$^{1}H-^{15}N$ HMQC for the Identification of Metal-Bound Histidines in ^{113}Cd-Substituted *Bacillus cereus* Zinc β-Lactamase

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Cadmium is often used as a useful NMR[1] probe to study zinc- or calcium-containing proteins;[2] we present here a method for identification of cadmium-coordinated histidine via $^{1}H-^{15}N$ HMQC, which does not require any Cd excitation pulse. The cross-peaks show an E-COSY[3] type of pattern which allows the measurement of the $^{3}J_{H-Cd}$ and $^{1}J_{N-Cd}$ coupling constants.

In many metalloproteins, notably those containing zinc or calcium, the natural metal is not conveniently detectable by NMR. To overcome this problem, substitution of the metal by cadmium has often been used. In particular, cadmium-113 NMR has often been used to study zinc-containing proteins;[2] the ^{113}Cd chemical shift is very sensitive to the nature, number, and geometric arrangement of the ligands within the coordination sphere. In many cases, the substitution of Zn^{2+} by Cd^{2+} has been shown to have only a modest effect on the catalytic activity of metalloenzymes.[4] ^{113}Cd resonances are commonly detected by direct observation (^{113}Cd has spin $^{1}/_{2}$ and a sensitivity 63% that of ^{13}C) or by inverse detection of ^{113}Cd scalar-coupled to ^{1}H. Cysteine, methionine, and histidine residues have been successfully identified as the coordinating ligands using $^{1}H-^{113}Cd$ HMQC, ^{113}Cd-edited $^{1}H-^{1}H$ COSY or $^{1}H-^{113}Cd$ heteroTOCSY experiments.[5−8] These inverse experiments require a time-delay for transfer of magnetization between ^{1}H and ^{113}Cd spin, and also a knowledge of the ^{113}Cd chemical shift, in view of the very large chemical shift range of ^{113}Cd, which can also make direct detection difficult. These prerequisites make the experiments difficult in many cases.

It would therefore be desirable to have a method by which the ligands of ^{113}Cd can be identified which does not rely on the polarization transfer between ^{1}H and ^{113}Cd spins. We describe here a method for identifying cadmium-coordinated histidines using a two-dimensional $^{1}H-^{15}N$ HMQC experiment which does not require Cd excitation pulses and which permits the identification of the histidine imidazole proton and nitrogen resonances of ^{113}Cd-bound imidazoles and the determination of the $^{3}J_{H-Cd}$ and $^{1}J_{N-Cd}$ coupling constants.

We demonstrate the method using the zinc β-lactamase from *Bacillus cereus* strain 569/H/9 (BCII). Zinc β-lactamases[9] in pathogenic bacteria, which can be plasmid-encoded, confer resistance toward all the β-lactam antibiotics and represent a real threat to antibiotic therapy because no inhibitors are presently available for clinical purposes.[10] The crystal structure[11] of β-lactamase BCII shows two zinc cations in the active site, one (site I) coordinated by three histidines and one water molecule, and the other (site II) coordinated by a histidine, a cysteine, an aspartate, and an unknown molecule, likely to be a carbonate ion. These two zinc ions can be replaced by ^{113}Cd with minimal change in the kinetic parameters of the enzyme. The ^{113}Cd spectrum of the enzyme showed two signals, at 143 and 266 ppm relative to 0.1 M $Cd(ClO_4)_2$; comparison with previously reported ^{113}Cd chemical shifts[12] indicates that these shifts are consistent with the same coordination for the cadmium as observed for zinc in the crystal.

Using a sample of uniformly ^{15}N-labeled ^{113}Cd-substituted β-lactamase BCII[13] dissolved in H_2O, a $^{1}H-^{15}N$ HMQC[16] experiment with a refocusing delay of about 17 ms, chosen as a compromise to enable simultaneous detection of both the direct ($^{1}J_{H-N}$ = 90 Hz) and long-range ($^{2}J_{H-N}$ = 5−10 Hz, $^{3}J_{H-N}$ = 2−3 Hz) $^{15}N-^{1}H$ correlations, gave spectra in which all the imidazole protons and nitrogens were detectable in a single experiment,[19] as shown in Figure 1a. Resonances from all seven histidine residues in the enzyme were observed; in four cases proton exchange with the bulk water was slow enough to allow

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(1) Abbreviations used: NMR, nuclear magnetic resonance; HMQC, heteronuclear mulitple quantum coherence; COSY, correlated spectroscopy; E-COSY, exclusive COSY.; TOCSY, total correlated spectroscopy; IPTG, isopropyl β-D-thiogalactopyranoside; FID, free induction decay; MES, 2-[*N*-morpholino]ethanesulfonic acid.

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(13) ^{15}N-labeled protein was expressed from plasmid pET/BCII in *Escherichia coli* BL21(DE3). Cells were grown at 30 °C in M9 minimal medium with 10 g of glucose and 1 g of $^{15}NH_4Cl$ as the only nitrogen source. Expression was induced by adding 0.5 mM IPTG at an OD_{600} of 1.00. After 16 h the cells were harvested by centrifugation and then broken by sonication; BcII was purified as described.[14] The cadmium enzyme is made by adding gradually, at room-temperature, 2 equiv (1.6 mM) of $^{112}CdCl_2$ or $^{113}CdCl_2$ (95.83%, Cambridge Isotope Laboratories MA) to 1 equiv of BCII apo-enzyme (0.8 mM) in 10 mM MES-Na, 100 mM NaCl, pH 6.4, 10% D_2O. The apo-enzyme was prepared as described.[15]

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(16) The $^{1}H-^{15}N$ HMQC experiment with pulsed field gradients was a modification of that described by Davis et al.[17] To detect broad signals in the nitrogen dimension, the original pulse sequence was shortened by removing a ^{15}N 180° refocusing pulse. A ^{1}H 90° selective (Gaussian) water flip-back pulse[18] just before the first ^{1}H pulse greatly improved the water suppression as well as the intensities of the imidazole one-bond NH cross-peaks. The pulse sequence used is: $g_1-\mathrm{wfb}_{\varphi 1}-90_{\varphi 1}(H)-\Delta-90_{\varphi 2}(N)-t_1/2-180_x(H)-t_1/2-g_2-180_x(N)-g_3-\tau_1-90_x(N)-\tau_2-g_4-$acq with phase cycling: $\varphi 1 = 2(x,-x)$; $\varphi 2 = 2(x)2(-x)$; receiver = $(x,-x,-x,x)$. The carrier frequencies are centered at 4.8 (water) and 160 ppm for ^{1}H and ^{15}N, respectively. The water flip-back (wfb) is a 90° water-selective pulse of 2.8 ms with a Gaussian profile. Delays are: Δ = 17 ms; $\tau_1 = \tau_{180}(H) + t_1^0$ (where $\tau_{180}(H)$ is the duration of the 180° (H) pulse and t_1^0 the initial incremental delay); $\tau_2 = \Delta-\tau_{g2}-\tau_{g3}-\tau_{g4}-\tau_{180}(N)-\tau_1$ (where τ_g are the duration of the gradient pulses). The duration and strengths of the gradients were: g_1: 1 ms, 9 G/cm; g_2: 0.5 ms, 27 G/cm; g_3: 0.5 ms, −27 G/cm; g_4: 0.5 ms, 5.6 G/cm. For each value of t_1, N- and P-type coherences were obtained by recording two data sets, the sign of the gradient g_4 being inverted for the second data set. The phase $\varphi 2$ was incremented by 180° along with the phase of the receiver for each t_1 increment. The spectral widths were 15 kHz (^{1}H) and 10 kHz (^{15}N), and the relaxation delay between scans was 1s. The data consisted of 150 complex points in the ^{15}N dimension and 1K complex points in the ^{1}H dimension, with 64 scans for each FID.

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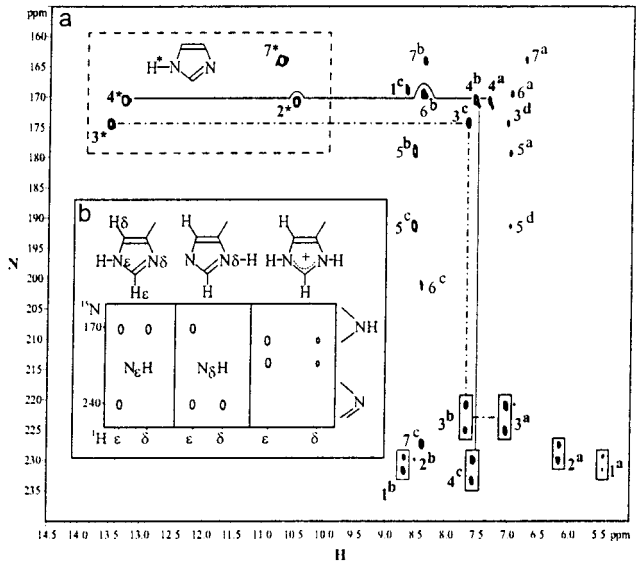

Figure 1. (a) The $^1H-^{15}N$ HMQC spectra of 0.8 mM uniformly ^{15}N-labeled ^{113}Cd-substituted β-lactamase BCII in 10 mM MES-Na, 100 mM NaCl, pH 6.4, 10% D_2O, recorded at 298 K on a Bruker DMX 500 MHz spectrometer. (b) Depicts how the imidazole tautomeric state can be determined with the help of the N−C−H spin patterns.[19] Histidine imidazoles are labeled from 1 to 7; direct H−N correlations are represented within the dashed box. Superscript letters on each histidine imidazole label indicate a specific type of N−C−H correlation (a: $N_\epsilon-C-H_\delta$, b: $N_\epsilon-C-H_\epsilon$, c: $N_\delta-C-H_\epsilon$, d: $N_\delta-C-H_\delta$). Boxes indicate where splittings are observed.

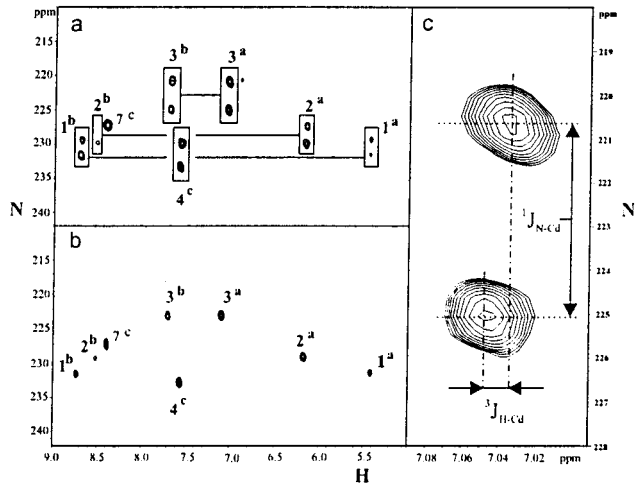

Figure 2. The $^1H-^{15}N$ HMQC spectra of 0.8 mM uniformly ^{15}N-labeled ^{113}Cd- (a,c) and ^{112}Cd-substituted (b) β-lactamase BCII in 10 mM MES-Na, 100 mM NaCl, pH 6.4, 10% D_2O, recorded at 298 K on a Bruker DMX 500 MHz spectrometer. **(**a,b**)** Comparison between (a) ^{113}Cd- and (b) ^{112}Cd-substituted β-lactamase BCII. **(c)** Expansion of the cross-peak pattern from one of the four cadmium-bound nitrogens; the $^1J_{N-Cd}$ and $^3J_{H-Cd}$ splittings are indicated. Peak labels as in Figure 1.

cross-peaks to be observed also for the exchangeable NHs (box marked with dashed lines in Figure 1). The imidazole $^{15}N-C-H$ cross-peaks of four of the histidines showed splittings in the ^{15}N dimension (Figures 1a and 2a) which were not observed in the spectra of the ^{112}Cd enzyme (Figure 2b) and which could thus be assigned to the direct $^1J_{N-Cd}$ coupling. This experiment thus makes it possible to identify the resonances of the four imidazoles bound to cadmium in the enzyme without applying ^{113}Cd pulses and without any previous knowledge of ^{113}Cd NMR parameters.

The same experiment also provides information on the protonation state and the tautomeric state of each imidazole ring from, respectively, the ^{15}N chemical shifts and the pattern of $^{15}N-C-H$ cross-peaks[19] (Figure 1b). Thus, for example, in the spectrum in Figure 1a the set of imidazole cross-peaks labeled 4 must arise from a cadmium-bound histidine which is in the $N_\epsilon H$ tautomeric state and must therefore be bound to the cadmium via N_δ; the same spectrum allows one to identify not only which histidines are bound to the cadmium but also which specific imidazole nitrogen is involved. There is in fact only one set of cross-peaks in the spectrum with the characteristics of a cadmium-bound $N_\epsilon H$ tautomer, and comparison with the crystal structure of the Zn-enzyme[11] allows assignment of the cross-peak pattern 4 to histidine 88. The histidine in the second metal binding site (His 210, cross-peak pattern 3) has also been assigned, using a Zn/Cd hybrid enzyme; details will be published elsewhere.

For the four cadmium-bound histidine residues, values of $^1J_{N-Cd}$ and $^3J_{H-Cd}$ could be measured from the $^{15}N-C-^1H$ cross-peaks as shown in Figure 2c, and we have attempted to relate the values of these coupling constants to structural features of the metal-binding site. The structure of the cadmium-substituted β-lactamase BCII is not yet available; however, in the related metallo-β-lactamase from *Bacteroides fragilis* the cadmium enzyme is essentially identical in structure to the zinc protein,[20] and we thus used the 1.85 Å structure of zinc β-lactamase BCII[11] as the basis for analysis of the coupling constants. In the case of the three-bond $^3J_{H-Cd}$, no clear-cut relation to structure could be discerned although it has been successfully used in the mono-metal iron−cadmium-substituted rubredoxin.[21] Similar difficulties have been reported in the bi-metal zinc cadmium-substituted DNA-binding domain of GAL4 and presumably arise from the complexity of the electronic structure of the metal.[22]

However, for the $^1J_{N-Cd}$ values, there are indications of a possible relationship to the structure around the cadmium center. The observed values ranged from 78 to 216 Hz, consistent with values of 190−210 Hz reported[23] for ^{15}N-sulfonamide complexes of ^{113}Cd-substituted carbonic anhydrase. The two cadmium-bound histidine residues whose assignments are known, His88 and His210, both have large values of $^1J_{N-Cd}$, 178 and 216 Hz respectively, and both have small values of the angle δ between the N−Zn vector and the imidazole plane, 7.8° and 4° respectively. The other two, as yet unassigned, histidine residues have larger values of δ (17.2° and 31°) and smaller values of $^1J_{N-Cd}$ (118 and 78 Hz) The present data are thus consistent with a relationship between the magnitude of $^1J_{N-Cd}$ and the extent to which the cadmium is out of the plane of the imidazole ring, with a large coupling constant corresponding to an in-plane metal. This is intuitively reasonable, but more data will be required to place this possible relationship on a firm footing, and to assess the influence of electronic effects on $^1J_{N-Cd}$.

In summary, we have shown that $^1H-^{15}N$ HMQC, with gradient coherence selection and water flip-back pulses, is the method of choice for identifying cadmium-bound histidines in metalloproteins. By selecting a suitable refocusing delay, it is possible to detect all the imidazole ^1H and ^{15}N resonances in the same experiment, conveniently identifying the metal ligands from the $^1J_{N-Cd}$ splitting, and their tautomeric state—and hence the individual nitrogen which binds the metal—from the pattern of N−C−H cross-peaks.

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