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Quantitative measurement of small through-hydrogen-bond and 'through-space' ^{1}H-^{113}Cd and ^{1}H-^{199}Hg J couplings in metal-substituted rubredoxin from *Pyrococcus furiosus*

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SUMMARY

A method is described for measurement of small unresolvable heteronuclear J couplings. The method is based on quantitative analysis of a phase-purged heteronuclear spin-echo difference spectrum, and is demonstrated for measuring ^{1}H-^{113}Cd and ^{1}H-^{199}Hg J couplings in metal-substituted rubredoxin (M_r ~5.4 kDa) from *Pyrococcus furiosus*. Couplings from cadmium to backbone amide protons that are hydrogen bonded to the Cys-S atoms directly bonded to Cd vary from smaller than 0.3 to 1.8 Hz; a 'through-space' coupling between Cd and the protons of an alanine methyl group was measured to be 0.3 Hz. Couplings to ^{199}Hg are significantly larger and fall in the 0.4–4 Hz range.

Recently, the presence of J couplings between backbone amide protons and ^{113}Cd in ^{113}Cd-substituted protein rubredoxin from *Pyrococcus furiosus* (an archaebacterium that grows optimally at 100°C) was shown by spin-echo difference and HMQC experiments (Blake et al., 1992a). These couplings are apparently mediated via hydrogen bonds to the S atoms of the cysteine residues coordinated to cadmium. In addition, evidence for a weak J interaction between the methyl protons of an alanine residue (A43) and ^{113}Cd was found, which could only arise via a 'through-space' mechanism. The present work describes how these unresolvable couplings can be quantified, and shows that the J couplings fall in the 0.3–1.8 Hz range. We also report the values of these

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couplings measured in ^{199}Hg-substituted rubredoxin and discuss their implication on the nature of the J coupling.

As described below, the heteronuclear J couplings can be measured from a quantitative spin-echo difference spectrum (Freeman et al., 1981), in which care has been taken to purge the effect of antiphase magnetization at the start of data acquisition (Sørensen and Ernst, 1983), and in which sensitivity is improved by removing the effect of homonuclear ^{1}H-^{1}H J modulation (Bax et al., 1989). The pulse sequence used in the present work is as follows:

$$\begin{array}{ll} ^1\text{H: (presat H}_2\text{O)...90}_x \quad \tau \quad 90_{\varphi 1}\text{..}\ \Delta \quad \Delta\, 90_{-\varphi 1}\text{.....}\tau\ \text{Acquire}\ (\psi) \\ ^{113}\text{Cd:} \qquad\qquad\qquad\qquad\quad 90_x 90_{\varphi 2} \qquad\quad 90_x \end{array}$$

with $\varphi_1 = x,y,-x,-y$, $\varphi_2 = 4(-x),4(x)$, and $\psi = 2(x,-x),2(-x,x)$. The ^{1}H carrier is positioned on the H_2O resonance, and the delay $2\Delta + 2\tau_{90}(Cd)$ ($\tau_{90}(Cd)$ is the 90° (Cd) pulse duration) between the $90_{\varphi 1}$ and $90_{-\varphi 1}$ ^{1}H pulses is adjusted to $\sim 1/[2(\delta_{HN} - \delta_{H_2O})]$ ($\Delta = 83$ μs, $\tau_{90}(Cd) = 13.5$ μs), such that the $90_{\varphi 1} - 2\Delta + 2\tau_{90}(Cd) - 90_{-\varphi 1}$ pulse pair functions as a 180° pulse for the amide protons of interest, without significantly affecting H_α protons that resonate close to the H_2O resonance. Consequently, there is no homonuclear J modulation of the amide signals at the start of data acquisition. The $90_x(^{113}Cd)$ purge pulse at the start of data acquisition serves to ensure a purely absorptive character of the signals from protons coupled to ^{113}Cd. For the case where $\varphi_2 = -x$, no heteronuclear J modulation occurs and the amplitude of the ^{1}H signal, detected during data acquisition, is proportional to:

$$S_0 = \exp(-2\tau/T_2) \tag{1a}$$

where T_2 is the transverse relaxation time of the observed proton. For $\varphi_2 = x$, just prior to the ^{113}Cd 90_x purge pulse, heteronuclear dephasing has occurred and the angle between proton multiplet components coupled to ^{113}Cd in the $|\alpha>$ and $|\beta>$ spin states equals $4\pi J_{H\text{-}Cd}\tau$. Consequently, after the $90_x(Cd)$ purge pulse the proton signal is attenuated by $\cos(2\pi J_{H\text{-}Cd}\tau)$, and the detected signal is proportional to

$$S_1 = \exp(-2\tau/T_2)\cos(2\pi J_{H\text{-}Cd}\tau) \tag{1b}$$

From S_0 and S_1, the value of $J_{H\text{-}Cd}$ can be extracted immediately:

$$(S_0 - S_1)/S_0 = 1 - \cos(2\pi J_{H\text{-}Cd}\tau) = 2\sin^2(\pi J_{H\text{-}Cd}\tau) \tag{2}$$

In the above discussion, a number of assumptions have been made. First, it was assumed that the $90_x 90_x(^{113}Cd)$ pulse pair causes perfect inversion of the Cd spin state, and that the $90_x 90_{-x}(^{113}Cd)$ pulse pair causes no inversion. In practice, RF inhomogeneity, resonance offset and miscalibration of the pulse width could decrease the intensity of the difference spectrum, $S_0 - S_1$. Based on tests performed with the same probehead, observing the methyl protons of $^{13}CH_3$-acetate, the difference spectrum is smaller by only ~3% compared to the ideal situation (using the same RF field strength (19 kHz) and resonance offset (100 Hz)). Second, it has been assumed that longitudinal relaxation of ^{113}Cd may be neglected during the spin-echo period of duration 2τ.

Considering that the coordination of the metal is nearly tetrahedral (Blake et al., 1992b; Day et al., 1992) the chemical shift anisotropy of the Cd nucleus should be small (Santos et al., 1991), and the ^{113}Cd T_1 is therefore expected to be at least on the order of ~1 s. For this case, assuming that $J_{H\text{-}Cd}\tau \ll 1$, it can be calculated that the finite life-time of the ^{113}Cd spin state attenuates the intensity of the spin-echo difference spectrum by a few percent at most. Finally, the difference spectrum is affected by the abundance of the ^{113}Cd isotope (91.6%), which decreases S_0-S_1 by 8.4%. As can be seen from Eq. 2, a 1% change in S_0-S_1 results in only a 0.5% change in J, and the above-mentioned corrections only have a very small effect on the accuracy of the coupling measured from the quantitative spin-echo difference experiment. Nevertheless, results from S_0-S_1 were corrected by multiplying them by 1.12, prior to calculation of J.

The method is demonstrated for a sample of ^{113}Cd-substituted *P. furiosus* rubredoxin (3 mM, 90% H_2O/10% D_2O), 25 mM acetate-d_3, pH 6.3, 250 mM NaCl, T = 45°C). Spectra were recorded on a Bruker AMX-600 spectrometer, operating at 600 MHz ^1H frequency. Figure 1A shows the reference spectrum, recorded with the pulse scheme described above, using 1200 scans, but using only the first four steps of the phase cycle. Figure 1B shows the spin-echo difference spectrum, recorded with the full 8-step phase cycle, using 120 000 scans. As can be seen from Eq. 1, for small unresolvable couplings the sensitivity of the spin-echo difference spectrum, S_0-S_1, is maximized for $\tau = T_2$,

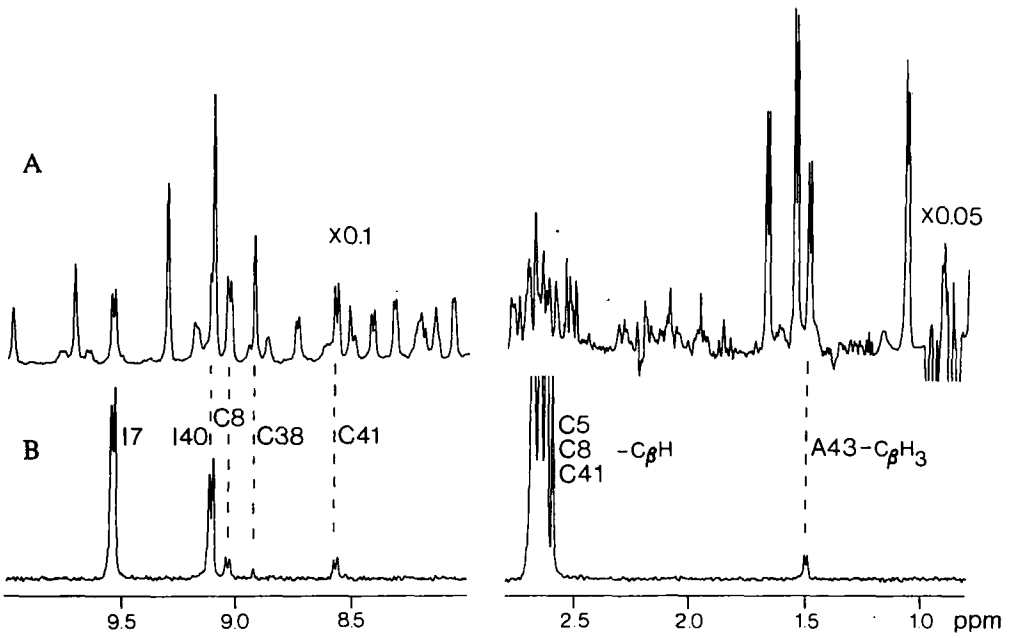

Fig. 1. Selected regions of the ^1H NMR spectrum of ^{113}Cd-substituted rubredoxin, acquired with the spin-echo sequence described in the text. (A) Spectrum obtained using the first 4 steps of the phase cycle, i.e., without affecting the ^{113}Cd spin state. The spectrum results from 1200 transients. (B) Spectrum obtained using the full 8-step phase cycle spin-echo difference experiment with identical parameters compared to (A) except for the number of transients (120 000 total, 60 000 with $\varphi_2 = x$, and 60 000 with $\varphi_2 = -x$). The total measuring time was ~36 h. The downfield and upfield regions of the ^1H spectrum shown in (A) are displayed at 5- and 2.5-fold higher magnification compared to (B), 0.1- and 0.05-fold after correction for the number of transients.

TABLE 1
RELATIVE INTENSITY OF THE SPIN-ECHO DIFFERENCE SPECTRUM $(S_0-S_1)/S_0$, AND THE MAGNITUDE OF $J_{H\text{-}Cd}$ AND $J_{H\text{-}Hg}$

Proton	$\tau = 70$ ms		$\tau = 35$ ms	
	$(S_0-S_1)/S_0$	$J_{H\text{-}Cd}$(Hz)[a]	$(S_0-S_1)/S_0$	$J_{H\text{-}Hg}$(Hz)[a]
I7-H_N	0.28 ± 0.02	1.85 ± 0.1	0.31 ± 0.03	3.9 ± 0.2
C8-H_N	0.027 ± 0.003	0.56 ± 0.03	0.105 ± 0.01	2.2 ± 0.1
C38-H_N	0.0082 ± 0.001	0.30 ± 0.03	0.007 ± 0.002	0.57 ± 0.1
I40-H_N	0.15 ± 0.05	1.34 ± 0.25	0.3 ± 0.1	3.8 ± 0.7
C41-H_N	0.027 ± 0.003	0.56 ± 0.03	0.08 ± 0.015	1.9 ± 0.2
A43-$C_\beta H_3$	0.0078 ± 0.001	0.29 ± 0.03	0.0085 ± 0.001	0.63 ± 0.04

[a] Calculated using Eq. 2, using a 3% correction for RF inhomogeneity and a correction for the isotopic abundance of ^{113}Cd (91.6%) and ^{199}Hg (90.9%).

but this optimum has a very flat maximum and can be changed by ±30% without significantly affecting the sensitivity of the experiment. The spectra shown in Fig. 1 were recorded with $\tau = 70$ ms. The downfield region of the difference spectrum shows the amide signals of I7, I40, C8 and C41, reported previously (Blake et al., 1992a), plus a weak resonance for C38 that previously was not observed because of signal cancellation associated with the ~4Hz $J_{HNH\alpha}$ modulation. The upfield region of the spectrum shows a clear spin-echo difference signal for the A43 methyl protons, in addition to some of the upfield $C_\beta H$ cysteine protons, which have a large 3-bond J coupling to Cd.

Table 1 lists the $(S_0-S_1)/S_0$ ratios, together with the $J_{H\text{-}Cd}$ value derived from this ratio using Eq. 2, after applying the 12% correction to $(S_0-S_1)/S_0$, as mentioned above. As can be seen from this table, the couplings are small and show significant variation. The largest values (1.8 and 1.3 Hz) are observed for I7-H_N and I40-H_N. These residues are located in the i+2 position in the Cys(i)-X(i+1)-X(i+2)-Cys(i+3) metal binding sequences in the protein. Smaller couplings (~0.55 Hz) are observed for C8 and C41, which are the cysteines in the i+3 position. The J coupling observed to C8-H_N, in the i position, is very weak (0.3 Hz).

The structure of zinc-substituted *P. furiosus* rubredoxin has recently been determined by NMR (Blake et al., 1992b) and was shown to be very similar to the crystal structure of the native protein (Blake et al., 1992c; Day et al., 1992). As observed originally in the X-ray structure of *Clostridium pasteurianum* rubredoxin, six backbone H_N protons appear to be involved in hydrogen bonds with cysteine S-atoms, forming Types-I, -II and -III NH-S turns (Adman et al., 1975). Four of these six (I7, C8, I40 and C41) show J coupling to Cd, suggesting that these hydrogen bonds contain significant covalent character. The largest couplings (1.8 and 1.3 Hz) are observed for the I7 and I40 amides that are involved in type-I NH-S tight turns. Weaker coupling (0.56 Hz) is observed for the C8 and C41 amides that are involved in type-III NH-S tight turns, and no coupling is detected for the amides of Y10 and A43 that are proposed to form type-II NH-S turns, indicating that these J values must be smaller than ~0.2 Hz in ^{113}Cd-rubredoxin. Interestingly, ^2H exchange experiments suggest that the Y10 and A43 amides may form the strongest hydrogen bonds (Blake et al., 1992a). Clearly, the magnitude of the J coupling is also modulated by factors other than the strength of the hydrogen bond. The very weak J coupling to C38-H_N, which is not hydrogen

bonded to S, is presumably a regular 5-bond J coupling. Most remarkable is the 0.3 Hz J coupling between the methyl protons of A43 and Cd. These nuclei are separated by 11 chemical bonds, and a 'through-space' J coupling mechanism (i.e., coupling mediated by direct overlap of the CH_3 orbitals and the ^{113}Cd and/or Cys-S orbitals) provides the only possible explanation for this coupling. Indeed, the NMR and X-ray structures indicate a very short distance (3.4 Å) between these methyl protons and the metal.

'Through-space' J couplings involving fluorine have commonly been observed in many organic compounds where the second nucleus is brought in close proximity to the fluorine (Mallory et al., 1992). However, to the best of our knowledge no such couplings between protons and metal nuclei have ever been reported. It is therefore essential to consider the possibility that the spin-echo difference spectrum could arise from dipolar cross correlation effects (Wimperis and Bodenhausen, 1989). In simple terms, the ^1H T_2 is determined primarily by the sum of the local fields. It is then conceivable that for a subset of the molecules with particular ^1H and ^{113}Cd spin states the sum of all dipolar local fields at the position of the proton of interest is very close to zero, giving rise to a very long T_2 for this subset, and causing the T_2 of the entire sample to be very non-exponential. However, if the ^{113}Cd spin state is inverted halfway through the spin-echo period, the sum of all local fields cannot be zero for the entire spin-echo period, decreasing the degree of non-exponentiality. The difference in this degree of non-exponentiality can give rise to a spin-echo difference (and HMQC) spectrum (Wimperis and Bodenhausen, 1989). We do not believe that this cross-correlation effect is responsible for the observed J couplings because the size of the dipolar ^1H-^{113}Cd coupling, corresponding to a ^1H-^1H dipolar coupling for two protons ~5 Å apart, appears too small for the relatively large effects observed. However, it is difficult to quantitate the exact magnitude of the cross-correlation effect as this requires detailed knowledge of the proton chemical shift anisotropy. Therefore we have resorted to a different solution for eliminating the remote possibility of cross correlation by also measuring the J values in ^{199}Hg-substituted rubredoxin.

Figure 2 shows the spectra recorded for a 6-mM sample of ^{199}Hg-substituted rubredoxin. Experimental conditions were the same as for Fig. 1, except that the data were obtained with a General Electric Omega PSG 600 spectrometer, using a 2-fold higher sample concentration, a τ delay of 35 ms and a 0.4 times smaller number of transients. The ^1H chemical shift patterns for the ^{199}Hg and ^{113}Cd adducts are very similar, indicating that their structures are also very similar (Blake et. al., unpublished data). All of the J couplings measured for the ^{199}Hg-substituted rubredoxin are at least 2-fold larger than for ^{113}Cd. If the spin-echo difference spectrum were due to cross correlation, a smaller effect would be expected for ^{199}Hg because of its 20% smaller magnetogyric ratio. This strongly indicates that the J coupling is of the regular electron orbital-mediated type. In addition, the I7-H_N J coupling to ^{199}Hg is sufficiently large to become partially resolved by strong resolution enhancement (inset Fig. 2B), which would not be possible in the case of cross correlation. The ^{199}Hg-H_N J splitting measured for the resolved doublet of doublets (3.9 Hz) agrees very well with the 3.9 ± 0.2 Hz measured from the spin-echo difference spectrum.

Interestingly, a weak coupling (0.4 Hz) to the Y10 H_N proton is also observed in Fig. 2, but signal overlap precludes the analogous coupling to be observed for A43 H_N. Thus, NH··S hydrogen-bond-mediated scalar coupling is now observed for all three types of NH··S turns.

The method presented here for quantitation of J couplings enables the measurement of couplings that are up to 10 times smaller than the natural line width of the proton involved. The accuracy is only limited by the signal-to-noise ratio of the difference spectrum, and as discussed

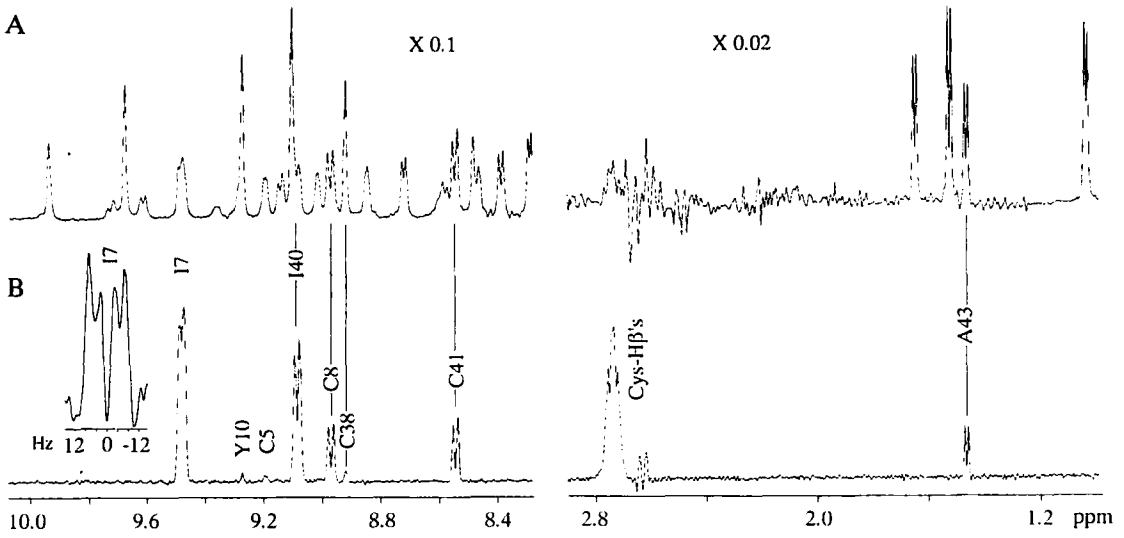

Fig. 2. Selected regions of the ^{1}H NMR spectrum of ^{199}Hg-substituted rubredoxin, acquired with the spin-echo sequence described in the text. Except for the differences mentioned in the text, the spectra were recorded under the same conditions as in Fig. 1. The downfield and upfield regions of the ^{1}H spectrum shown in (A) are displayed at 0.1- and 0.02-fold magnification compared to (B), after correction for the number of transients. Inset: ^{1}H-^{199}Hg J splitting for I7-H_N resolved by strong resolution enhancement.

above, by parameters such as T_1 of the second nucleus, pulse imperfection, and isotopic abundance. The method is conceptually closely related to a recently proposed method for measurement of ^{13}C-^{13}C long-range J couplings in ^{13}C-enriched proteins (Bax et al., 1992). Note that E-COSY-based techniques (Griesinger et al., 1986; Worgötter et al., 1988), which frequently are used for measurement of unresolvable J couplings, are not applicable to the J_{HN-X} problem, because this latter approach requires a third nucleus with a resolved J coupling to both other nuclei.

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