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Sensitivity Enhancement of Multidimensional NMR Experiments by Paramagnetic Relaxation Effects

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Abstract: One of the main goals of NMR method development is to increase the sensitivity of multidimensional NMR experiments or reduce the required acquisition time. In these experiments, more than 80% of the NMR instrument time is spent on the recycle delay, where the instrument idles to wait for the recovery of proton magnetization. In this study, we report a method of using paramagnetic relaxation effects to shorten the recycle delays required in multidimensional NMR experiments of biological macromolecules. This approach significantly reduces the NMR instrument time required. Ni^{2+} ion, complexed with the chelating molecule DO2A, is used to decrease the proton T_1 relaxation time of biological macromolecules without the significant line-broadening effects that are associated with most paramagnetic ions. The Ni(DO2A) also significantly decreases the T_1 relaxation time of water, thus providing additional sensitivity gain by eliminating the saturation of labile amide resonances.

Introduction

A bottleneck in NMR studies of biological macromolecules is the time it takes to acquire multidimensional NMR spectra, because of the intrinsic low sensitivity of nuclear spins. Sensitivity enhancement through better instrumentation (i.e., better probe designs) or experimental designs (i.e., nonlinear sampling) is one of the main focuses in improving NMR technology. Solution NMR experiments, particularly those of biological macromolecules, are mostly designed to start with ^{1}H-magnetization and end with ^{1}H-detection, because ^{1}H is usually the most sensitive nucleus of these molecules. Recycle delays, during which the instrument idles to wait for the recovery of magnetization, are approximately one second, which correlates to the proton T_1 relaxation time.[1] The recycle delays consume 80% or more of the NMR instrumental time in solution NMR experiments.

Several approaches to shorten the recycle delays have been reported. Pervushin and co-workers[2] have recently developed a longitudinal ^{1}H relaxation optimized TROSY method to reduce recycle delays for large macromolecules. An alternative approach to shorten the recycle delay is to use paramagnetic reagents. Pervushin and co-workers[3] used Gd^{3+} to reduce the ^{13}C T_1 and to shorten the recycle delay in carbon-detected experiments. Wüthrich and co-workers,[4] used 1 mM Gd^{3+} to reduce the proton T_1 of water from more than 3 s to 0.3 s, resulting in a significant signal-to-noise ratio increase of the labile amide protons. However, Gd^{3+} complexes can cause significant line broadening effects for protons due to Gd^{3+}'s long electronic relaxation time (T_{1e} and T_{2e} have similar values, in the range of nano to micro seconds),[5] and thus cannot be used for most proton-detection solution NMR experiments with magnetization starting on proton.

In this study, we demonstrate that the use of Ni(DO2A) (a neutral, chelated form of Ni^{2+}) can significantly enhance the longitudinal relaxation rates of macomolecular and water proton resonances without a significant line-broadening effect. This is because Ni^{2+}, in contrast to Gd^{3+}, has a much shorter electronic relaxation time (in the range of pico to nano seconds).[5] Thus, the addition of Ni(DO2A) allows the use of much shorter recycle delays for solution NMR experiments that start on protons and end with proton detection. This approach significantly increases the sensitivity of NMR spectra by allowing more transients in the same NMR instrument time and by eliminating water saturation, which enhances the sensitivity of labile amide protons.

Theory

In the presence of paramagnetic species in solution, the spin relaxation rates of nuclear spins are given by (neglecting the chemical exchange term)[4,6]

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$$R_1 = R_{1d} + c \cdot R_{1p} \quad (1)$$

$$R_2 = R_{2d} + c \cdot R_{2p} \quad (2)$$

where R_{1d} and R_{2d} are the longitudinal and transverse relaxation rates, respectively, of nuclear spins in the absence of paramagnetic effects, R_{1p} and R_{2p} represent the longitudinal and transverse relaxation enhancement, respectively, induced by the unpaired electron, and c is the concentration coefficient of the paramagnetic species. The relaxation enhancements induced on carbon and nitrogen nuclei can be neglected in this study, because of their much smaller gyromagnetic ratios compared to protons'.

If the interaction between the unpaired electron and nuclear spin is purely dipolar (no specific interactions between the macromolecule and the paramagnetic compound), the paramagnetic relaxation enhancement terms can be described by[7]

$$R_{1p} = \frac{2S(S+1)\gamma_I^2 g^2 \beta^2}{15r^6}\left[\frac{\tau_{c2}}{1+(\omega_I-\omega_S)^2\tau_{c2}^2} + \frac{3\tau_{c1}}{1+\omega_I^2\tau_{c1}^2} + \frac{6\tau_{c2}}{1+(\omega_I+\omega_S)^2\tau_{c2}^2}\right] \quad (3)$$

$$R_{2p} = \frac{S(S+1)\gamma_I^2 g^2 \beta^2}{15r^6}\left[4\tau_{c1} + \frac{\tau_{c2}}{1+(\omega_I-\omega_S)^2\tau_{c2}^2} + \frac{3\tau_{c1}}{1+\omega_I^2\tau_{c1}^2} + \frac{6\tau_{c2}}{1+\omega_S^2\tau_{c2}^2} + \frac{6\tau_{c2}}{1+(\omega_I+\omega_S)^2\tau_{c2}^2}\right] \quad (4)$$

where S is the electronic spin number, γ is the nuclear gyromagnetic ratio, g is the electronic g factor, β is the Bohr magneton, r is the distance between an electron and nuclear spins, ω_I and ω_S are the respective Larmor frequencies of protons and electrons, and τ_{c1} and τ_{c2} are the proton effective correlation times in the longitudinal and transverse directions, respectively. They are determined by the protein rotational correlation time τ_r and the longitudinal and transverse electronic relaxation time T_{1e} and T_{2e} as follows:

$$1/\tau_{c1} = 1/T_{1e} + 1/\tau_r \quad (5)$$

$$1/\tau_{c2} = 1/T_{2e} + 1/\tau_r \quad (6)$$

To simplify analysis, we neglected the exchange terms (e.g., the contribution from the finite residential time of the paramagnetic species) in eqs 5 and 6, according to Wûthrich and coworkers.[4] This contribution can be included in the coefficient c in eqs 1 and 2. Although $T_{1e} \geq T_{2e}$, in many cases T_{1e} and T_{2e} have similar values.[8] We assume $T_{1e} = T_{2e}$ in our following analysis.

In this study, we used Ni(DO2A) to enhance the T_1 relaxation rates of protons in solutions of proteins. The electronic relaxation time of Ni^{2+} (as well as some other metal ions such as V^{3+} and low spin Fe^{3+}) at $10^{-13}-10^{-10}$ sec[5] is much smaller than the rotational correlation time of a macromolecule (τ_r). Assuming τ_r is approximately 10 ns (i.e., a small to medium size protein), τ_{c1} and τ_{c2} are dominated by T_{1e} (or T_{2e}) at 500 MHz or above, and thus eqs 3 and 4 reduce to

$$R_{1p} \approx R_{2p} = \frac{20S(S+1)\gamma_I^2 g^2 \beta^2}{15r^6} T_{1e} \quad (7)$$

In this case, R_{1p} and R_{2p} have similar values, and thus these ions cause similar enhancement in longitudinal and transverse relaxation rates. This is particularly useful for biological macromolecules, since their proton T_1 relaxation times are much longer than their T_2 relaxation times, and thus T_1 can be shortened significantly without a significant effect on T_2. For example, typical proton T_{1d} and T_{2d} are approximately 1 s and 20 ms, respectively, for a small to medium size protein. If the concentration of a paramagnetic reagent is adjusted so that $c \times R_{1p}$ and $c \times R_{2p}$ are approximately 5 Hz, T_1 is shortened from 1 s to 167 ms, but T_2 is only shortened from 20 to 18.2 ms. Therefore, these paramagnetic ions can decrease the proton longitudinal relaxation time dramatically (more than 80%) with a very little effect on line broadening (only 10%).

Gd^{3+}, Mn^{2+}, and Cu^{2+} can be used to enhance ^{13}C R_1 of macromolecules[3] and the proton R_1 of the water resonance.[4,9] However, these ions have long electronic relaxation times ($10^{-8}-10^{-6}$ sec),[5] which are larger than the rotational correlation times of biomolecules; thus, τ_{c1} and τ_{c2} are dominated by τ_r. In such cases, eqs 3 and 4 are reduced to

$$R_{1p} = \frac{S(S+1)\gamma_I^2 g^2 \beta^2}{15r^6} \times \frac{6\tau_r}{1+\omega_I^2\tau_r^2} \quad (8)$$

$$R_{2p} = \frac{S(S+1)\gamma_I^2 g^2 \beta^2}{15r^6}\left[4\tau_r + \frac{3\tau_r}{1+\omega_I^2\tau_r^2}\right] \quad (9)$$

In this situation, R_{1p} is much smaller than R_{2p}, and therefore, these ions will cause significant line-broadening effects if one wants to obtain detectable longitudinal relaxation enhancement. Thus, Gd^{3+} ions are good candidates when line broadening effects are needed, such as in mapping the binding interface,[10,11] but not useful for shortening proton T_1 relaxation times of proteins and nucleic acids.

Materials and Methods

Sample Preparation. All experiments were carried out using SUMO-1, a 12 kDa ubiquitin-like protein. ^{15}N and $^{13}C/^{15}N$-labeled SUMO-1 with N-terminal His_6-tag was expressed and purified as described previously.[12] DO2A·2.8HCl·1.1H_2O was synthesized as described.[13] NMR samples contained 1 mM SUMO-1 in 10% D_2O/90% H_2O, 20 mM Tris buffer at pH 7.2. Either EDTA or DO2A·2.8HCl·1.1H_2O was used to form a chelate complex with Ni^{2+} or Mn^{2+} by directly titrating the aqueous chelate into a solution of $NiSO_4$ or $MnCl_2$. To ensure that all Ni^{2+} was complexed by the chelate, DO2A or EDTA was used in 30% molar excess. After titration, the pH was adjusted to the corresponding pH of the protein sample. These solutions were then titrated to the protein sample used in the T_1 and T_2 measurements. For the sensitivity enhancement experiments with different Ni(DO2A) concentrations, desalted Ni(DO2A) was used to minimize the salt effects on experimental sensitivity. Anhydrous $NiCl_2$ (60 mg) and 200 mg of the ligand (5% in excess) were dissolved in 5

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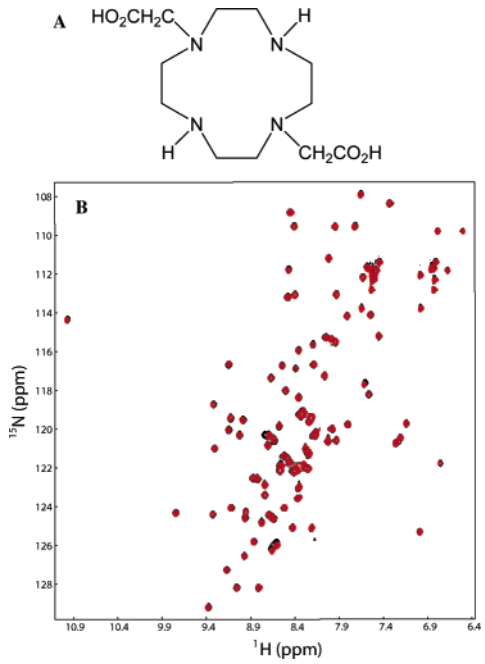

Figure 1. (A) The chemical structure of DO2A. (B) The HSQC spectra of SUMO-1 alone (black) and in the presence of (red) 20 mM Ni(DO2A).

mL of water, and OH-form anion-exchange resin (Dowex 1 × 8−100) was added in small portions with shaking until the pH of the mixture reached 7. The purple solution was allowed to stand at room-temperature overnight and was then freeze-dried to give 165 mg of a purple solid.

NMR Measurements. All NMR spectra were obtained at 25 °C on a Bruker Avance 500 equipped with a room-temperature probe or a Bruker Avance 600 spectrometer with a cryo-probe. Proton longitudinal and transverse relaxation times were measured using the nonselective inversion−recovery and the spin−echo (non-CPMG) method, respectively. To eliminate the radiation damping effect for the measurement of T_1 relaxation time, weak gradients were applied continually during the T_1 relaxation delay (the time between the 180° inversion pulse and the read-out pulse). The spectra were processed using NMRPipe[14] and analyzed using NMRView.[15]

Results and Discussion

Choice of the Paramagnetic Ion and the Chelate Molecule. As we have shown in the Theory section, metal ions with fast electronic relaxation rates, such as Ni^{2+}, can enhance proton longitudinal relaxation of proteins and nucleic acids without significantly affecting proton transverse relaxation. We therefore tested such effects experimentally. To prevent specific binding of Ni^{2+} to proteins, we used a neutral chelated form of Ni^{2+}. We first tested the common chelate, EDTA (ethylenediaminetetraacetic acid). However, with the $Ni(EDTA)^{2-}$ complex, a few residues of SUMO-1 showed significant line broadening and were unobservable in the ^1H-^{15}N HSQC spectra, indicating specific interactions of the paramagnetic species with the protein. This is possibly due to electrostatic interactions, because $Ni(EDTA)^{2-}$ has a negative charge.

We then tested DO2A (1,7-dicarboxymethyl-1,4,7,10-tetraazacyclododecane; Figure 1A) as a chelating molecule for Ni^{2+}. DO2A forms stable, 1:1 chelates with most transition metal and lanthanide ions.[13,16] Ni^{2+} binds to DO2A so strongly (stability constant log $K_f = 16.5$)[17] that it does not bind the His_6-tag, as confirmed by the following experiment: 0.2 mM free $NiSO_4$ in 1 mM SUMO-1 sample caused significant line broadening effects on the N-terminal residues adjacent to the N-terminal His_6-tag (data not shown), whereas no residue showed such significant line-broadening effect in 50 mM Ni(DO2A). Another advantage of choosing DO2A as the ligand is that the Ni(DO2A) complex has a net zero charge, which minimizes potential electrostatic interactions of the paramagnetic complex with proteins or nucleic acids. Figure 1B shows that, unlike EDTA complex, Ni(DO2A) did not induce significant line broadening effects and chemical shift changes on SUMO-1. A further advantage of using DO2A is that the uncharged Ni(DO2A) has low conductivity, allowing it to be used in cryoprobes at high concentrations without suffering reduced sensitivity.[18]

Effect of Ni^{2+} on Proton T_1 and T_2 Relaxation Times. We investigated how the proton T_1 and T_2 relaxation times of different SUMO-1 residues were affected at different concentrations of Ni^{2+} and Mn^{2+} complexes (see Figure 2A,B). Ni(DO2A) at 20 mM decreased the amide proton T_1 relaxation times dramatically for the residues in the unstructured termini and loops, but had little effect on the residues of well structured regions. The T_2 relaxation times were not significantly affected for all residues. Mn(DO2A) at 3 mM induced a similar degree of proton R_1 enhancement as 20 mM Ni(DO2A), but it significantly decreased the proton T_2 relaxation times for most residues. Ni(DO2A) at 50 mM decreased the proton T_2 for residues in the flexible terminal and loop regions, while its effect on the residues in the structured region was negligible. However, it induced the largest proton R_1 enhancement among three samples with the longest T_1 around 0.5 s.

The overall R_2 enhancements afforded by 50 mM Ni(DO2A) were much smaller than those afforded by 3 mM Mn(DO2A). These results show that Ni(DO2A), but not Mn(DO2A), can be used to decrease proton T_1 relaxation times of macromolecules significantly with little line-broadening effects because the electronic relaxation time of Ni^{2+} is faster than the rotational correlation time of biological macromolecules. In contrast, metal ions with slow electronic relaxation times, such as Mn^{2+} and Gd^{3+} (as well as organic free radicals, such as TRMPO[19,20]), enhance R_2 relaxation rates more significantly, and thereby induce line-broadening.[10,11] Our data also showed that the magnitude of T_1 enhancement is not uniform throughout the protein, and depends strongly on the solvent accessibility of the individual residues. This is likely due to the fact that solvent accessible residues are also more accessible by the paramagnetic complex (see the section on labile and nonlabile protons below).

Time Reduction and Sensitivity Enhancement of NMR Experiments using Paramagnetic Ions. Because Ni(DO2A) can enhance T_1 relaxation with a little effect on T_2 relaxation,

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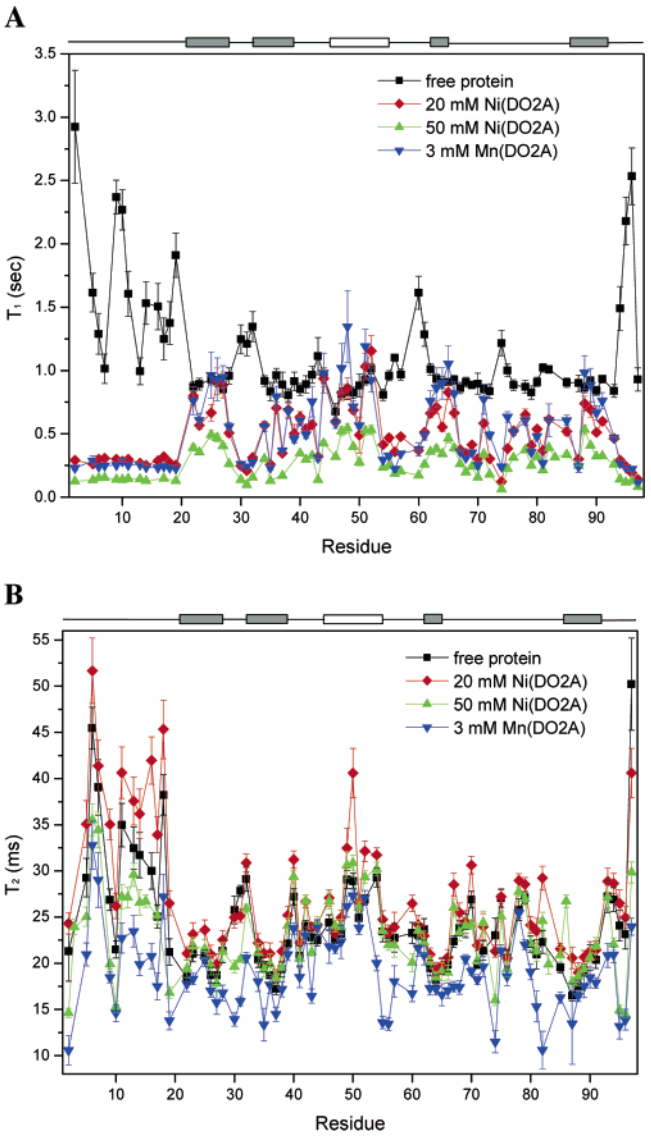

Figure 2. (A) The proton T_1 and (B) T_2 of SUMO-1 residues in the presence of different paramagnetic complexes and concentrations. The secondary structures are shown on the top of the figure: gray bar, β strand; open bar, α-helix.

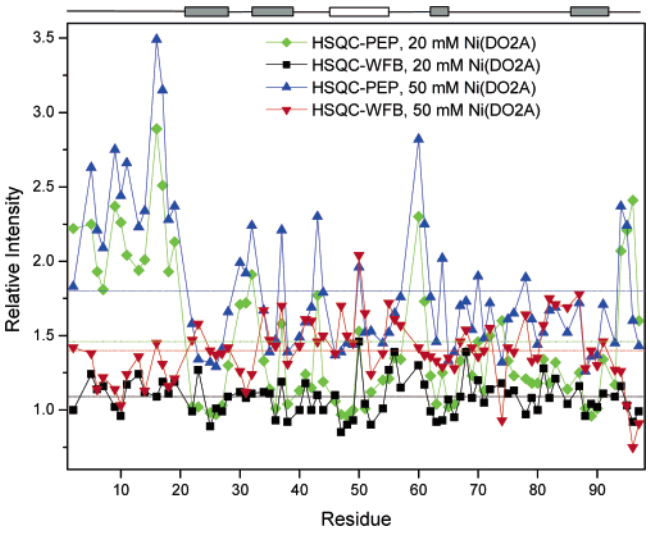

Figure 3. Relative sensitivities of different HSQC experiments in the presence of different concentrations of Ni(DO2A), normalized to the same NMR time with 0.5 s recycle delays. The average relative sensitivities are plotted as follows: black, HSQC−WFB, 20 mM Ni(DO2A); red, HSQC−WFB, 50 mM Ni(DO2A); green, HSQC−PEP, 20 mM Ni(DO2A); blue, HSQC−PEP, 50 mM Ni(DO2A). The secondary structures are shown on the top of the figure: gray bar, β strand; open bar, helix.

Table 1. Experiments Using 0.5 Second Recycle Delays in the Presence of Various Paramagnetic Complex Concentrations Were Compared to the Equivalent Experiments Using 1 sec Recycle Delays and No Paramagnetic Complex[a]

experiment	concentration of Ni^{2+} (mM)	enhancement (%)
HSQC−WFB	0	−9
	20	9
	50	40
HSQC−PEP	0	−14
	20	46
	50	80
HNCA-WFB	20	7
	50	86
CBCA(CO)NH−WFB	20	30
	50	114

[a] Average sensitivity enhancements are summarized.

we examined the amount of NMR instrument time that could be saved by reducing the inter-scan recycle delays. This also allows sensitivity enhancement by allowing more transients within the same instrumental time. The effects of Ni(DO2A) was tested using two types of experiments: the first with water-gate to suppress the water resonance, in combination with a water flip-back pulse[21] to keep the water magnetization on +Z (WFB), and the second using the sensitivity enhancement scheme[22] with water dephased by gradients before acquisition (PEP). Figure 3 shows the relative sensitivities of enhanced $^1H-^{15}N$ HSQC experiments in the presence of different concentrations of Ni(DO2A) using 0.5 s recycle delays. The relative sensitivities are the peak intensities of the experiments using 0.5 s recycle delays, expressed relative to those without Ni(DO2A) using 1.0 s recycle delays and normalized to the same total NMR instrumental time. In the absence of Ni(DO2A), there was a slight loss of sensitivity by using 0.5 s recycle delay and twice the number of scans (Table 1). However, in the presence of the Ni^{2+} complex, the experiments with 0.5 s recycle delays lead to significant sensitivity enhancements that were dependent on the concentration of the paramagnetic ion (Figure 3 and Table 1).

As predicted by the differential R_1 enhancements, the sensitivity enhancement is not the same for all residues of the protein. In experiments with water flip-back pulses, the terminal and loop regions have the least sensitivity gain and sometimes sensitivity loss. Because these residues have longer T_2 and higher solvent accessibility, they experience significant reductions of the T_2 relaxation times (Figure 2B), and hence much less sensitivity gains. However, because these residues have high signal intensities to begin with, their resonances remain strong even with the reductions of their T_2 relaxation times. In fact, the shortened T_2 relaxation times were beneficial in producing more spectral evenness. However, in experiments without water flip-back pulses, the terminal and loop regions experience the

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Table 2. Proton T_1 of Water With and Without the Paramagnetic Complexes

complexes	T_1 of water (sec)
none	3.2
20 mM Ni^{2+}	0.31
50 mM Ni^{2+}	0.13
3 mM Mn^{2+}	0.26

most significant sensitivity gain, due to the effect of water magnetization on these labile residues.

The Differential Effect of the Paramagnetic Ion on Nonlabile and Labile Protons. Sensitivity enhancement is much larger in PEP type experiments (without water flip-back) than that in the WFB type experiments (with water flip-back pulse), especially for residues in the terminal and loop regions. This is likely due to the fact that in WFB type experiments, exchange between water protons (always on + Z) and the protein amide protons enhances the amide proton longitudinal relaxation. Thus the WFB type experiments benefit less from amide proton T_1 shortening in the presence of Ni(DO2A). For the PEP type of experiments, the presence of Ni(DO2A) also induces much faster relaxation of the water magnetization (see Table 2), and thus enhances sensitivity not only by enhancing T_1 relaxation of amide protons but also by reducing water resonance saturation.

We also examined sensitivity enhancement produced by using Ni(DO2A) in 3-D triple-resonance experiments. We measured the relative sensitivities of HNCA-WFB and CBCA(CO)NH−WFB with 20 and 50 mM Ni(DO2A) using 0.5 s recycle delays (Figure 4). The HNCA-WFB experiment showed a similar extent of sensitivity enhancement as the HSQC−WFB experiment, while the CBCA(CO)NH−WFB experiment presented a much larger sensitivity gain. The difference in sensitivity gain between HNCA-WFB and CBCA(CO)NH−WFB can be ascribed to the different effects of water on labile and nonlabile resonances. For experiments with magnetization starting from amide protons, as in HNCA-WFB, the exchange between water (along +Z) and amide protons helps to decrease the amide proton T_1 significantly, which is independent of the paramagnetic effect. In contrast, the CBCA(CO)NH−WFB experiment originates on nonlabile H_α and H_β resonances, which are affected less by the water resonance, and therefore benefits more strongly from the presence of Ni(DO2A). To confirm the difference in water effects on amide and aliphatic protons, we simultaneously measured the amide and aliphatic proton T_1 with water magnetization kept on +Z or saturated. With water saturated, the amide and aliphatic protons relaxed at very similar rates. However, when the water magnetization was kept on +Z, the T_1 of aliphatic protons changed very little, while the amide protons relaxed faster, especially among residue in unstructured regions (data not shown). Therefore, these results indicate that Ni(DO2A) can also decrease the T_1 relaxation times of nonlabile aliphatic resonances significantly.

In conclusion, we have demonstrated that appropriate paramagnetic metal ions and chelate molecules can be used to enhance proton longitudinal relaxation rates of proteins with minimal line-broadening effects. Using 50 mM Ni(DO2A), 80−114% sensitivity enhancement (corresponding to a 70%-75% NMR time reduction) can be obtained by shortening inter-scan recycle delays. The degree of sensitivity enhancement by a paramagnetic complex depends on solvent accessibility of the amino acid residues, lability of the protons where magnetization originates, and whether the experiment saturates water resonances. The approach described in this study is particularly useful for proteins with large segments of unstructured regions, which usually give strong signals with severe truncation artifacts, by producing more even spectra. The Ni(DO2A) complex can reduce both the T_1 and T_2 of these residues, and hence their truncation artifacts, while providing sensitivity enhancement for other residues in the well-structured regions at the same time. Another benefit of Ni(DO2A) is the shortening of water proton T_1 without causing significant line-broadening effects for residues in structured regions. The Ni^{2+} complex can also be used in combination with SEA-TROSY (Solvent Exposed Amides with TROSY)[23,24] to study the surface residues of very large proteins (>300 residues) and improve the performance of SEA-TROSY techniques.

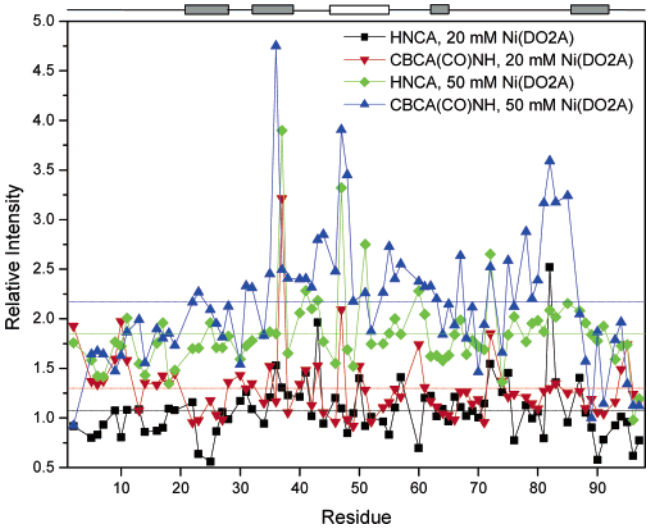

Figure 4. Relative sensitivities per unit of NMR instrument time of HNCA-WFB and CBCA(CO)NH experiments in the presence of different concentrations of Ni(DO2A), using 0.5 s recycle delays. The average relative sensitivities are plotted in the figure as follows: black, HNCA-WFB, 20 mM Ni(DO2A); red, CBCA(CO)NH−WFB, 20 mM Ni(DO2A); Green, HNCA-WFB, 50 mM Ni(DO2A); blue, CBCA(CO)NH−WFB, 50 mM Ni-(DO2A). The secondary structures are shown on the top of the figure: gray bar, β strand; open bar, helix.

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