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Assignment of ^{1}H, ^{13}C, and ^{15}N signals of oxidized *Clostridium pasteurianum* rubredoxin*

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Biological context

Rubredoxins belong to a class of iron–sulfur proteins that contain one high-spin iron tetrahedrally coordinated to the sulfur atoms of four cysteine residues. The paramagnetic character of the iron center in both oxidized [Fe(III)] and reduced [Fe(II)] rubredoxin causes the NMR signals of 12 residues near the iron–sulfur cluster to be shifted away from the normal diamagnetic region, and these signals have been studied extensively in this laboratory (Xia et al., 1995; Wilkens et al., 1997). Whereas high-resolution X-ray crystal structures have been determined for Fe(III) and Fe(II) rubredoxins, no solution NMR structures have been reported. Standard methods for NMR structure determination are insufficient for highly paramagnetic proteins, and this laboratory is investigating approaches to extract enough information from both the paramagnetic (hyperfine-shifted) and diamagnetic spectra of rubredoxin for structural characterization. In this study, the 54 amino acid rubredoxin from *Clostridium pasteurianum* was produced by recombinant means in *Escherichia coli* (the recombinant protein abbreviated here as Rdx) and labeled uniformly with ^{15}N and ^{13}C for examination by multidimensional NMR methods. Extensive backbone and aliphatic side-chain resonance assignments have been determined for the diamagnetic portions of the ^{1}H, ^{15}N and ^{13}C spectra of oxidized Rdx. This approach led to assignments for 31 residues in Fe(III) Rdx (backbone ^{15}N resonances for 10 residues have not been accounted for in either the multinuclear NMR data or the hyperfine-shifted spectra).

Methods and Results

NMR samples of oxidized [*U*-^{15}N]- and [*U*-^{15}N,^{13}C]-Rdx were prepared as previously described (Xia et al., in preparation). All samples contained ~4–6 mM rubredoxin in 50 mM phosphate buffer, pH 6.0. NMR experiments were recorded with the parameters shown in Table 1 at 25 °C on Bruker DMX500 and DMX600 spectrometers equipped with triple-resonance $^{1}H/^{13}C/^{15}N$ probes and triple-axis pulsed field gradient capabilities. Quadrature detection in the indirectly detected dimensions was obtained either by the States-TPPI (Marion and Wüthrich, 1983) or echo/anti-echo method. Gradient pulses as a combination of X- and Z-gradients at the 'magic angle' (Warren et al., 1993; Van Zijl et al., 1995) were used for coherence selection in experiments with sensitivity enhancement (SE) to maximize water suppression.

All Fourier transformation of NMR data was performed with FELIX95 (Molecular Simulations, San Diego, CA, U.S.A.). For 3D data, the indirect dimension with the least amount of digitization was extended with linear prediction by no more than 50% of the orig-

*These data have been deposited in BioMagResBank (http://www.bmrb.wisc.edu) under BMRB accession number 4051.
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Abbreviations: Rdx, recombinant *Clostridium pasteurianum* rubredoxin produced in *Escherichia coli* and reconstituted with iron; HSQC, heteronuclear single quantum coherence; SE, sensitivity enhancement; INEPT, insensitive nuclear enhancement by polarization transfer.

TABLE 1
PARAMETERS FOR NMR EXPERIMENTS USED IN THE $^{1}H/^{15}N/^{13}C$ ASSIGNMENTS OF OXIDIZED RUBREDOXIN

Experiment	^{1}H			D2			D3			Matrix dimensions[c]	Mixing time[d]	BMRB[e]
	SF (MHz)[a]	SW (Hz)	N*[b]	Nucleus	SW (Hz)	N*	Nucleus	SW (Hz)	N*			
HNCO[f]	600.13	6944.44	512	$^{13}C'$	2000	60	^{15}N	2000	36	512×256×128		1
HNCACB[f]	600.13	6944.44	512	$^{13}C^{\alpha\beta}$	10000	60	^{15}N	2000	40	512×256×128		
C(CO)NH[f]	600.13	6944.44	512	^{13}C	10416.7	64	^{15}N	2000	40	512×256×128		
^{15}N TOCSY[g]	600.13	6944.44	512	^{1}H	6944.44	128	^{15}N	2000	40	512×512×128	53	3
$^{15}N/^{1}H$ HSQC	499.84	8333.33	1K	^{15}N	1666.67	256				512×512		
$^{13}C/^{1}H$ CT-HSQC	600.13	8333.33	1K	^{13}C	5000	128				1024×512		

[a] ^{1}H frequency used for this experiment. 499.84 and 600.13 denote the DMX500i and DMX600 instruments, respectively, at NMRFAM (University of Wisconsin–Madison).
[b] Number of complex points collected in this indirect dimension.
[c] Final processed matrix size.
[d] Time in ms for isotropic mixing times.
[e] BMRB pulse program library accession number.
[f] Experiments used for backbone assignments.
[g] To complete side-chain assignments.

inal data size. An 85° shifted squared sinebell window function was applied to each FID prior to zero-filling to the final matrix size, Fourier transformation, and phase correction. The initial values for incremented delays in multidimensional experiments were set in a manner that allowed predictable phasing in each dimension and minimized roll and offset of the baseline (Bax et al., 1991).

All ^{1}H dimensions were referenced to internal DSS (2,2-dimethyl-2-silapentane-5-sulfonate). ^{13}C and ^{15}N dimensions were indirectly referenced to DSS as previously described (Wishart et al., 1995). Chemical shifts for all cross peaks were tabulated with PPFLX (http://www.nmrfam.wisc.edu/roger/Software/peakpick/pp_main.html). Referenced outputs from PPFLX for each experiment could be compiled into separate databases to be searched for assignment information in a semi-automated manner. This was accomplished using PERL (Wall and Schwartz, 1991) scripts written to produce potential assignments for each cross peak by matching to the reference database of ^{1}H and ^{15}N shifts from the 2D HSQC.

Extent of assignments and data deposition

Sequence-specific assignments for oxidized rubredoxin were obtained from the HNCACB and CCONH data. Hyperfine shifts affect 12 backbone ^{15}N signals arising from the two CXXCGX motifs that provide covalent bonds to the iron (Xia et al., in preparation). No signals from these 12 residues are observed in any of the 2D or 3D NMR spectra. Assignments were obtained for residues K2-Y4, N14-W37, and E51-E54, which accounts for all but 10 of the non-hyperfine-shifted residues. The missing residues presumably experience extreme line broadening due to electron–nuclear dipolar relaxation, as all are found to be within 11 Å of the iron in the crystal structure (Watenpaugh et al., 1980). Chemical shift assignments for oxidized Rdx have been deposited at BioMagResBank under the accession number given in the first footnote on page 415; pulse sequences have been deposited under the accession numbers in Table 1.

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