

Selective ^{15}N labeling and direct observation by NMR of the active-site glutamine of Fe-containing superoxide dismutase

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Summary

The glutamine in position 69 is one of only three conserved active-site amino acid differences between Fe- and Mn-containing superoxide dismutases (SODs). We have refined the conditions for extremely selective labeling of the side chains of glutamine with ^{15}N , and thus obtained dramatically simplified spectra, despite the large size of Fe-SOD. The improved resolution afforded by such highly specific labeling permits the use of direct ^{15}N detection to observe and assign Gln 69, even though its distance to the paramagnetic Fe^{2+} is only 5 Å. Selective glutamine side-chain labeling is inexpensive and has general utility for large (and paramagnet-containing) proteins.

^{15}N is a very informative NMR nucleus because its chemical shift is very sensitive to chemical environment, nitrogen is a natural component of proteins and nucleic acids and is directly bonded to labile and chemically active protons. The side chains of Gln and Asn play important roles in protein structure and activity, as they can both donate and accept hydrogen bonds, and in so doing are often found to stabilize metal binding sites (Kiefer et al., 1995). In the case of iron-containing superoxide dismutase (Fe-SOD), Gln 69 in the active site is proposed to be a key determinant of metal ion specificity (Carlioz et al., 1988; Parker and Blake, 1988; Stoddard et al., 1990). However, the metal ions of many metallo-enzymes are paramagnetic at least part of the time. Although paramagnetism can facilitate NMR observation of groups nearby in favorable cases (Emptage et al., 1981; Unger et al., 1985; Bertini et al., 1994; Cheng and Markley, 1995), it all too often relaxes the NMR resonances of protons in the active site to the extent that they can no longer be observed (Miller et al., 1992). Paramagnetic $1/T_2$ relaxation is mediated by Curie, dipolar and contact mechanisms (Bertini and Luchinat, 1986). All three mech-

anisms depend on the square of the gyromagnetic ratio (γ) of the nucleus being observed (and relaxed) and dipolar and Curie $1/T_2$ relaxation also depend on the -6 th power of the distance (r) to the paramagnet. Because the γ of ^{15}N is only one tenth that of ^1H , ^{15}N nuclei should be 100 times less sensitive than ^1H to paramagnetic relaxation. Thus, for nuclei not connected through bonds to the paramagnet, ^{15}N should be observable when it is less than half as far from the paramagnet as ^1H , potentially providing the possibility of directly observing chemical events that occur in paramagnet-containing enzyme active sites which are inaccessible by ^1H NMR (Oh and Markley, 1990).

Unfortunately, the resonances of Gln and Asn are often relatively poorly resolved and their specific assignment generally requires prior assignment of the backbone. To facilitate observation and assignment of Gln side chains of particular interest, we have produced samples in which ^{15}N is incorporated exclusively into Gln side chains. In principle, this is inexpensive to do because Gln is synthesized from Glu by addition of an $-\text{NH}_2$ group, derived directly from NH_4^+ (see, for example, Metzler, 1977).

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Abbreviations: Asn, asparagine; Asp, aspartate; Gln, glutamine; Glu, glutamate; HSQC, heteronuclear single-quantum coherence spectroscopy; INEPT, Insensitive Nuclei Enhanced by Polarization Transfer; NOE, nuclear Overhauser enhancement; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SOD, superoxide dismutase; WEFT, water eliminated Fourier transform.

However, the side-chain nitrogen of Gln is the entry-point into most nitrogen metabolism. Thus, ^{15}N placed in the side chain of Gln is transferred extremely readily to all other amino acids, unless the latter are supplied in the medium and their biosynthesis is suppressed (Muchmore et al., 1989). It is particularly difficult to suppress label incorporation into the side chain of Asn. Not only does Asn incorporate $^{15}\text{NH}_4^+$ into the side chain via $\text{Asp} + \text{NH}_4^+ \rightleftharpoons \text{Asn}$, but even if ^{15}N -labeled Gln is supplied, Asn will incorporate ^{15}N released by the $\text{Gln} \rightleftharpoons \text{Glu} + \text{NH}_4^+$ reaction. Moreover, provision of large amounts of ^{14}N -Asn to suppress ^{15}N -Asn synthesis results in release of $^{14}\text{NH}_4$ by $\text{Asn} \rightleftharpoons \text{Asp} + ^{14}\text{NH}_4^+$, which in turn is incorporated into Gln and dilutes the labeling of the desired position. Thus, in order to achieve highly selective, yet inexpensive labeling of Gln, we have used an auxotrophic strain of *E. coli* in which the incorporation of NH_4^+ into Asn is prevented.

Selective labeling is particularly useful in the study of larger proteins, circumventing increased resonance overlap and increasingly laborious total assignment when a few well-chosen resonances are sufficient to address the questions of interest. Fe-SOD is a particularly difficult case, as its (dimer) molecular weight is 42 kDa and the active site contains a paramagnetic Fe ion. The vast majority of NMR studies of Fe-containing proteins have dealt with heme Fe, often in low-spin states. By contrast, the non-heme Fe of Fe-SOD is high-spin in both oxidation states. However, the Gln implicated in determining the metal ion specificity of Fe-SOD is one of only three glutamines in the molecule, so selective labeling methods offer immediate access to the residue of interest, as well as the opportunity to exploit the low γ of ^{15}N to circumvent the paramagnetism of Fe.

Mn-SODs closely resemble Fe-SODs with respect to amino acid sequence and have superimposable global folds (Stallings et al., 1984; Parker and Blake, 1988). However, most Fe- or Mn-SODs are less than 10% active with the other metal ion bound (Ose and Fridovich, 1979; Beyer et al., 1989; Yamakura et al., 1995). One of the few conserved differences between Mn-SODs and Fe-SODs is the position in the amino acid sequence of the conserved active-site Gln (Carlioz et al., 1988; Parker and Blake, 1988; Stoddard et al., 1990). This Gln is believed to form hydrogen bonds with the conserved tyrosine and the metal-coordinated solvent, and thus could modulate the activities of both these potential proton donors, as well as the metal ion itself.

Fe-SOD was overexpressed from the plasmid pHS1-8 (Carlioz and Touati, 1986) in *E. coli* strain JK120, which is $\text{AsnA}^- \text{AsnB}^-$ and thus an Asn auxotroph (Parker and Friesen, 1980), to obtain selective ^{15}N labeling of Gln side chains only. We have optimized the growth conditions with respect to the levels of NH_4^+ , Asp, Glu, Asn supplied, as well as the anaerobicity of the growth, in order to simultaneously maximize the yield of Fe-SOD per liter of

medium, favour the incorporation of ^{15}N into Gln side chains and minimize the incorporation of ^{15}N into any other protein positions. JK120(pHS1-8) were grown in a defined medium containing the same salts and half the concentrations of the amino acid and bases as described by Muchmore et al. (1989), but using 0.2 g Asn, 0.225 g $^{15}\text{NH}_4\text{Cl/l}$, no Gln, and 10 times the concentration of micronutrients. Microaerobic cultures were grown to an OD_{600} of 1.2 and harvested. To label the side chains of both Gln and Asn, *E. coli* QC774 (sodA^- , sodB^- (Carlioz and Touati, 1986)) bearing pHS1-8 was grown microaerobically in the above medium, omitting Gln and including 0.03 g Asn and 0.112 g $^{15}\text{NH}_4\text{Cl/l}$ (see also Tate et al., 1992) to an OD_{600} of ≈ 1.6 . Uniformly ^{15}N -labeled Fe-SOD was expressed from *E. coli* QC774(pHS1-8), grown microaerobically in minimal medium incorporating 0.1 g/l $^{15}\text{NH}_4\text{Cl}$, to an OD_{600} of 0.75. Erlenmeyer flasks with a capacity of 4 l, containing 4 l of medium, were sparged with argon, sealed with airtight stoppers and incubated at 37 °C under slow shaking. The least dense (minimal) culture yielded 0.85 g of cell paste per liter, from which 20 mg of Fe-SOD per liter of culture was purified as described by Slykhouse and Fee (1976), and Sorkin and Miller (1997). Fe-SOD was >98% pure based on SDS-PAGE, had specific activities of 5500 units/mg protein or greater and retained full activity after NMR measurements.

NMR samples of Fe-SOD suspended in 50 mM phosphate buffer, pH 7.5 with 25 mM NaCl in 10% D_2O /90% H_2O were reduced to the Fe^{2+} state (unless the oxidized state is specified) using a sevenfold excess of ascorbate or a twofold excess of dithionite under an inert atmosphere, and sealed. The pH after reduction was determined to be ≈ 7 from the chemical shifts of internal NMR pH indicators (Sorkin and Miller, 1997).

The HSQC at the top of Fig. 1 shows the resonances of all the amides in reduced uniformly ^{15}N -labeled Fe-SOD, except those near the active site, which are so close to Fe that they relax too quickly to be observed by indirect methods. Although the resonances are actually quite well dispersed, many are not resolved because of their large number and the size of Fe-SOD. The number of resonances present indicates that the two monomers of Fe-SOD are spectroscopically identical. The analogous spectrum of Fe-SOD labeled with ^{15}N in the side chains of Gln and Asn demonstrates that there is relatively little leak of the label into the backbone (Fig. 1, middle). However, not all of the expected 17 resonance pairs are observed, indicating that direct detection is necessary to observe active-site resonances, yet the lack of dispersion in the ^{15}N dimension indicates that such ^{15}N spectra will be poorly resolved.

Combined labeling of Gln and Asn side chains has been demonstrated before (Tate et al., 1992); however, it is very difficult to incorporate high levels of ^{15}N in Gln

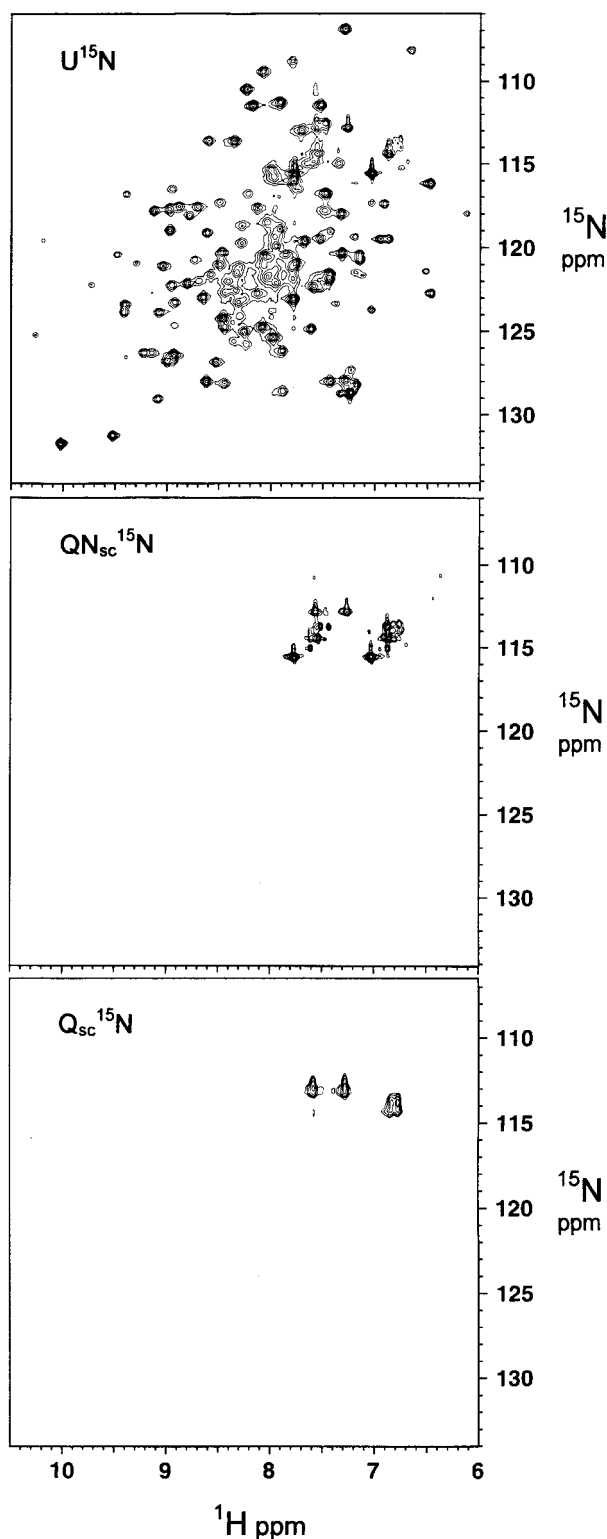


Fig. 1. HSQC spectra of uniformly ^{15}N -labeled Fe-SOD (top), Fe-SOD labeled in the side chains of Asn and Gln (middle) and Fe-SOD labeled exclusively in the side chains of Gln (bottom). NMR spectra were collected at 25 °C on a Unityplus spectrometer at 500 MHz for ^1H and 50 MHz for ^{15}N . ^1H - ^{15}N HSQC spectra were collected with sweep widths of 1650 Hz for ^{15}N and 6500 Hz for ^1H , with GARP decoupling of ^{15}N during acquisition. Chemical shifts are referenced to water at 4.78 ppm for ^1H and external $^{15}\text{NH}_4\text{Cl}$ (2.9 mM) in 1 M HCl at 20 °C at 24.93 ppm (Levy and Lichter, 1979).

side chains without obtaining it in Asn side chains as well, since Asn is produced from $\text{NH}_4^+ + \text{Asp}$ (as well as $\text{Gln} + \text{Asp} \rightarrow \text{Glu} + \text{Asn}$). To reduce both incorporation of ^{15}N into new Asn as well as release of $^{14}\text{NH}_4^+$ from Asn supplied, we have expressed SOD in a strain of *E. coli* in which both of the enzymes that synthesize Asn from Asp are inactivated (Parker and Friesen, 1980). Fe-SOD expressed in the auxotrophic *E. coli* retains only two pairs of amide side-chain signals in a spectrum that is dramatically simplified relative to the spectrum of uniformly ^{15}N -labeled Fe-SOD (Fig. 1, bottom). The Asn side chains each incorporate ^{15}N to at most 10% of the level incorporated into Gln side chains, based on the invisibility of even the stronger Asn signals in samples labeled in Gln only. Thus, this method permits a very clean discrimination between Asn and Gln (permitting their identification in Asn and Gln labeled samples). Fe-SOD is expected to have three pairs of Gln side-chain resonances, not two. However, since Gln 69 is approximately 5 Å from Fe, whereas the other two Gln are more than 14 Å away (Lah et al., 1995), Gln 69 is likely subject to paramagnetic relaxation and shifting, and the two pairs of signals in the HSQC spectrum most probably correspond to Gln 32 and Gln 109.

Although direct observation of ^{15}N is considerably less sensitive than indirect observation via HSQC, it permits collection of spectra over a larger spectral width than indirect methods, in which pulse imperfections from numerous pulses accrue for those resonances a long way from the carrier frequency. Additionally, direct observation can permit detection of ^{15}N nuclei not attached to protons or ^{15}N attached to protons that relax too fast to transmit magnetization effectively to and from their bound nitrogen.

The ^{15}N spectrum of uniformly ^{15}N -labeled reduced Fe-SOD (Fig. 2a) is dominated by the signals of the backbone amides, which overlap the side-chain amides. The latter are revealed in the sample labeled in the Gln and Asn side chains (Fig. 2b), with the poor dispersion anticipated. Although some ^{15}N -labeled backbone amide positions are apparent as a hump under the side-chain signals, they are weaker than the side-chain amide signals and consideration of the fact that there are 185 backbone amides and only 17 side-chain amides indicates that the leak is relatively slight.

A signal at 158 ppm is retained in samples labeled in Gln side chains only, which also show two strong side-chain amide signals, at 113 and 114 ppm (Fig. 2c, Table 1). In an otherwise identical ^{15}N spectrum collected without ^1H decoupling, all three signals display the 88 Hz 1:2:1 splitting expected of side-chain amides (Fig. 2d). Thus, all three strong signals appear to derive from Gln. The weaker signal intensity extending from approximately 116 to 130 ppm is shown to derive from backbone amides by its dependence on the duration of the refocusing delay

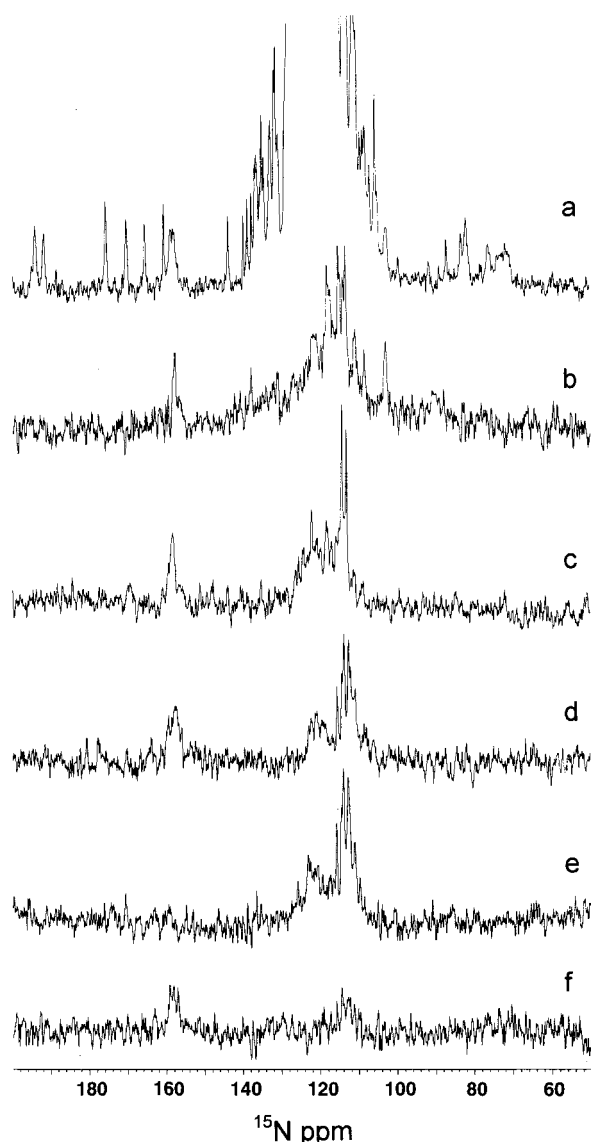


Fig. 2. ^1H -decoupled ^{15}N spectra of (a) 2 mM uniformly ^{15}N -labeled Fe-SOD (20 000 scans) and (b) 1 mM Fe-SOD labeled in the side chains of Asn and Gln (50 000 scans), obtained using a 1.0 s relaxation delay, and (c) 2 mM Fe-SOD labeled exclusively in the side chains of Gln (20 000 scans) obtained using a 3 s relaxation delay. (d) Non-decoupled ^{15}N spectrum of a different preparation of Fe-SOD, labeled exclusively in the side chains of Gln (50 000 scans); (e) non-decoupled ^{15}N spectrum of oxidized Fe-SOD (40 000 scans); and (f) non-decoupled ^{15}N WEFT spectrum of Fe-SOD (60 000 scans). ^{15}N NMR spectra were collected at 25 °C using a 5 mm broadband probe, sweep widths of 200–600 ppm, 20–23.5 μs excitation pulses, a 0.3 s acquisition time and a ^{15}N carrier frequency between 120.5 and 150.5 ppm. When ^1H was decoupled, WALTZ decoupling was applied only during signal acquisition using a 2 kHz decoupling field, centered at 7 ppm ^1H for the sample labeled only in Gln and at 10 ppm for the other two. Super-WEFT spectra (Inubishi and Becker, 1983) of ^{15}N were obtained using a 46.3 μs inversion pulse followed by a 0.6 s delay during which fast-relaxing ^{15}N signals fully recovered but most ^{15}N signals decayed to close to zero magnetization, a 90° observe pulse and 0.25 s acquisition, followed by a 0.6 s relaxation delay. A line broadening of 10 Hz was applied to all spectra. Typical normal and WEFT ^{15}N spectra were collected in 14 and 21 h, respectively. Fe-SOD samples other than the one used for (e) all contained reduced Fe-SOD.

in INEPT experiments. In ^1H -to- ^{15}N INEPT (Morris and Freeman, 1979) spectra, the fact that the intensity between 116 and 130 ppm is maximized by a total refocusing delay of $1/2J$, positive but smaller after delays of $1/4J$ or $3/4J$ and negative after a delay of $5/4J$, identifies it as due to NH groups and thus backbone amides (Fig. 3, Table 2). By contrast, the signals at 113 and 114 ppm are positive after refocusing for $1/4J$ and $5/4J$, negative after refocusing for $3/4J$ and close to null after $1/2J$, indicating that they correspond to NH_2 groups and thus side-chain amides. (The resonance at 158 ppm was not observed in any of these experiments, probably because the protons associated with it relax within the delays employed; vide infra.) Thus, the intensity between 116 and 130 ppm corresponds to ^{15}N leakage into backbone positions and only two side-chain amides are significantly ^{15}N labeled in the region of 70–170 ppm. Since there are three glutamines in Fe-SOD, either the third Gln is not visible and the strong resonance at 158 ppm is some other functionality, or the resonance at 158 ppm is the signal of the third Gln. The former is less likely due to the virtual absence of signals from any functionalities other than backbone amides and Gln side-chain amides, and the significantly weaker labeling of backbone amides compared to Gln side-chain amides. Thus, we conclude that the resonance at 158 ppm is the third Gln. Based on integrated signal areas, we conclude that we have labeled 90% of the Gln side-chain amide positions, 2–10% of the Asn side-chain amide positions (depending on the preparation) and approximately 1% of the backbone amide positions.

The signals at 113 and 114 ppm have ^{15}N chemical shifts identical to those of the two pairs of side-chain amide signals observed in the HSQC (Fig. 1, bottom), confirming their assignment to Gln side chains. However, the chemical shift of 158 ppm is very unusual for a Gln side chain. Nevertheless, the area of the peak at 158 ppm is approximately half the combined areas of the resonances at 113 and 114 ppm in the absence of decoupling,

TABLE 1
CHEMICAL SHIFTS OF Gln RESONANCES IN REDUCED AND OXIDIZED Fe-SOD

Assignment (tentative)	Chemical shift (ppm) ^a			
	Reduced Fe-SOD		Oxidized Fe-SOD	
	^{15}N	^1H	^{15}N	^1H
Gln 69	158.14			
Gln 32 ^b	113.99	6.83, 6.76	114.08	6.92, 6.85
Gln 109 ^b	112.78	7.57, 7.27	112.79	7.56, 7.25

^a ^1H and ^{15}N chemical shifts are ± 0.03 ppm.

^b Specific assignments to Gln 32 versus Gln 109 are based on the greater sensitivity to Fe oxidation state of the chemical shifts and the ^1H - ^{15}N NOE of the resonance at 114 ppm, coupled with the fact that Gln 32 occurs in the monomer–monomer interface, which is bridged by a hydrogen bond network that also encompasses the active sites. Thus, the assignments are strictly tentative.

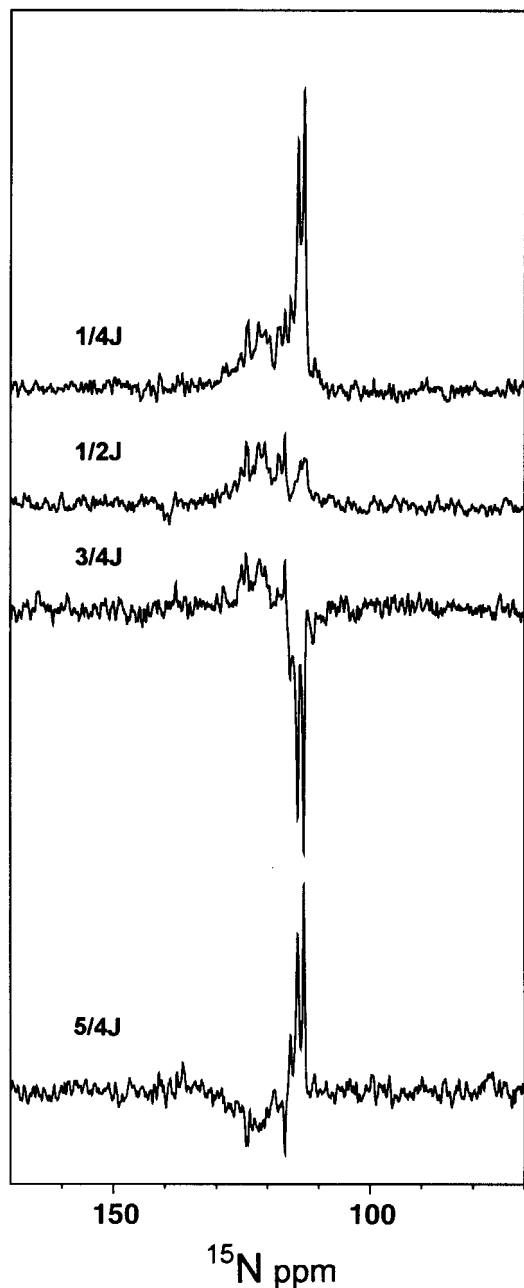


Fig. 3. ^1H -decoupled ^{15}N INEPT spectra of reduced Fe-SOD, labeled in the Gln side chains only. The spectra obtained using total refocusing delays of $1/4J$, $2/4J$, $3/4J$ and $5/4J$ are presented along with the expected relative signal intensities for NH_2 and NH groups. All spectra were obtained at 25°C using a 5 mm broadband probe, a total delay of 5.6 ms for dephasing of ^1H with respect to ^{15}N , integral multiples of $1/4J=2.8$ ms total time for refocusing ^{15}N with respect to ^1H , a 0.3 s acquisition time with 2 kHz GARP ^1H decoupling centered at 6.9 ppm ^1H , and a 3 s relaxation delay. For each spectrum, 3000 scans were acquired and 10 Hz line broadening was applied prior to Fourier transformation. Anticipated relative signal intensities for NH and NH_2 groups are tabulated in Table 2; the experimental intensities also decrease with increasing refocusing delay, due to relaxation.

suggesting that it represents one N position labeled to a similar degree as the two Gln side chains. Also, the resonance at 158 ppm shows a 1:2:1 splitting of 88 Hz, which

TABLE 2
ANTICIPATED RELATIVE INTENSITIES OF ^{15}N SIGNALS OBTAINED FROM ^1H -TO- ^{15}N INEPT SPECTRA^a

Functionality	Refocusing delay ^b			
	$1/4J$	$1/2J$	$3/4J$	$5/4J$
$-\text{NH}_2$	$1/2$	0	$-1/2$	$1/2$
$-\text{NH}$	$1/\sqrt{2}$	1	$1/\sqrt{2}$	$-1/\sqrt{2}$

^a Relative intensities do not take into account relaxation during the refocusing delay.

^b Total refocusing delay, i.e., sum of the pair of delays flanking the simultaneous 180° pulses.

is less well resolved but otherwise similar to the structure of the other two Gln signals (Fig. 2d). Thus, the properties of the signal at 158 ppm other than its chemical shift are consistent with its being the third Gln.

If the atypical chemical shift of 158 ppm reflects paramagnetic contributions to chemical shift due to the proximity of this Gln to Fe^{2+} , it should be accompanied by paramagnetic contributions to relaxation as well. This could explain the observed absence of signals at 158 ppm from HSQC spectra (not shown). The resonance at 158 ppm is indeed visibly broader than the resonances at 113 and 114 ppm, indicating that it has a shorter T_2 . The resonance at 158 ppm is also significantly enhanced relative to the resonances at 113 and 114 ppm in a WEFT spectrum (Inubishi and Becker, 1983), indicating that the T_1 of the former is shorter than those of the latter (Fig. 2f). Thus, although these comparisons are not quantitative, they suggest that the resonance at 158 ppm represents a Gln close to Fe^{2+} , which also provides a plausible explanation for the chemical shift of 158 ppm for a Gln side chain.

Finally, we observed the effect on the ^{15}N spectrum of oxidizing the Fe. X-ray crystallography indicates that this does not significantly change the structure of Fe-SOD, and only causes small movements of the side chains of amino acids in the active site (Lah et al., 1995). However, oxidation of Fe from d^6 to d^5 has profound effects on the electronic spin, which in Fe-SOD changes from $S=2$ to $S=5/2$, and acquires considerably longer spin relaxation times, due to the orbital non-degeneracy of the ^6A electronic state formed (Bertini and Luchinat, 1986). The long spin relaxation times make Fe^{3+} a much more potent relaxer of NMR nuclei, including ^{15}N , whereas the greater symmetry of the ^6A electronic state makes it much less likely to contribute much to chemical shifts of non-ligand atoms (Bertini and Luchinat, 1986). Paramagnetic contributions to relaxation and chemical shift drop off of as $1/r^6$ and $1/r^3$, respectively, and therefore are only expected to affect nuclei close to Fe. Comparison of the ^{15}N spectra of reduced (Fe^{2+})- and oxidized (Fe^{3+})-SOD shows that the resonance at 158 ppm moves significantly (and/or is severely broadened) in oxidized Fe-SOD, as it is no longer visible at 158 ppm (Fig. 2e), whereas the reson-

ances at 113 and 114 ppm shift only slightly upon oxidation of Fe. Thus, the resonance at 158 ppm is assigned to Gln 69 (5 Å from Fe) and the resonances at 113 and 114 ppm are collectively assigned to Gln 32 and Gln 109 (more than 14 Å away).

In view of the strongly shifted ^{15}N resonance of Gln 69, and the implied strong paramagnetic effects on the side-chain protons as well, it is interesting that the ^{15}N nonetheless displays a resolved coupling (88 Hz) to two protons. This indicates that both amide protons have a T_1 longer than $1/88 = 12$ ms. Whether or not the protons also display resolved coupling to ^{15}N depends on their T_2 values. The ^1H spectrum has been observed (Ming et al., 1994; Miller and Sorkin, 1997) and shows resonances >2000 Hz wide, which probably correspond to ligands, as well as resonances up to 650 Hz wide, which are probably due to non-ligand protons, based on the few assignments available (Ming et al., 1994). Thus, the protons have short T_2 values. However, paramagnetic $1/T_2$ relaxation in Fe-SOD is dominated by the Curie contribution, based on the magnetic field dependence of the line widths (J.C. Lansing and A.-F. Miller, manuscript in preparation), and the Curie mechanism does not contribute to $1/T_1$ relaxation (Bertini and Luchinat, 1986). For example, even a ligand proton with a resonance 460 Hz wide has a selective T_1 of 8.6 ms. Thus, the combination of the relative insensitivity of ^{15}N to paramagnetic line broadening and the dominance of ^1H relaxation by a mechanism that does not contribute to $1/T_1$ can explain the resolution of coupling to ^1H in the ^{15}N signal of Gln 69.

In conclusion, we have optimized the conditions for inexpensive but highly selective ^{15}N labeling of the side chains of Gln, avoiding even ^{15}N incorporation into the side chains of Asn, by exploiting an Asn auxotrophic expression system. Selective labeling of Gln side chains greatly facilitates their observation and resolution in one- and two-dimensional NMR spectra and has general applicability to a wide range of systems. The resonance of Gln 69 of Fe-SOD is distinguished from those of the other two glutamines on the basis of its strongly paramagnetically shifted resonance, its broad lines, its shortened T_1 and its sensitivity to the oxidation state of Fe. This assignment lays the foundation for NMR investigations of the contribution of Gln 69 and its analog, Gln 141, to metal-ion specificity in Fe-SOD and Mn-SOD.

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