically on the order of 1 ms for carbon polarization, whereas in solution longer INEPT transfer delays (5 ms) are required and interfere with the intrinsic relaxation timescale of the spin system ($T_2 = 10$ ms at 10 Å from the metal center, according to Figure S1 in the Supporting Information). Furthermore, cross-polarization transfer is mediated by the whole proton bath and thus is limited to a less extent by the large paramagnetic enhancement of the protons surrounding the metal center. As a result, one can thus afford to exploit the proton nuclei as the source of polarization, with the corresponding gain in sensitivity. Moreover, the timescale of typical spin-diffusion mixing, and any other recoupling sequence (a few hundreds of μs), minimizes the impact of paramagnetic relaxation during the pulse sequence, even close to the metal center, as compared to liquids.

A sample of ^{13}C,^{15}N-labeled, oxidized dimeric stabilized SOD (10 mg) was obtained from ProtEra srl (Sesto Fiorentino, Italy) and crystallized from a PEG solution in citrate buffer at pH 5 (Figure 1).

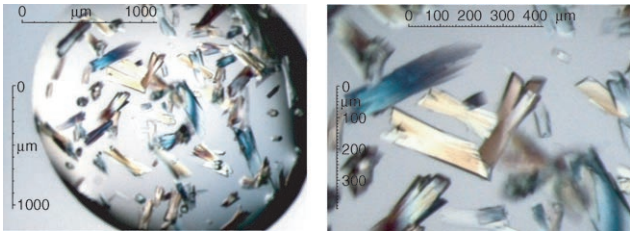

Figure 1. SOD protein microcrystals of the type used to prepare the sample for solid-state NMR experiments. The microcrystals, which were typically about 100 μm thick, were directly centrifuged at 2000 *g* into a 3.2-mm Bruker rotor by using protocol described elsewhere.[16] The sample used in this study contained about 10 mg of protein.

We recorded a series of homonuclear ^{13}C–^{13}C PDSD correlation experiments,[13] with mixing times ranging from 10 ms to 200 ms, supplemented by a refocused INADEQUATE spectrum (Figure 2).[17] Despite the very crowded nature of the spectra, the data were clear enough to allow the identification of the ^{13}C spin systems for each residue. Sequential connectivities could be determined from the inspection of 2D experiments involving ^{15}N nuclei, where ^{15}N–$^{13}C^{\alpha}$ and ^{15}N–$^{13}C^{\beta}$ cross-peaks are obtained through the combination of either inter- (NCACB, Figure 3a) or intra-residue (NCOCACB, Supporting Information) ^{15}N–^{13}C-specific transfer and the following recoupling of ^{13}C spins within each amino acid.[16] Although chemical shifts vary somewhat between the liquid and solid phases, the availability of an assigned solution-state counterpart can be very useful as a guide in assigning solid-state systems (even if variations are such that direct transfer is clearly not possible). In this case, the solution counterpart is not available for this protein in this oxidation state. However, since the copper center is only slightly anisotropic and produces mostly negligible pseudocontact shifts, the solution resonance frequencies available for the reduced (diamagnetic) dimer[9] were useful here as guidelines to aid in the whole assignment process.

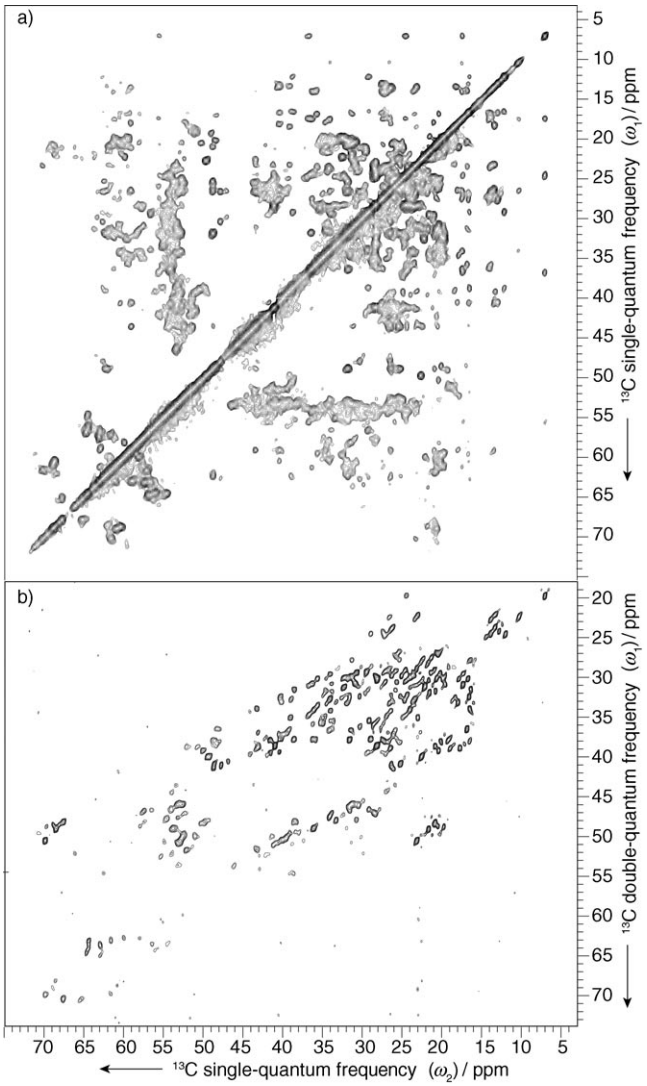

Figure 2. Expansions of the aliphatic regions of a) a ^{13}C–^{13}C PDSD spectrum of SOD recorded at 15 kHz MAS (mixing time 50 ms), and b) a ^{13}C refocused INADEQUATE aliphatic DQ correlation spectrum.

As a result, we could detect almost all the backbone and side-chain ^{13}C spins, including histidine residues coordinating to the copper center, therefore extracting information as close as 5 Å from the metal center ($^{13}C^{\alpha} = 98\%$, $^{13}C^{\beta} = 98\%$, $^{13}C^{\text{sidechain}} = 95\%$; owing to spectral crowding, the assignment of the carbonyl region requires dedicated experiments, for instance a 3D NCOCA experiment,[18] or alternatively spin-state-selective[19] or homodecoupled experiments,[20] and was not attempted here).

Figures 3b and c show two regions of the ^{13}C–^{13}C spin-diffusion spectrum which are well-resolved: the region containing C^{β}/C^{γ} correlations of aromatic residues clearly shows signals coming from phenylalanine residues 45 and 64, which are detectable on the monomer in solution only with a specifically designed experimental setup.[12] Similarly, serine residues 134 and 142 and threonine residues 116, 135, and 137,

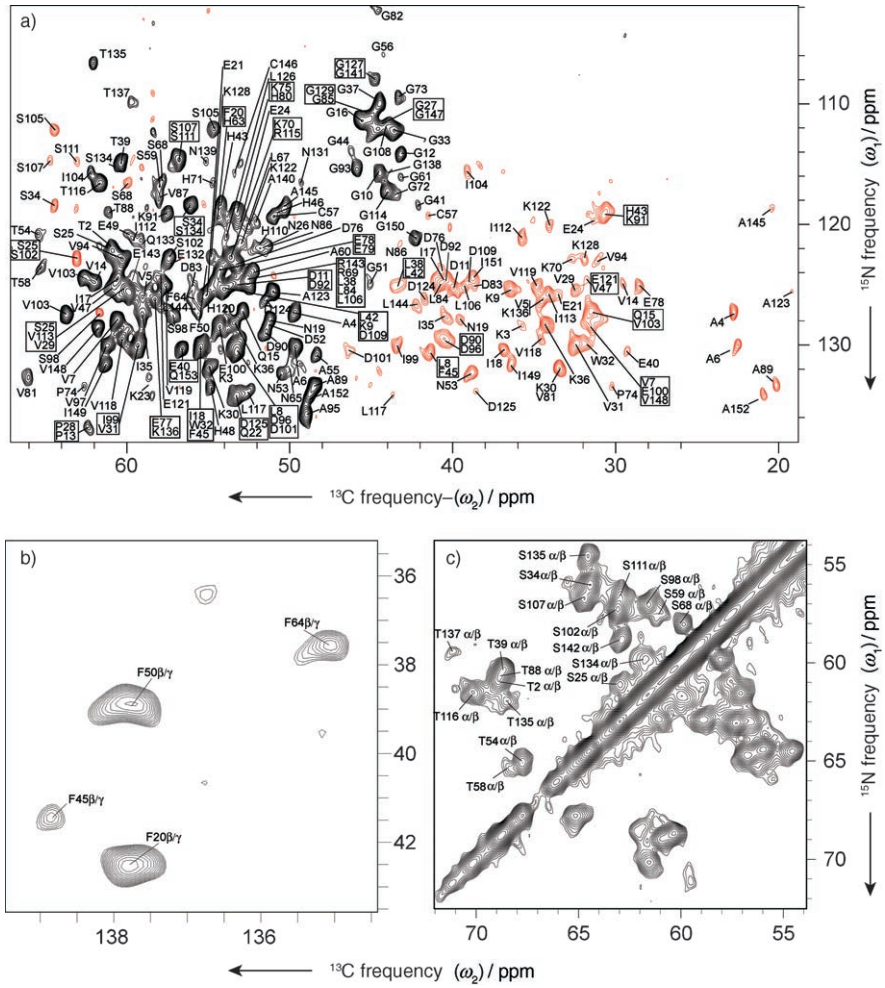

Figure 3. a) NCACB DQ correlation spectrum. Positive peaks (black contours) correspond to ^{15}N–^{13}C one-bond correlation, while negative peaks (red contours) to two-bond correlations. Signals are labeled according to their assignment. b, c) Extracts from the ^{13}C–^{13}C PDSD spectrum of Figure 2: phenylalanine C^{β}–C^{γ} region (b) and Thr/Ser C^{α}–C^{β} region (c).

which are all close to the copper ion (< 9 Å), are easily observable among all the threonine and serine C^{α}/C^{β} correlations.

In summary, it is remarkable that such a complete characterization can be achieved within a rather limited experimental time (for example, 12–24 h for each of the ^{13}C–^{13}C PDSD spectra allowing spin-system assignments). The advantage with respect to the liquid-state approach is all the more striking when we note that a ^{13}C–^{13}C NOESY spectrum requires a much longer experimental time. This characterization is highlighted by the fact that this is the first NMR study of the oxidized dimeric form of SOD, which has never been achieved in solution. This system constitutes the largest crystalline protein assigned to date.

In conclusion, ^{13}C–^{13}C homonuclear and ^{15}N–^{13}C heteronuclear NMR correlation experiments, which have recently been shown to allow sequential assignment in diamagnetic microcrystalline protein samples, provide a powerful tool for the study of paramagnetic macromolecules in crystals as presented here. The solid-state NMR approach combines carbon detection and efficient transfer of longitudinal magnetization, and is able to probe the close proximities of the metal center. Notably, in solids, the detection of signals from atoms close to the metal will open an avenue for the measurement and the exploitation of a range of anisotropic effects which are averaged out in solution, thus offering data with a potentially higher information content for the study of metallobiomolecules. Even greater impact of solid-state NMR may be expected for those systems where paramagnetic relaxation is independent from electronic relaxation and relies uniquely on molecular tumbling (Curie mechanism),[21] such as cobalt-containing proteins or lanthanide-substituted systems.

Experimental Section

See the Supporting Information for full experimental procedures about sample preparation, solid-state NMR spectra acquisition and assignment, and the list of the assigned shifts.

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