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# Zinc-substituted *Desulfovibrio gigas* desulfiredoxins: Resolving subunit degeneracy with nonsymmetric pseudocontact shifts

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## Abstract

*Desulfovibrio gigas* desulfiredoxin (Dx) consists of two identical peptides, each containing one [Fe-4S] center per monomer. Variants with different iron and zinc metal compositions arise when desulfiredoxin is produced recombinantly from *Escherichia coli*. The three forms of the protein, the two homodimers [Fe(III)/Fe(III)]Dx and [Zn(II)/Zn(II)]Dx, and the heterodimer [Fe(III)/Zn(II)]Dx, can be separated by ion exchange chromatography on the basis of their charge differences. Once separated, the desulfiredoxins containing iron can be reduced with added dithionite. For NMR studies, different protein samples were prepared labeled with <sup>15</sup>N or <sup>15</sup>N + <sup>13</sup>C. Spectral assignments were determined for [Fe(II)/Fe(II)]Dx and [Fe(II)/Zn(II)]Dx from 3D <sup>15</sup>N TOCSY-HSQC and NOESY-HSQC data, and compared with those reported previously for [Zn(II)/Zn(II)]Dx. Assignments for the <sup>13</sup>C $\alpha$  shifts were obtained from an HNCA experiment. Comparison of <sup>1</sup>H–<sup>15</sup>N HSQC spectra of [Zn(II)/Zn(II)]Dx, [Fe(II)/Fe(II)]Dx and [Fe(II)/Zn(II)]Dx revealed that the pseudocontact shifts in [Fe(II)/Zn(II)]Dx can be decomposed into inter- and intramonomer components, which, when summed, accurately predict the observed pseudocontact shifts observed for [Fe(II)/Fe(II)]Dx. The degree of linearity observed in the pseudocontact shifts for residues  $\geq 8.5$  Å from the metal center indicates that the replacement of Fe(II) by Zn(II) produces little or no change in the structure of Dx. The results suggest a general strategy for the analysis of NMR spectra of homo-oligomeric proteins in which a paramagnetic center introduced into a single subunit is used to break the magnetic symmetry and make it possible to obtain distance constraints (both pseudocontact and NOE) between subunits.

**Keywords:** NMR; pseudocontact shifts; desulfiredoxin; [Fe-4S] center; paramagnetic protein; *Desulfovibrio gigas*

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**Abbreviations:** Cp, *Clostridium pasteurianum*; Dg, *Desulfovibrio gigas*; Dx, desulfiredoxin from *Desulfovibrio gigas*; Rd, rubredoxin; HSQC, heteronuclear single quantum coherence; NOESY, nuclear Overhauser effect spectroscopy; TOCSY, total correlation spectroscopy.

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The simplest iron–sulfur protein isolated from sulfate reducing bacteria is rubredoxin (Rd). The rubredoxins are low molecular weight (6–7 kD) proteins containing one iron center (Sieker et al. 1994). The metal atom is bound by four cysteine residues with a conserved binding sequence of the type: -C-X-Y-C-. . . . -C-X-Y-C-. In the oxidized native state, the iron is high-spin Fe(III) with  $S = 5/2$ , and in the reduced state, the iron is high-spin Fe(II) with  $S = 2$ . The center has a distorted tetrahedral geometry. Several X-ray structures are available for Rds from various bacterial sources in both oxidation states (Watenpaugh et al. 1980; Frey et al. 1987; Dauter et al. 1992; Day et al. 1992).



addition, owing to the dimeric nature of Dx, a mixed metal form containing both Fe and Zn is also produced. The three forms can be separated by ion exchange chromatography as a result of their charge differences. We report here NMR investigations of [Fe(II)/Fe(II)]Dx, [Fe(II)/Zn(II)]Dx, and [Zn(II)/Zn(II)]Dx carried out with protein samples labeled uniformly with  $^{15}\text{N}$  and with  $^{15}\text{N} + ^{13}\text{C}$ . These studies have resulted in extensive  $^1\text{H}$ ,  $^{15}\text{N}$ , and  $^{13}\text{C}^\alpha$  assignments for these molecules. In addition, because of the diamagnetism of zinc, the results have enabled the dissection of the intra- and intersubunit pseudocontact shifts for nuclei  $>8.5 \text{ \AA}$  distant from iron centers. The intra- and intersubunit  $^1\text{H}$  and  $^{15}\text{N}$  pseudocontact shifts were found to be strictly additive; this indicates that substitution of Fe(II) with Zn(II) does not lead to significant changes in the positions of atoms in the protein  $>8.5 \text{ \AA}$  from the metal center.

## Results

Figure 2 shows  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra of [Zn(II)/Zn(II)]Dx, [Fe(II)/Zn(II)]Dx, and [Fe(II)/Fe(II)]Dx. Distinct patterns of chemical shifts are clearly observed for the three different combinations of Zn- and Fe-containing Dx species, shown in Figure 3A–C. Because [Zn(II)/Zn(II)]Dx is a completely symmetric dimer, with identical local environments in the two monomers, a single set of resonances is observed for the 35 backbone NH groups of the 36-amino acid monomer as well as for the  $\text{NH}_2$  groups of the side chains of N2, Q14, and Q36 (Fig. 2A). The  $^1\text{H}$  and  $^{15}\text{N}$  chemical shifts for [Zn(II)/Zn(II)]Dx were assigned in a straightforward manner on the basis of previously reported  $^1\text{H}$  chemical shifts (Goodfellow et al. 1996) and the 3D  $^{15}\text{N}$ -edited NOESY and TOCSY spectra.

The  $^1\text{H}$ - $^{15}\text{N}$ -HSQC spectrum of the mixed metal form of Dx (Fig. 2B) shows 50 backbone NH cross peaks and 5  $\text{NH}_2$  side-chain cross peaks. The larger number of peaks present in this spectrum is due to the symmetry of the dimer being

broken by the nonequivalence of the two metal centers. From previous studies of *CpRd*, “bleaching” of  $^1\text{H}$ - $^{15}\text{N}$  cross peaks through the effects of paramagnetic broadening is expected for groups closer than about  $8.5 \text{ \AA}$  to an iron center (Prantner et al. 1997; Volkman et al. 1997). Application of this principle to the structure of Dx, as shown in Figure 3B, suggests that each iron center will “bleach” residues in both the iron-containing subunit as well as the other subunit. The extent of paramagnetic broadening from one Fe atom can be estimated using the X-ray structure: residues 1–8, 16–27, 32, and 34–36 of the Fe-containing monomer radius and residues 1–21 and 28–36 from the Zn containing monomer are more than  $8.5 \text{ \AA}$  from the Fe (a total of 54, including five side-chain  $\text{NH}_2$  groups). The number of expected (52 backbone/five side chain) and observed (50 backbone/five side chain) cross peaks is very similar. Simple comparisons between the spectra of [Zn(II)/Zn(II)]Dx and [Fe(II)/Zn(II)]Dx led to assignments for residues 3–6, 19–26, 35, and 36 in the iron-containing subunit and residues 8–13, 29, 30, 34–36 in the zinc-containing subunit. All the remaining peaks in the  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectrum of [Fe(II)/Zn(II)]Dx were assigned on the basis of 3D  $^{15}\text{N}$ -HSQC-TOCSY and  $^{15}\text{N}$ -HSQC-NOESY data.

[Fe(II)/Fe(II)]Dx also is a symmetric dimer; however, the paramagnetism of the iron centers obliterates signals from residues close to the iron centers, and only 17 backbone NH cross peaks and cross peaks from the  $\text{NH}_2$  groups of two side chains are seen (Fig. 2C). Residues in Dx with  $^{15}\text{N}$  and  $^1\text{H}$  atoms more than  $8.5 \text{ \AA}$  away from both Fe atoms include 1–8, 16–21, 32, and 34–36 (18 residues, including two residues with side chain  $\text{NH}_2$  groups) (Fig. 3C). It became apparent that the chemical shifts observed in the  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectrum of [Fe(II)/Fe(II)]Dx could be predicted by adding the pseudocontact shifts from each of the subunits of [Fe(II)/Zn(II)]Dx. For example, Figure 3D shows an expansion of the two cross peaks of [Fe(II)/Zn(II)]Dx assigned to L19 compared to the single L19 resonance observed in

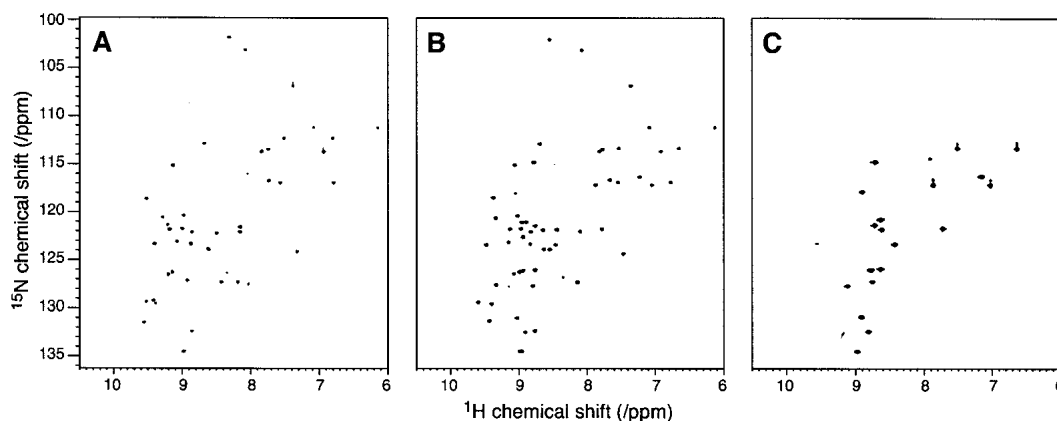
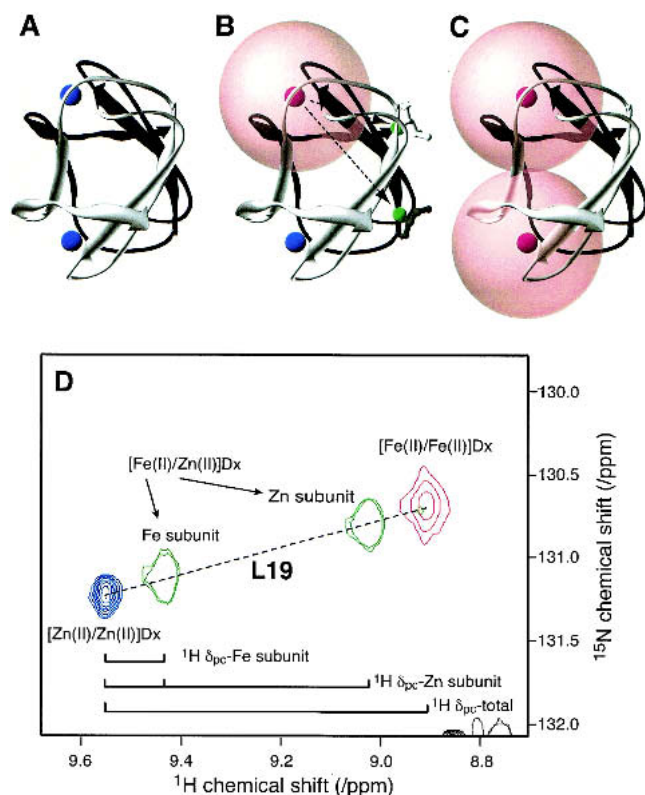


Fig. 2.  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra at pH 7.2 and 303 K of (A) [Zn(II)/Zn(II)]Dx, (B) Dx [Fe(II)/Zn(II)]Dx, and (C) [Fe(II)/Fe(II)].



**Fig. 3.** The metal composition of desulfurodoxin affects backbone  $^1\text{H}$  and  $^{15}\text{N}$  chemical shifts. The X-ray structure of desulfurodoxin (PDB ID: 1DXG) (Archer et al. 1995) is used to represent the three combinations of Fe and Zn species, (A)  $[\text{Zn(II)/Zn(II)}]\text{Dx}$ , (B)  $[\text{Fe(II)/Zn(II)}]\text{Dx}$ , and (C)  $[\text{Fe(II)/Fe(II)}]\text{Dx}$ . In each case, one subunit of the dimeric desulfurodoxin is colored black, and the other is colored white. Zn and Fe atoms are colored blue and red, respectively. In (B), the Fe and Zn atoms are bound to the black and white protein subunits, respectively. Spheres of radius 8.5 Å are centered on the Fe atoms, indicating the regions within which NMR signals are usually broadened beyond detection in 2D HSQC spectra. Fewer resonances should be observed from the homodimer containing Fe at both metal sites than from the heterodimer containing one Zn(II) and one Fe(II). In the case of  $[\text{Fe(II)/Fe(II)}]\text{Dx}$ , the environments of corresponding nuclei in both subunits are equivalent; in the case of  $[\text{Fe(II)/Zn(II)}]\text{Dx}$ , the environments of corresponding  $^{15}\text{N}$  nuclei in each subunit are different, as indicated for L19 (green) in (B). (D) Enlarged region of the superimposed  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra of (red)  $[\text{Fe(II)/Fe(II)}]\text{Dx}$ , (green)  $[\text{Fe(II)/Zn(II)}]\text{Dx}$ , and (blue)  $[\text{Zn(II)/Zn(II)}]\text{Dx}$ . The cross peaks assigned to Leu19 are indicated in the figure. The proton pseudocontact shifts ( $^1\text{H } \delta_{\text{pc}}$ ) are indicated for the iron and zinc subunits of  $[\text{Fe(II)/Zn(II)}]\text{Dx}$  and for  $[\text{Fe(II)/Fe(II)}]\text{Dx}$ . Note that these are additive and that the additivity holds also for the  $^{15}\text{N}$  pseudocontact shifts, as indicated by the colinearity of the set of four cross peaks.

the spectrum of  $[\text{Zn(II)/Zn(II)}]\text{Dx}$ . The two distinct resonances observed for L19 in  $[\text{Fe(II)/Zn(II)}]\text{Dx}$  arise due to their different positions in the structure relative to the single Fe atom, as illustrated in Figure 3. In this instance, a larger pseudocontact shift is observed for L19 in the Zn subunit than for the corresponding residue of the Fe subunit (Fig. 3D), consistent with their relative distances from the paramagnetic center (Fig. 3B). Addition of the pseudocontact

shifts for each subunit in of  $[\text{Fe(II)/Zn(II)}]\text{Dx}$  (in both the  $^1\text{H}$  and  $^{15}\text{N}$  dimensions) yielded the position of the cross peak in the spectrum of  $[\text{Fe(II)/Fe(II)}]\text{Dx}$  assigned to L19. The rationale for this is that nuclei in each symmetrical monomer of  $[\text{Fe(II)/Fe(II)}]\text{Dx}$  experience both intrasubunit and intersubunit pseudocontact shifts. All peaks in the  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectrum of  $[\text{Fe(II)/Fe(II)}]\text{Dx}$  could be assigned by this approach.

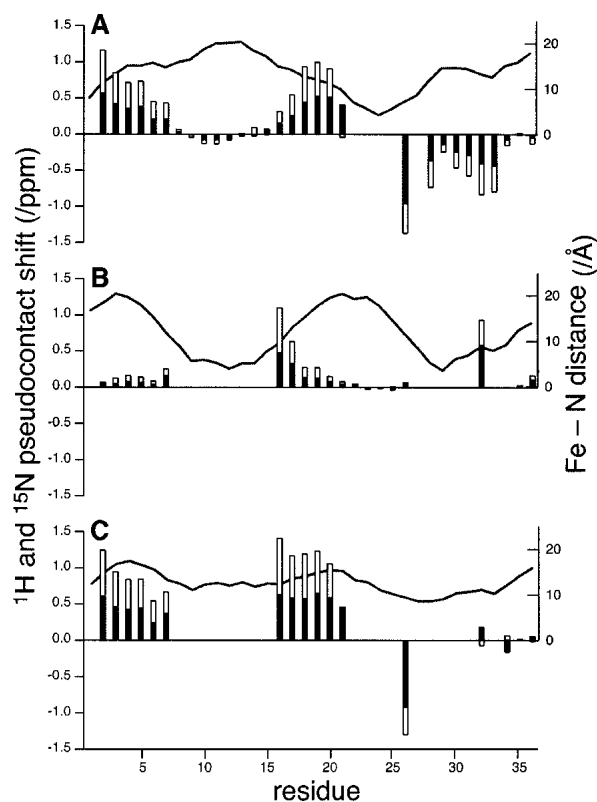
Intra- and intersubunit pseudocontact contributions to the  $^1\text{H}^\alpha$  and  $^{13}\text{C}^\alpha$  chemical shifts of Dx were obtained through analysis of NMR spectra of samples labeled uniformly with  $^{15}\text{N}$  and  $^{13}\text{C}$ . Comparison of the  $^1\text{H}^\alpha$  and  $^{15}\text{N}$  assignments described above for  $[\text{Fe(II)/Zn(II)}]\text{Dx}$  with the 3D HNCA spectrum of  $[\text{Fe(II)/Zn(II)}]\text{Dx}$  led to assignments for its  $^{13}\text{C}^\alpha$  shifts. Constant time  $^1\text{H}$ - $^{13}\text{C}$  HSQC data sets were recorded for  $[\text{Zn(II)/Zn(II)}]\text{Dx}$ ,  $[\text{Fe(II)/Zn(II)}]\text{Dx}$ , and  $[\text{Fe(II)/Fe(II)}]\text{Dx}$ . The  $^1\text{H}^\alpha$ - $^{13}\text{C}^\alpha$  cross peaks in the HSQC spectra of  $[\text{Zn(II)/Zn(II)}]\text{Dx}$  and  $[\text{Fe(II)/Zn(II)}]\text{Dx}$  were assigned by reference to the assignments described above and along with previous  $^1\text{H}^\alpha$  assignments for  $[\text{Zn(II)/Zn(II)}]\text{Dx}$  (Goodfellow et al. 1996). The  $^1\text{H}$ - $^{13}\text{C}$  cross peaks showed the same additivity of intrasubunit and intersubunit pseudocontact shifts observed with the  $^1\text{H}$ - $^{15}\text{N}$  cross peaks. This enabled the transfer of chemical shift assignments to the spectrum of  $[\text{Fe(II)/Fe(II)}]\text{Dx}$ .

Figure 4 displays differences between the  $^1\text{H}$  and  $^{15}\text{N}$  chemical shifts of the diamagnetic Dx species ( $[\text{Zn(II)/Zn(II)}]\text{Dx}$ ) and those of the iron-containing Dx species (the Zn(II) subunit of  $[\text{Fe(II)/Zn(II)}]\text{Dx}$ , the Fe(II) subunit of  $[\text{Fe(II)/Zn(II)}]\text{Dx}$ , and homodimeric  $[\text{Fe(II)/Fe(II)}]\text{Dx}$ ) plotted as a function of the residue number. Also shown in the figure are distances between the iron and each nitrogen atom whose chemical shift is analyzed (in the case of  $[\text{Fe(II)/Zn(II)}]\text{Dx}$  this is the mean distance to both irons). Interestingly, the magnitudes of shift differences in the Zn-containing subunit of  $[\text{Fe(II)/Zn(II)}]\text{Dx}$  (Fig. 4A) are generally larger than those for the Fe subunit (Fig. 4B). This is partly due to the fact that more residues of the Fe subunit are 'bleached,' owing to their close proximity to the paramagnetic center. The pattern of shift perturbations for  $[\text{Fe(II)/Fe(II)}]\text{Dx}$  (Fig. 4C) is quite different from those of either subunit of  $[\text{Fe(II)/Zn(II)}]\text{Dx}$  (Fig. 4B), but correlates with their sum, as described above.

## Discussion

Chemical shift differences between the observed peaks in the  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra of the three forms of Dx are expected to arise from pseudocontact shifts plus possible differences in protein conformation. Pseudocontact shifts have a  $1/r^3$  dependence on the distance between the observed nucleus and the metal atom. Thus, resonances showing the smallest chemical shift differences are likely to arise from groups farthest from the iron center(s). Chemical shift





**Fig. 4.** Chemical shifts of the paramagnetic, iron-containing desulfuredoxin species of desulfuredoxin relative to those of the diamagnetic species ( $[\text{Zn(II)/Zn(II)}]\text{Dx}$ ) plotted as a function of residue number  $^1\text{H}$  (black bars) and  $^{15}\text{N}$  (white bars): (A) Zn-containing monomer of  $[\text{Fe(II)/Zn(II)}]\text{Dx}$ . (B) Fe-containing monomer of  $[\text{Fe(II)/Zn(II)}]\text{Dx}$ . (C)  $[\text{Fe(II)/Fe(II)}]\text{Dx}$ . The lines trace the distances (in Å) between the iron and the nitrogen whose chemical shift is analyzed; in the case of  $[\text{Fe(II)/Fe(II)}]\text{Dx}$ , this distance is the mean distance to both irons.

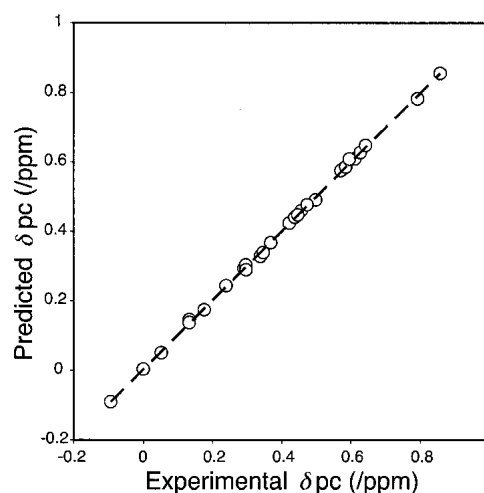
differences between the completely diamagnetic  $[\text{Zn(II)/Zn(II)}]\text{Dx}$  species and the three types of paramagnetically perturbed Dx subunits (Fig. 4) are generally consistent with this assertion, but in some instances residues equidistant from the  $\text{Fe}^{2+}$  center show dramatically different shift perturbations. For example, the backbone N atoms of residues 5 and 34 in the Zn subunit of  $[\text{Fe(II)/Zn(II)}]\text{Dx}$  are each 15.1 Å from the  $\text{Fe}^{2+}$ , but are shifted 0.35 and  $-0.08$  ppm from their positions in the HSQC spectrum of  $[\text{Zn(II)/Zn(II)}]\text{Dx}$ . The presence of both positive and negative shift differences also clearly illustrates the lack of spherical symmetry in the dipolar effects of the paramagnetic center (Guiles et al. 1996; Volkman et al. 1999).

Ordinarily, detailed knowledge of the magnitude, rhombicity, and orientation of the magnetic susceptibility tensor is required for structural interpretation of pseudocontact shifts. In this case, however, the accuracy with which the sum of  $[\text{Fe(II)/Zn(II)}]\text{Dx}$  inter- and intramonomer pseudocontact shifts predicts the measured shifts in  $[\text{Fe(II)/Fe(II)}]\text{Dx}$  provides a direct measure of structural similarity

for the different Dx species. Any large differences between the experimental shifts of  $[\text{Fe(II)/Fe(II)}]\text{Dx}$  and those predicted from adding the intra- and intersubunit pseudocontact shifts of  $[\text{Fe(II)/Zn(II)}]\text{Dx}$  to the diamagnetic shifts of  $[\text{Zn(II)/Zn(II)}]\text{Dx}$  would indicate that structural changes result from substitution of a Zn atom for an Fe atom. Figure 5 shows the correlation between the experimental and predicted  $^1\text{H}^{\alpha}$  and  $^1\text{H}^{\text{N}}$  chemical shifts for  $[\text{Fe(II)/Fe(II)}]\text{Dx}$ . The slope is very close to unity, and the off-line deviations are small. As mentioned above, only signals from residues 2–6, 16–21, 26, 32, and 34–36 are observable in the HSQC spectra of  $[\text{Fe(II)/Fe(II)}]\text{Dx}$ , owing to the bleaching effect of the iron atoms. The close agreement between predicted and observed shifts indicates that structural changes resulting from replacement of Fe(II) by Zn(II) are minimal for those atoms observed, that is, those farther than  $\sim 8.5$  Å from the site of the metal replacement.

How close can a backbone amide be to Fe(II) and still be observed? According to the X-ray structure of Dx, L26 of the Zn(II) subunit is 7.7 Å from the iron in the Fe(II) subunit; its signal was observed, albeit as a weak peak. This result for Dx is comparable with that for reduced Cp Rd in which a  $^1\text{H}-^{15}\text{N}$  HSQC signal was observed from I12 whose  $\text{H}^{\text{N}}$  is 7.6 Å from the iron (Prantner et al. 1997; Volkman et al. 1997). On the other hand, being farther from the iron does not guarantee that the signal will be observed. No signal was observed for K8 of the Fe(II) monomer of  $[\text{Fe(II)/Zn(II)}]\text{Dx}$ , which is 9.5 Å from Fe(II).

The finding that the structures of the different Dx species are similar suggests that it will be feasible to use the wealth of experimental pseudocontact shifts determined for  $[\text{Fe(II)/Zn(II)}]\text{Dx}$  as constraints for refinement of the solution structure of Dx allowing the geometry at the metal center, and



**Fig. 5.** Correlation between the observed ( $\delta_{\text{PC}}^{\text{exp}}$ ) and calculated ( $\delta_{\text{PC}}^{\text{pred}}$ )  $^1\text{H}^{\text{N}}$  and  $^1\text{H}^{\alpha}$  pseudocontact shifts for  $[\text{Fe(II)/Fe(II)}]\text{Dx}$ . The dashed line represents the linear best fit, with a slope of 0.9941 and correlation coefficient of 0.9994.

subsequently the H-bonding network at the center, to be probed. This will require knowledge of the orientation and magnitude of the magnetic susceptibility anisotropy ( $\Delta\chi$ ) of the paramagnetic center (Banci et al. 1998b; Wang et al. 1998).  $\Delta\chi$  can be determined by measuring magnetic field-dependent  $^1\text{H}$ - $^{15}\text{N}$  and/or  $^1\text{H}$ - $^{13}\text{C}$  residual dipolar couplings and fitting them to the relatively low-resolution X-ray structure for Dx (Tjandra et al. 1996; Ottiger and Bax 1998; Volkman et al. 1999). Work along these lines is in progress.

Desulforedoxin is an example of an oligomeric protein containing identical subunits. Structural information for such molecules is hindered by the magnetic equivalence of signals from each subunit, which complicates the assignment of observables (such as NOEs) to intrasubunit and intersubunit interactions. One way of breaking this symmetry is to produce oligomers containing subunits with different stable isotope labeling patterns (e.g., a dimer one with  $^{13}\text{C}$ - and one with  $^{15}\text{N}$ -labeled subunit). The results reported here suggest another strategy: production of an oligomer containing one subunit with a paramagnetic center that produces pseudocontact shifts. The paramagnetism will break the degeneracy between chemical shifts of the paramagnetic-containing subunit and those of the other subunits. Further, the pseudocontact shifts can provide additional intra- and intersubunit structural constraints.

## Materials and methods

Competent BL21(DE3) cells were transformed with the pT7-7 cloning vector containing the *dsr* gene (Czaja et al. 1995). A 25- $\mu\text{L}$  aliquot of a fresh LB broth culture containing ampicillin (0.1 mg/mL) was used to inoculate 25 mL of M9 medium prepared with  $^{15}\text{N}$ -ammonium chloride and supplemented with 0.5% glucose (replaced with  $^{13}\text{C}$ -glucose for the doubly labeled sample), 0.5  $\mu\text{g}/\text{mL}$  thiamine, 4% trace metals solution, and 0.1 mg/mL ampicillin. The trace metals solution contained the following (per liter): 69.9 g  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ ; 1.84 g  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ ; 640 mg  $\text{H}_3\text{BO}_3$ ; 400 mg  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ ; 180 mg  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ ; 40 mg  $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ ; 3.40 g  $\text{ZnCl}_2$ ; 6.05 g  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ ; 8 ml HCl 37%. Six milliliters of the resultant culture of an overnight growth at 37°C with shaking was used to inoculate six liters of an identical minimal medium. Expression of the *dsr* gene and purification of Dx were performed as previously described (Czaja et al. 1995). The yields were: 6.5 mg of  $[\text{Fe(III)/Fe(III)]Dx}$ ,  $A_{280}/A_{507} = 1.13$ ; and 5.4 mg of  $[\text{Fe(III)/Zn(II)]Dx}$ ,  $A_{280}/A_{507} = 1.58$ .  $[\text{Zn(II)/Zn(II)]Dx}$  was prepared as described previously (Goodfellow et al. 1996).

Owing to the reduced paramagnetism ( $S = 2$ ) in the Fe(II) state compared to the Fe(III) state, the iron-containing proteins were investigated in their reduced states. The proteins were reduced by adding one or two crystals of dithionite to a previously degassed (~45 min) NMR sample under argon.

Each purified protein was exchanged through ultrafiltration (YM3 membrane, Amicon) with 100 mM potassium phosphate buffer, at pH 7.2. The sample was concentrated to 450  $\mu\text{L}$  in 90%/10%  $\text{H}_2\text{O}/\text{D}_2\text{O}$ . The final protein concentration was 1.6 mM for  $[\text{Fe(II)/Fe(II)]Dx}$  and 1 mM for  $[\text{Fe(II)/Zn(II)]Dx}$ .

All spectra were acquired at a temperature of 303 K on the Bruker DMX 750 spectrometer at the National Magnetic Reso-

nance Facility at Madison, WI, at a  $^1\text{H}$  Larmor frequency of 750.13 MHz. Chemical shifts were referenced to external DSS (2,2 dimethyl-2-silapentane-5-sulfonate) as 0 ppm ( $^1\text{H}$ ), and indirectly for  $^{15}\text{N}$  and  $^{13}\text{C}$  (Markley et al. 1998).  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra were acquired using the sequence of Wagner (Talluri and Wagner 1996) with water suppression via a 3-9-19 sequence with gradients (Piotto et al. 1992). Quadrature detection in the indirectly detected dimensions was obtained with the States-TPPI method (Marion and Wüthrich 1983). Each two-dimensional spectrum consisted of 2 K complex points in  $t_2$  and of 512 increments in  $t_1$  with spectral widths of 10,000 and 2778 Hz (at 750 MHz), respectively. A  $^{13}\text{C}$  CT-HSQC sequence (Tjandra and Bax 1997) was used to obtain the  $^1\text{H}/^{13}\text{C}$  chemical shifts. Spectra contained 1 K complex points in  $t_2$  and 220 increments in  $t_1$  with spectral widths of 10,000 and 6250 Hz (at 750 MHz), respectively. Quadrature detection was achieved using the echo-antiecho method (Kay et al. 1992). The HNCA spectrum was acquired at 750 MHz using a sequence with gradient sensitivity enhancement (Kay et al. 1994) and contained 1 K complex points in  $t_3$ , 64 increments in  $t_2$  and ( $^{15}\text{N}$  dimension with echo-antiecho selection) 120 increments in  $t_1$  ( $^{13}\text{C}$  dimension with States detection). Spectral widths were 10,000 ( $^1\text{H}$ ), 2500 ( $^{15}\text{N}$ ), and 6250 ( $^{13}\text{C}$ ) Hz. All data were weighted with squared cosine functions and processed using NMRPipe (Delaglio et al. 1995). The Sparky software program (T. D. Goddard and D. G. Kneller, SPARKY 3, University of California, San Francisco) was used in determining spectral assignments.

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