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^{113}Cd-^{1}H heteroTOCSY: A method for determining metal–protein connectivities

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SUMMARY

^{113}Cd-^{1}H NMR correlation experiments have been extremely useful for determining the amino acid ligands that form metal-binding sites in proteins. To date, the majority of these methods have used heteronuclear multiple-quantum transfer as the basis for establishing correlations. In this paper, we demonstrate the feasibility of using correlation methods that employ heteronuclear cross-polarization (heteroTOCSY) as viable alternatives. Additionally, we couple heteroTOCSY with selective excitation and transfer procedures to take advantage of the small number of heteronuclei usually present in metalloprotein systems. One- and two-dimensional experiments are presented as examples of these techniques.

INTRODUCTION

^{113}Cd NMR spectroscopy has been used to probe the structure of metalloproteins for over 15 years (reviewed by Coleman (1993)). The spin 1/2 isotopes ^{113}Cd and ^{111}Cd have been substituted at zinc, calcium, copper, iron and magnesium binding sites in proteins utilizing ligands ranging from all sulfur to all oxygen as well as combinations of sulfur, nitrogen and oxygen donors. To identify the amino acid side chains that are involved in coordinating the cadmium atom(s), ^{113}Cd-^{1}H heteronuclear correlation experiments typically have been used. To date, the majority of these methods have utilized nonselective rf pulses in combination with periods of free precession to accommodate heteronuclear coherence transfer, as in the HMQC experiment (Frey et al., 1985).

An example of a metal–protein system that was initially characterized by these techniques is the Cys_6Zn_2 binuclear cluster motif. Originally identified in the DNA binding domain of the yeast transcription factor GAL4 (Pan and Coleman, 1990a,b), it has since been found to be conserved in over 20 proteins, including LAC9 from *Kluyveromyces lactis* (Pan et al., 1990). Figure 1 shows

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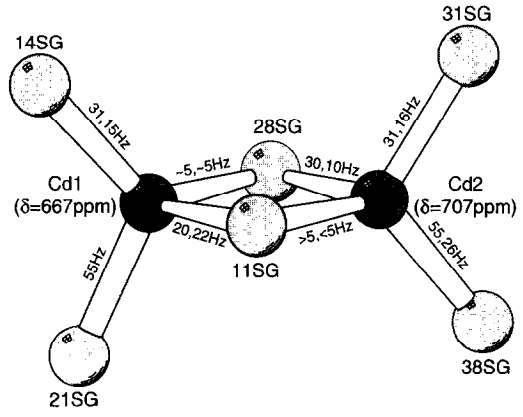

Fig. 1. Metal–protein connectivities in the Cd_2GAL4(62*) binuclear cluster. A similar arrangement exists in LAC9(61), with the analogous cysteines at amino acid positions 95, 98, 105, 112, 115 and 122, with ^{113}Cd chemical shifts of 691 and 706 ppm. Dark grey spheres represent cadmium atoms, light grey spheres represent the γ sulfur atoms of the metal-binding cysteine side chains. Atomic coordinates are derived from Cd_2GAL4(65) structures deposited in the Brookhaven Protein Data Bank (1d66: Marmorstein et al., 1992; 125d: Baleja et al., 1992). $^3J_{^{113}Cd-^1H^\beta}$ coupling constants are listed along the bond connecting the cadmium to the cysteine which contains the β proton involved in the coupling; these values were previously determined by examination of ^{113}Cd-induced splittings in ^1H COSY and $^{113}Cd/^1H$ HMQC spectra (Gardner et al., 1991). The molecular graphics in this figure were produced using the MOLSCRIPT software package (Kraulis, 1991).

a diagram of the six cysteines coordinating two cadmium ions in place of the biologically present zinc, using the ligands established by ^{113}Cd-^1H HMQC and ^{113}Cd-filtered ^1H COSY techniques in GAL4(62*) (Gadhavi et al., 1991; Gardner et al., 1991). Additionally shown are the values of the coupling constants for the three-bond ^{113}Cd-$^1H^\beta$ ($^3J_{Cd-H}$) couplings as resolved from 2D ^1H COSY spectra. It is apparent that a given cadmium nucleus has relatively large coupling constants to the protons on three of the four liganding cysteine residues. The magnitude of the $^3J_{Cd-H}$ coupling constant varies over a range of 5 to 55 Hz; comparing these values to the Cd-S-C-H^β dihedral angles from the X-ray and NMR structures of Cd_2GAL4(65) (Baleja et al., 1992; Marmorstein et al., 1992) shows them to be consistent with the 'Karplus-like' relationship established by work on Cys_4 ^{113}Cd- and ^{199}Hg-substituted rubredoxins (Henehan et al., 1993; Blake et al., 1994) and $^{113}Cd_7$-substituted metallothionein (Zerbe et al., 1994).

Although free precession-based techniques worked successfully to characterize the metal–protein interactions in the GAL4 system, problems were encountered when attempting to extend this work to LAC9, another member of the Cys_6Zn_2 class. Thus, we turned to a previously unexplored alternative for establishing ^{113}Cd-^1H correlations, using heteronuclear cross-polarization methods, as exemplified by the heteroTOCSY or HEHAHA experiments (Müller and Ernst, 1979; Bearden and Brown, 1989; Zuiderweg, 1990; Brown and Sanctuary, 1991; Ernst et al., 1991). This class of experiments is well suited for application to ^{113}Cd-substituted proteins for a

Abbreviations: GAL4(62*): the N-terminal 62 amino acids of the GAL4 protein (Met^1 to Leu^{61}) with a D62E mutation introduced during cloning; LAC9(61): a 61-residue internal fragment of the LAC9 protein (Lys^{85} to Arg^{144}, with an N-terminal methionine residue added during cloning).

variety of reasons. Cadmium-113 compounds are commercially available in isotopically enriched forms (>90% abundance), and the cadmium nucleus has a relatively high gyromagnetic ratio ($\gamma = -5.95 \times 10^7$ rad T^{-1} s^{-1}). The second fact makes transfers to ^1H observable without a preliminary proton→cadmium transfer step, as is necessary for other heteronuclei such as ^{15}N. Additionally, previous studies have suggested that heteronuclear cross-polarization methods are superior to coherence transfer methods for transferring magnetization from rapidly relaxing nuclei to more slowly relaxing nuclei (Majumdar et al., 1993; Richardson et al., 1993). In our systems, the cadmium nuclei generally relax significantly faster than the protons to which they are coupled (Gardner et al., 1991; K.H. Gardner and J.E. Coleman, unpublished results). The efficiency of polarization transfer by heteronuclear cross-polarization methods has also been found to be less dependent on the values of heteronuclear coupling constants (Zuiderweg, 1990); this should benefit our studies, given the wide range of $^3J_{Cd\text{-}H}$ we expect in these systems (Fig. 1). Finally, one can observe remote couplings simply by extending the heteroTOCSY mixing period without adding additional relay steps, facilitating the assignment of entire side chains.

Heteronuclear cross-polarization transfer has been incorporated into several multidimensional NMR experiments for use on proteins or nucleic acids, which have relatively large numbers of heteronuclei (^{13}C,^{15}N,^{31}P) present (Kellogg, 1992; Kellogg and Schweitzer, 1993; Majumdar et al., 1993; Richardson et al., 1993). However, most metalloproteins bind a small number of metals, reducing the potential for chemical shift overlap among the heteronuclei. This situation allows the reduction of spectral resolution in the ^{113}Cd dimension of multidimensional experiments and provides the opportunity to reduce the dimensionality of experiments through the use of selective excitation (Kessler et al., 1986). Both of these modifications allow for reduced acquisition times or the acquisition of subspectra with improved signal-to-noise ratios. The benefits of selective excitation can be extended to systems with multiple heteronuclei present, as will be demonstrated below.

In this paper we demonstrate the application of selective heteroTOCSY experiments to the $Cys_6{}^{113}Cd_2$ binuclear cluster of the transcription factor LAC9 and compare the results to those generated by standard free precession methods. Additionally, we apply this technique to the GAL4 cluster that we have previously characterized by using coherence transfer methods. We present the development of three increasingly selective forms of the basic experiment, each one applicable to different biological systems.

MATERIALS AND METHODS

Sample preparation

GAL4(62*) and LAC9(61) were overexpressed in *Escherichia coli* and purified as previously described (Pan and Coleman, 1989,1990a; Pan et al., 1990). The proteins were purified in the zinc forms; typical purifications yielded protein with approximately 1.5 equiv of zinc per protein. ^{113}Cd was substituted for zinc in the metal binding sites by incubating protein samples with a three- to fivefold molar excess of $^{113}CdCl_2$ (95% isotopically enriched in ^{113}Cd) for 24 h, followed by dialysis against metal-free buffer to remove the free metal. After this exchange, samples contained 2.0 equiv of cadmium and negligible amounts of zinc, as determined by flame atomic absorption. The protein samples used for NMR were 3–5 mM protein in 50 mM sodium phosphate or 10 mM acetic acid-d_3 (CD_3COOH), pH 5.7.

NMR experiments

All NMR experiments were performed using a Bruker (Billerica, MA) AM-500 spectrometer at 25 °C, operating at field strengths of 500.13 MHz for protons and 110.93 MHz for ^{113}Cd. All experiments were conducted in a 5 mm inverse-tunable probe using the decoupler channel for ^{1}H presaturation and rf pulses. The proton and cadmium rf field strengths were matched to satisfy the Hartmann–Hahn matching condition ($\gamma_I B_I = \gamma_S B_S$). These values were matched to within +/− 10% by use of variable attenuators with 1 dB resolution. Heteronuclear cross-polarization was achieved using DIPSI-2 mixing sequences (Shaka et al., 1988), simultaneously applied to both proton and cadmium channels at rf field strengths discussed in the figure legends. Chemical shift scales were referenced with 1.0 M $Cd(ClO_4)_2$ (0 ppm ^{113}Cd) and H_2O (4.77 ppm ^{1}H) at 298 K.

Experimental parameters for most spectra are described in the figure legends. For selective heteroTOCSY experiments, utilizing proton carriers positioned off the H_2O resonance, phase-ramped DANTE pulse trains (Kay et al., 1989) were used to presaturate the water resonance. Typical 1D ^{113}Cd-^{1}H heteroTOCSY experiments used 1000 to 4000 scans with a relaxation delay of approximately 1.6 s between scans; this resulted in measurement times of between 30 min to 2 h.

Data processing

All NMR data was processed using the FELIX software package (Version 2.1, Biosym Technologies, San Diego, CA) on a Sun Sparc2 workstation. Data processing for ^{113}Cd-detected spectra consisted of linear prediction of the first two data points in the FID and 20 Hz exponential line-broadening prior to Fourier transformation; 1D ^{1}H-detected experiments used the same linear prediction with 3 Hz exponential line-broadening, unless otherwise specified.

RESULTS AND DISCUSSION

Nonselective ^{113}Cd-^{1}H heteroTOCSY experiments applied to the $Cys_6^{113}Cd_2$ binuclear cluster of LAC9

As initially designed, the heteroTOCSY is a nonselective experiment that consists of a hard ^{113}Cd $\pi/2$ pulse, followed by a heteronuclear polarization transfer sequence simultaneously applied to protons and cadmium (Fig. 2a). Transverse heteronuclear magnetization is initially generated and then transferred to neighboring coupled nuclei by the mixing sequence. We chose to use a DIPSI-2 mixing sequence (Shaka et al., 1988) on the basis of its superior transfer characteristics, as demonstrated both in simulations and in practice (Brown and Sanctuary, 1991; Ernst et al., 1991). The mixing sequence is run with an rf field strength wide enough to adequately cover the frequencies of all heteronuclei and their coupled protons. This 1D ^{113}Cd-^{1}H heteroTOCSY technique was applied to the $^{113}Cd_2$ cluster of LAC9(61). The resulting spectrum (Fig. 3b) demonstrates a dramatic simplification compared to a nonselective 1D ^{1}H spectrum of the same protein (Fig. 3a). The heteroTOCSY spectrum shows a number of resonances that arise from polarization transfer from the ^{113}Cd nuclei to the protons of the coordinating cysteine residues, including β, α and amide protons. The spin system topology can be explored by lengthening the duration of the heteronuclear mixing period and observing the buildup of peaks in 1D spectra. The nonselective 1D experiment is ideally suited for use on systems involving a lone heteronucleus, such as a lone CdS_4 site as found in Cd-substituted rubredoxin.

By comparing the performance of the heteroTOCSY experiment to several free-precession methods, it is evident that the heteroTOCSY has superior characteristics for identifying ^{113}Cd-

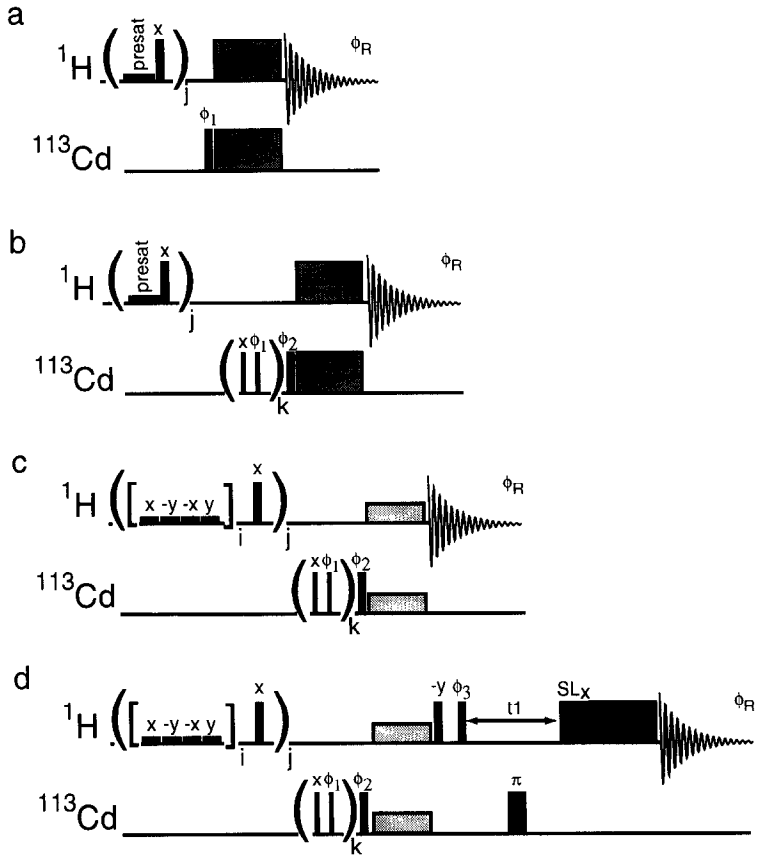

Fig. 2. ^{113}Cd-^{1}H hetero TOCSY pulse sequences. Large dark shaded boxes represent DIPSI-2 (Shaka et al., 1988) heteronuclear mixing sequences applied at high power (≥5 kHz rf field strength); lower power mixing sequences (at power levels discussed in the text) are indicated by lower height and lighter shading. All DIPSI-2 sequences were applied along the +/−x-axis. Thin black boxes represent π/2 pulses; other pulse widths are listed above the pulse. (a) Nonselective heteroTOCSY. j Cycle: 40–50 ms of presaturation of the H_2O resonance, followed by a 120° hard pulse to ensure complete saturation of all proton resonances; the cycle is repeated for a total duration of approximately 1.5 s, making the typical value for j between 30 and 40. Phase cycling for sequence a: $\phi_1 = \phi_R = (y,-y)$. (b) Semiselective heteroTOCSY. k Cycle: DANTE-Z (Boudot et al., 1989), using 22.5° pulses (k = 4), separated by delays of 1/(4D) s, where D is the separation between cadmium resonances in Hz; this delay ensures null excitation of the second cadmium. (c) Selective heteroTOCSY. i Cycle: off-resonance DANTE (ORDANTE; Kay et al., 1989) to presaturate H_2O while the carrier is positioned off the water resonance. Pulses were delivered for 1/(4D) s, where D is the separation between the carrier and H_2O in Hz, and separated by 3 μs delays to accommodate phase switching. i Was a multiple of four and was set to generate 40–50 ms of ORDANTE pulsing. (d) 2D selective heteroTOCSY-TOCSY. A 1 ms Z-filtration delay (Sørensen et al., 1984; Rance, 1987) is applied prior to the t_1 increment to facilitate switching power levels for the ^{1}H channel. A 1 ms trim pulse was applied along the x-axis (SL_x pulse) following the t_1 period. Phase cycling for sequences b, c and d: $\phi_1 = (x,-x)$; $\phi_2 = (y,y,-y,-y)$; $\phi_3 = (y)$ + TPPI; $\phi_R = (y,-y,-y,y)$.

coupled protons in the Cd_2LAC9(61) system (Fig. 4). All peaks in the heteroTOCSY spectrum have equivalent or better signal-to-noise ratios than those observed in spectra generated by the other two experiments. Furthermore, a larger number of peaks are evident in the heteroTOCSY spectrum; these arise from correlations to some of the cysteine H^α protons via proton–proton

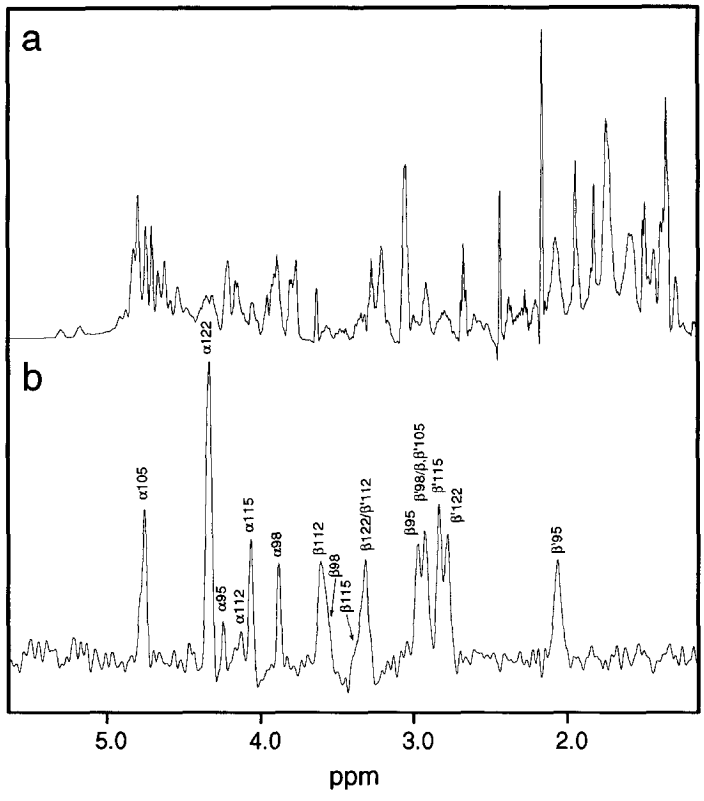

Fig. 3. Comparison between nonselective 1D ^{1}H spectrum and nonselective heteroTOCSY of $^{113}Cd_2$LAC9(61). (a) 1D ^{1}H nonselective spectrum of $^{113}Cd_2$LAC9(61). 32 Scans were acquired, using 1.5 s of presaturation of the water resonance, followed by a 90° nonselective pulse on the proton channel. (b) Nonselective heteroTOCSY of $^{113}Cd_2$LAC9(61), acquired using the sequence of Fig. 2a. A DIPSI-2 heteromixing sequence was applied for 28.5 ms, using a 4.0 kHz mixing field. A total of 2000 scans were acquired (approximately 1 h acquisition time). Carriers were positioned at the center of each spectrum (699 ppm for ^{113}Cd, 4.77 ppm (H_2O) for ^{1}H).

homonuclear transfers from H^{β} protons with direct ^{113}Cd-^{1}H heteronuclear couplings. Although correlations of this kind could potentially be observed by equipping the INEPT and HMQC experiments with additional homonuclear relay steps, such correlations are present in heteroTOCSY spectra without any modification, as the DIPSI-2 sequence will efficiently transfer magnetization along homonuclear and heteronuclear pathways.

Other researchers have found similar improvements in signal-to-noise ratios for heteroTOCSY-based methods over free-precession techniques. Increases in signal-to-noise ratio of up to 100% have been observed in $^{13}C \rightarrow {}^{1}H$ transfers (Zuiderweg, 1990; Majumdar et al., 1993); similar results have been measured for $^{13}C \rightarrow {}^{15}N$ (Richardson et al., 1993), where magnetization is transferred through couplings of the same magnitude as the carbon linewidth. These increases have been generally attributed to the fact that heteroTOCSY experiments will be more robust to a variety of detrimental factors, including rf and magnetic field inhomogeneity and relaxation processes (Zuiderweg, 1990). It is apparent from our studies (Fig. 4) that increases of similar magnitude are possible with $^{113}Cd \rightarrow {}^{1}H$ transfers.

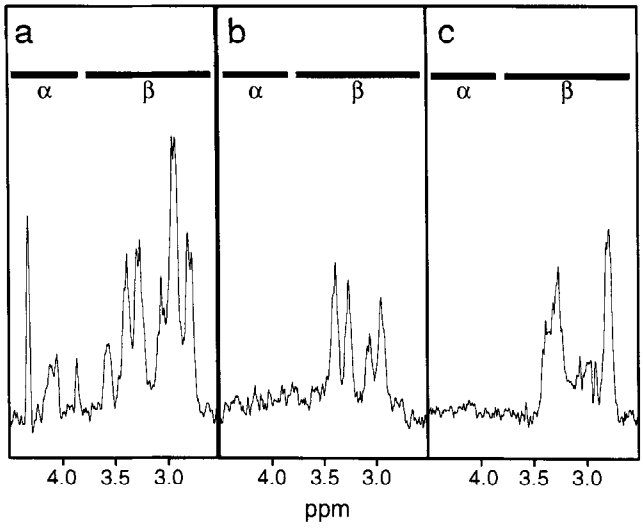

Fig. 4. Comparison between different methods of identifying ^{113}Cd-coupled protons in $^{113}Cd_2$LAC9(61). All spectra were acquired using 1000 scans. All experimental parameters for heteronuclear transfer were optimized for a J = 65 Hz coupling. Regions marked with an α represent cysteine H^{α} protons; cysteine H^{β} protons are in the β region. (a) Nonselective heteroTOCSY, using the sequence of Fig. 2a and a 15.2 ms mixing time (= 1/J). (b) Refocussed INEPT (Sørensen and Ernst, 1983), using delays of 3.8 ms (= 1/4J) for the initial transfer and 2.1 ms (= 1/7J) for refocussing. (c) HMQC (Frey et al., 1985) with delays of 7.5 ms (= 1/2J) for the evolution of heteronuclear coupling.

Semiselective ^{113}Cd-^{1}H heteroTOCSY experiments: Selective excitation

To separately identify the cysteine residues bonded to each of the two ^{113}Cd ions of LAC9(61), we modified the nonselective heteroTOCSY (Fig. 2a) by replacing the initial ^{113}Cd π/2 preparation pulse with a selective one (Fig. 2b), generating an experiment analogous to the softTOCSY (Kessler et al., 1986). We used the DANTE-Z selective excitation scheme (Boudot et al., 1989), as this pulse scheme could be easily implemented and gave excellent selectivity between the ^{113}Cd resonances, even when they were separated by only 15 ppm (1.66 kHz at 11.7 T) as in the case of LAC9(61). The performance of the DANTE-Z scheme was demonstrated by the selective excitation of one of the two ^{113}Cd resonances in LAC9(61) (Figs. 5a and b).

We refer to this experiment as a semiselective heteroTOCSY; although the initial magnetization is restricted to one heteronucleus, the subsequent mixing sequence is applied with an rf field strength that covers both ^{113}Cd resonances. While the DANTE-Z sequence serves as a frequency-selective preparation pulse, the subsequent DIPSI-2 mixing sequence has a broad excitation bandwidth (5.7 kHz). These pulse sequences are capable of transferring magnetization through both homonuclear and heteronuclear pathways. Hence, if bridging ligands between cadmium ions are present that facilitate ^{113}Cd-^{113}Cd coupling, the mixing sequence will transfer ^{113}Cd magnetization between cadmium spins, thus ruining the selective excitation and complicating the interpretation of results from a semiselective heteroTOCSY experiment. In our Cys_6Cd_2 systems, two of the cysteinyl sulfurs bridge between the cadmium atoms, creating a two-bond pathway for such a transfer. This is clearly demonstrated in Fig. 5c, where the excitation, selectively applied to one cadmium of the LAC9(61) binuclear cluster, can be transferred to the other by application of a DIPSI-2 mixing sequence applied exclusively to ^{113}Cd. This problem is exacerbated by the

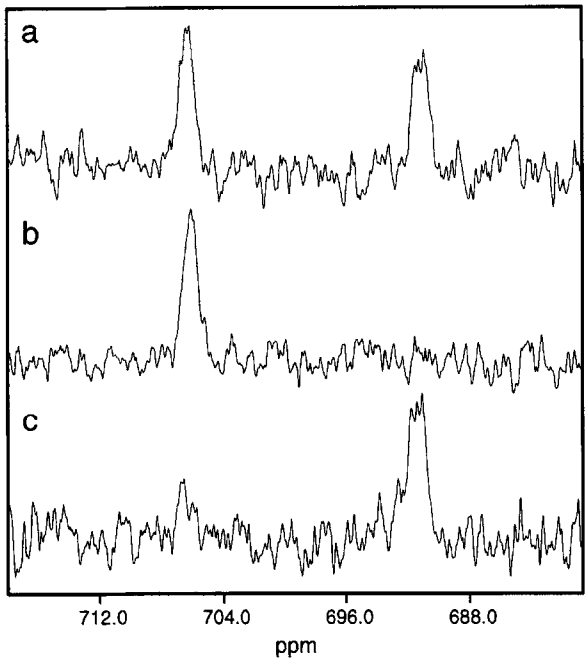

Fig. 5. One-dimensional ^{113}Cd NMR spectra of $^{113}Cd_2$LAC9(61). (a) Nonselective excitation; 3325 transients were acquired, using a 60° pulse width and a 0.4 s delay between pulses. (b) DANTE-Z-based selective excitation of the 706 ppm ^{113}Cd resonance. The DANTE-Z scheme consisted of a (22.5°(x)-151 μs-22.5°(+x/−x)-151 μs) train, sequentially delivered four times, followed by a 90° nonselective ^{113}Cd pulse that was CYCLOPS cycled (x,y,−x,−y). The receiver was cycled by the product of the second DANTE-Z pulse and CYCLOPS (x,−x,y,−y,....). The cadmium carrier was positioned at 706 ppm. (c) DANTE-Z selective excitation, followed by DIPSI-2 mixing applied to the ^{113}Cd channel. DANTE-Z was applied as in part b; the DIPSI-2 sequence was applied for 30 ms with a 5.7 kHz field strength centered at 706 ppm on the ^{113}Cd channel.

fact that homonuclear transfer proceeds at a rate twice as fast as that of heteronuclear transfer if the homo- and heteronuclear coupling constants are of similar magnitude (Ernst et al., 1991):

$$\text{homonuclear } (^{113}\text{Cd-}^{113}\text{Cd or } ^{1}\text{H-}^{1}\text{H}): I_x \rightarrow 0.5(1 + \cos[2\pi Jt])I_x + 0.5(1 - \cos[2\pi Jt])S_x \quad (1)$$

$$\text{heteronuclear } (^{113}\text{Cd-}^{1}\text{H}): I_x \rightarrow 0.5(1 + \cos[\pi Jt])I_x + 0.5(1 - \cos[\pi Jt])S_x \quad (2)$$

Peaks arising from a ^{113}Cd→^{113}Cd→^{1}H pathway were observed in semiselective heteroTOCSY experiments conducted on LAC9(61) at reduced (but still significant) signal-to-noise level compared to peaks resulting from the desired ^{113}Cd→^{1}H pathway (data not shown). Given this potential for misinterpretation, this method would be most appropriate to apply to systems with multiple, noncoupled heteronuclei where bridging ligands are definitely not present (i.e., CdN_2S_2 fingers from zinc finger proteins).

Selective ^{113}Cd-^{1}H heteroTOCSY experiments: Selective excitation and transfer

The selectivity of the heteroTOCSY experiment can be further improved by reducing the rf

field strength of the DIPSI-2 mixing sequence, such that it is on the order of the separation between the ^{113}Cd resonances (in Hz). Previous studies of the off-resonance heteronuclear transfer properties of mixing sequences such as DIPSI-2 (Brown and Sanctuary, 1991; Ernst et al., 1991) have demonstrated that these sequences are ineffective at transfer for frequencies >0.5 ($\gamma B_1/2\pi$) Hz away from the carrier. We can exploit this fact in our experiments to achieve frequency-selective transfer, and thus generate the experiment that we term the selective heteroTOCSY (Fig. 2c). Such an experiment is applicable to the most complicated metal–protein systems with bridging ligands (i.e., metallothioneins and transcription factors of the GAL4 class).

The guidelines for choosing the optimal mixing field strength are (i) the separation of the ^{113}Cd resonances of interest and (ii) the range of resonance frequencies of the ^{113}Cd-coupled protons. The former dictates the upper limit of the rf field strength, while the latter established the lower limit. In our most stringent case, LAC9(61), the two ^{113}Cd resonances are 1.6 kHz apart, necessitating the use of a TOCSY field strength less than 3 kHz to avoid any ^{113}Cd-^{113}Cd homonuclear transfer. To further reduce the mixing field strength and yet maintain heteronuclear transfer, the proton carrier was moved to 3.5 ppm, thus reducing the maximum frequency difference between the carrier and a proton involved in transfer to 750 Hz. This allowed the rf field strength during the mixing period to be set to $\omega_1 = 1.5$ kHz.

Note that several other methods have been developed for increasing the selectivity of TOCSY-based transfers, chiefly in homonuclear systems. The simplest alternative is to replace the mixing sequence with a selective spin-lock, applied to both nuclei (Glaser and Drobny, 1989,1991; Ernst et al., 1991). This method is extremely dependent on frequency offset and is thus of somewhat limited utility. Other methods have been developed for selective homonuclear transfer through computer-based optimization of either phase-modulated pulse trains (Glaser and Drobny, 1989) or amplitude-shaped TACSY pulses (Schmidt et al., 1993). These sequences can be designed to facilitate selective transfer between distinct subsets of nuclei and could potentially be extended into heteronuclear systems to yield new sequences with similar selectivity.

An additional benefit of the increased frequency selectivity of the selective heteroTOCSY experiments is a potential increase in the signal amplitude of the detected protons. Under the influence of a mixing field, magnetization from one ^{113}Cd nucleus will distribute amongst N other coupled nuclei (^{113}Cd or ^{1}H). Therefore, magnetization will be diluted over a total of N+1 destinations, including the original source (Ernst et al., 1991). By increasing the selectivity of the transfer, the value of N can be reduced and the magnetization transferred to the destination spins can be enhanced proportionally. The limit of this sensitivity gain can be achieved by restricting the mixing sequence bandwidth to a very narrow range for both protons and heteronuclei, for example through the use of a spin-lock transfer mechanism or similarly selective method.

Employing the above methods, the selective heteroTOCSY experiment can be made completely selective, with no detectable transfer to protons on the cysteinyl residues primarily coupled to the second ^{113}Cd (Fig. 6). Thus, this method appears to be the best option for use on systems with multiple heteronuclei that share bridging ligands.

Structural features of the ^{113}Cd binuclear cluster of LAC9(61) as determined by selective ^{113}Cd heteroTOCSY spectra

As indicated above, the presence of the bridging sulfur ligands in the binuclear cadmium cluster gives rise to two-bond ^{113}Cd-S-^{113}Cd couplings ($^2J_{Cd\text{-}Cd}$). In the case of cadmium-substituted

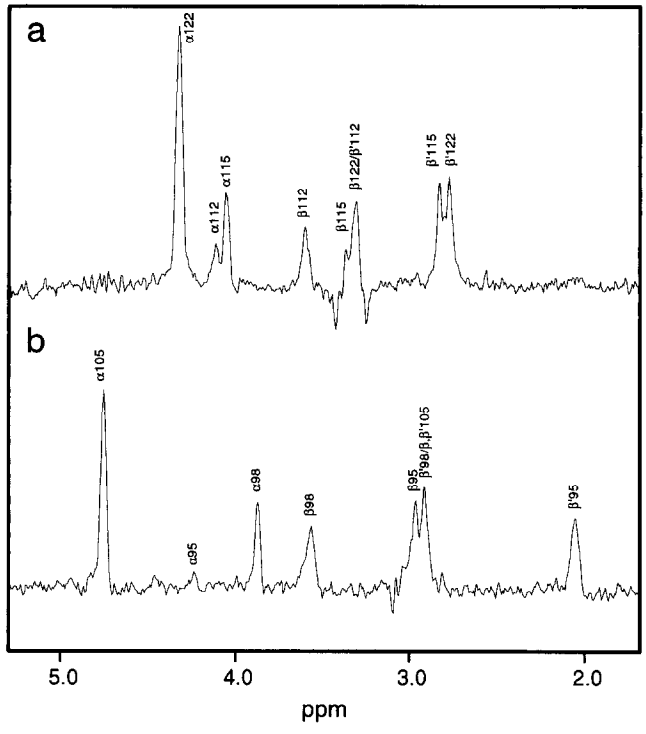

Fig. 6. Selective heteroTOCSY experiments, applied to $^{113}Cd_2$LAC9(61). The spectra were acquired using the scheme described in Fig. 2c, with the proton carrier at 3.5 ppm and the cadmium carrier as described below. 4000 Scans were taken for each spectrum, for an acquisition time of approximately 2 h apiece. A 2.2 kHz DIPSI-2 heteronuclear mixing field was applied for 13.1 ms. (a) ^{113}Cd carrier placed at 706 ppm. (b) ^{113}Cd carrier placed at 691 ppm.

metallothioneins in which the ^{113}Cd resonances are relatively narrow, ^{113}Cd-^{113}Cd coupling between the metal nuclei of the cluster has been resolved directly in 1D ^{113}Cd and 2D ^{113}Cd-^{113}Cd COSY spectra; these $^2J_{Cd\text{-}Cd}$ coupling constants range from 20 to 45 Hz (Frey et al., 1985). In contrast, the cadmium resonances in GAL4(62*) and LAC9(61) are significantly broader ($\Delta\nu_{1/2} \sim 140$ Hz at 110.9 MHz) and these two-bond couplings are not directly resolvable. However, from the mixing time dependence of transfer between ^{113}Cd nuclei in homonuclear cadmium TOCSY experiments (Fig. 5) using Eq. 1, the $^2J_{Cd\text{-}Cd}$ value is estimated to be 22 Hz.

Given the fact that the $^2J_{Cd\text{-}Cd}$ and the $^3J_{Cd\text{-}H}$ coupling constants (Fig. 1) in the ^{113}Cd cluster systems are of similar magnitude, it is apparent that any significant extension of the mixing period in a nonselective heteronuclear mixing sequence can quickly destroy the selectivity of the ^{113}Cd preparation pulse by transferring magnetization between the cadmium nuclei. These peaks can be identified by comparing semiselective and selective spectra or by examining the mixing time dependence of the transfer; the intermediate homonuclear transfer step introduces a delay that generally distinguishes these peaks from the more direct transfers.

To avoid these pitfalls in identifying the protons in the liganding cysteines in LAC9(61), we have therefore used the 1D selective heteroTOCSY experiment. This entailed collecting a series of these spectra at different mixing times and observing the rate of heteronuclear transfer. The results from these experiments are summarized in the assignments shown in Fig. 6. Transfers to

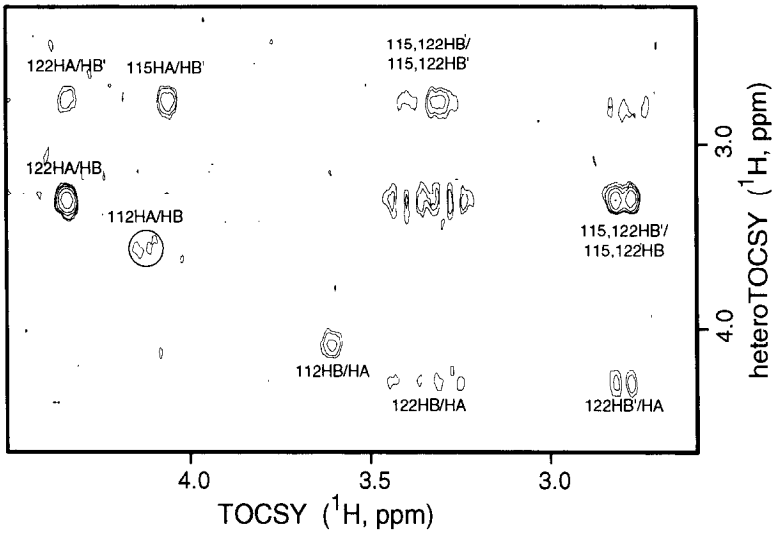

Fig. 7. 2D selective heteroTOCSY-TOCSY. This spectrum was acquired using a 2.1 kHz heteroTOCSY rf field strength, applied for 13.5 ms, and a 5.8 kHz homoTOCSY rf field strength, switched on for 19.7 ms. The acquired data set was 56 × 512 data points, using a spectral width of 2500 Hz in each dimension; 1600 scans were taken in each t_1 experiment. Data processing consisted of linear prediction of the t_1 data (50% extension) and 60°-shifted sine-squared apodization in each dimension before Fourier transformation into a 128 × 1K matrix.

all of the coupled cysteine β protons were observed with a 13 ms mixing time; further transfers to α protons in those spin systems were observed within 25 ms. However, transfer was not observed from either cadmium to the bridging protons with the smallest coupling constants in this system; short relaxation times appear to be hampering effective transfer through the expected small $^3J_{Cd\text{-}H}$ coupling constants (estimated to be ≤5 Hz).

The experiments described here are useful not only as 1D techniques, but also as components for multidimensional studies. An example of such an application is the 2D selective hetero-TOCSY-TOCSY (Fig. 2d). The first half of this experiment consists of a selective heteroTOCSY experiment, indirectly detecting the chemical shifts of the H^α and H^β protons from cysteines that are bonded to the selectively excited cadmium. The second half of the experiment is a standard proton TOCSY, which extends the connectivity information through the protons in the side chain. This results in a pattern of correlations, indicating the J coupling pattern of protons in side chains liganding the cadmium; these results are analogous to those that would be observed using an $\omega_1{}^{113}$Cd half-filtered TOCSY (Otting et al., 1986). This experiment was applied to the LAC9(61) cluster (Fig. 7), and the results were used to confirm the assignments reached with the 1D methods. This experiment can be viewed as a selective version of the 3D heteroTOCSY-TOCSY proposed by Kellogg (Kellogg, 1992) or the recently described PHH heteroTOCSY-TOCSY experiment (Wang et al., 1994).

Structural features of the ^{113}Cd binuclear cluster of GAL4(62) as determined by selective ^{113}Cd heteroTOCSY spectra*

We undertook a similar series of experiments on $^{113}Cd_2$GAL4(62*) to confirm the behaviour of the heteroTOCSY experiment on a metalloprotein system that has been previously characterized

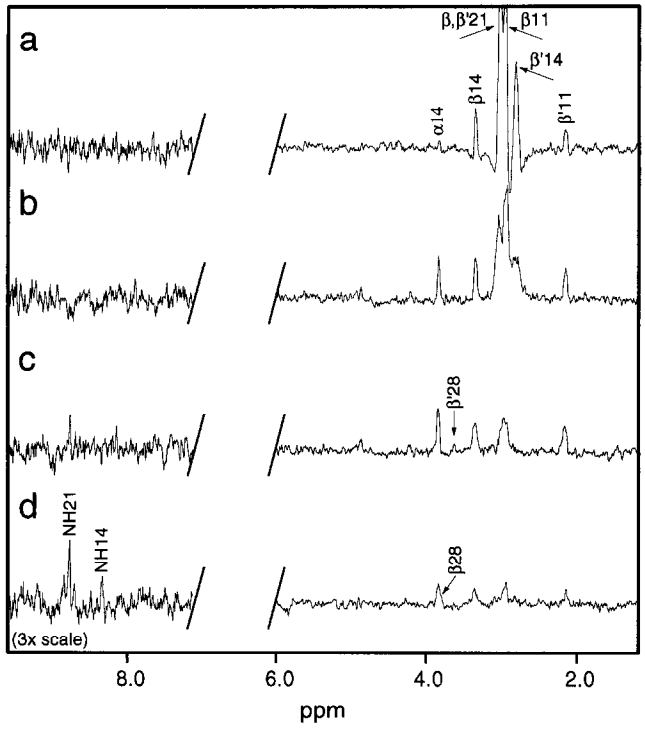

Fig. 8. Mixing time dependence of transfer peaks in selective heteroTOCSYs of $^{113}Cd_2$GAL4(62*). Data was acquired using the scheme of Fig. 2b, with DIPSI-2 applied at 5.3 kHz field strength and carriers at 666 ppm (^{113}Cd) and H_2O (4.77 ppm, ^{1}H). 4200 Scans were acquired for each spectrum, at approximately 2 h collection time each. A 5 Hz exponential line-broadening was applied. (a) Mixing time 5.4 ms; (b) Mixing time 16.3 ms; (c) Mixing time 27.1 ms; (d) Mixing time 48.8 ms.

by HMQC and X-filtered methods (Gadhavi et al., 1991; Gardner et al., 1991). Additionally, the ^{113}Cd resonances in GAL4(62*) are significantly further apart than in the LAC9 system, as GAL4(62*) has resonances at 666 and 707 ppm (Δ = 4.4 kHz at 11.7 T). This allows use of larger rf field strengths in the DIPSI-2 transfer steps, while still maintaining their frequency selectivity for a lone ^{113}Cd nucleus. A series of selective heteroTOCSY experiments at different mixing times is presented (Fig. 8) to demonstrate transfer from the 666 ppm cadmium resonance. As was observed in the LAC9(61) system, transfer to all the strongly coupled β protons on liganding cysteines was observed at the shortest mixing time attempted (5.4 ms), followed by transfer to H^α protons in these spin systems within the next 10–20 ms. However, in contrast to the LAC9(61) system, transfers from the cadmium resonance to the weakly coupled bridging cysteine H^β protons were observed (C28β,β'). The proton assignments (NH, H^α, H^β) of the cysteine residues of Cd_2GAL4(62*) from these experiments are consistent with previously published work (Gardner et al., 1991). They are also consistent with the assignments of various Zn_2GAL4 fragments under several different conditions (Gadhavi et al., 1990; Gardner et al., 1991; Shirakawa et al., 1993).

The larger rf field strength of the DIPSI-2 mixing sequence in the GAL4(62*) selective heteroTOCSY experiments facilitated transfer through the entire spin system of the ^{113}Cd-liganding cysteines, including the backbone amide protons (Fig. 8). Correlation to the backbone NH is

most prominently observed for Cys^{21}; this residue is shown by the structures of GAL4(65) (Baleja et al., 1992; Kraulis et al., 1992; Marmorstein et al., 1992) to have a non-alpha-helical conformation that should have a fairly large $^3J_{HN\alpha}$ coupling constant. Transfer to amides in helical regions (C14) were observed at lower intensities (Fig. 8), probably due in part to the smaller coupling constant between the α and amide protons. These relays through the cysteine spin systems highlight the benefits of the homonuclear transfers that were a problem with the semiselective heteroTOCSY experiments; whereas ^{113}Cd-^{113}Cd homonuclear transfers were detrimental to determining the cysteine–cadmium connectivity, ^1H-^1H transfers extend the connectivity information to potentially allow for the identification of all protons in a liganding amino acid.

CONCLUSIONS

The application of the ^{113}Cd-^1H heteroTOCSY technique to ^{113}Cd-substituted proteins has proven to be a convenient means of identifying the ligands to the cadmium ions; the nature of the magnetization transfer facilitates the assignment of the entire spin system of metal-liganding amino acids. Furthermore, it is a more sensitive experiment at detecting ^{113}Cd-^1H correlations in the Cys_6Cd_2 system, a point which bears further testing in other metal-binding systems. With the various degrees of selectivity for the ^{113}Cd-^1H heteroTOCSY experiment demonstrated in this work, a group of methods are available for studying metalloprotein systems binding single or multiple metal ions. Given that metals are widely used in both structural and catalytic roles in macromolecules, a large number of systems are known in which this technique can be fruitfully applied.

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