and $k_r^{I'}$ = 0.072–0.079 mdyne Å^{-1}, respectively. This agreement lends some credance to the proposition that the low-intensity asterisked bands in the far-IR spectra of $Na_{56}Y$ and $Na_{86}X$ may be associated with the symmetrical counterparts of the main, asymmetric site II and I′ E-type Na^+ cation vibrations. Raman experiments are underway in our laboratory to investigate this idea further.

Clearly similar ideas to those expounded for sites I′ and II apply to site III cation modes. For example, in the case of site III″ cations, the local symmetry of the site is C_s and is expected to display 2A′ + A″ ν_{MO_3} cation modes, of which the asymmetric A″ is likely to be the most intense but with some measureable IR activity from at least one of the A′ symmetric modes. Finally one should note that "second-order" intensity effects relating, for example, to Jahn–Teller cation site distortions for transition-metal 2+ ions, reductions in site symmetry orginating from the Si/Al distribution in the zeolite lattice, and small deviations from ideal site symmetries (e.g., site I, $O_h \rightarrow D_{3d}$), can give rise to slight IR activity to cation modes which would normally be IR silent.

Conclusions. This paper demonstrates that far-infrared spectroscopy is an extremely sensitive direct probe of the cation locations in faujasite zeolites. It is an extremely powerful partner to X-ray diffraction techniques and succeeds in locating previously unidentified cations. In terms of a fuller understanding of the catalytic behavior of zeolites, this is clearly of pivotal importance. The main conclusions of this paper can be summarized as follows:

1. A local molecule **GF**-matrix approximation for computing the vibrational frequencies of cation modes for metal faujasite zeolites can satisfactorily explain the frequency ordering I > I′ > III′ for alkali-metal 1+ and I > III″ > I′ for transition-metal 2+ ion-exchanged faujasite.

2. Site III transition-metal ion vibrations occur at much higher frequencies than one would predict by using the Brodskii approach and site III frequencies for alkali-metal zeolites. Hence the assignment of III″ for the former and III′ for the latter is favored.

3. Far-infrared spectroscopy can identify cation occupancy of sites that have previously remained unnoticed.

4. From the data gathered in this first study, one feels optimistic that the transition bond dipole moment approach to the analysis of the far-IR intensities of metal cation vibrational modes in faujasites carries considerable potential as a valuable adjunct to the Brodskii or **GF**-frequency method for assigning metal cation IR bands and site locations in the zeolite framework. Furthermore, the intensity method offers new opportunities to establish actual site populations of exchangeable metal cations in the zeolite, thereby complimenting X-ray crystallographic techniques.

5. A local molecule **GF**-matrix and transition bond dipole moment analysis of the expected frequencies and intensities of IR-active "symmetrical" metal cation vibrations of three-coordinate sites (in relation to their "asymmetrical" counterparts) alerts one to the realization that weak bands and unexplained absorptions might have their origin in just these kinds of symmetrical modes and therefore they should not be neglected when evaluating the far-IR spectra of metal ion-exchanged zeolites.

However, numerous new and fascinating questions have been raised by this work, and many problems remain which will require close attention in future studies of metal ion-exchanged zeolites.

Acknowledgment. The financial assistance of the Natural Sciences and Engineering Research Council of Canada's Strategic Grants Programme and the Connaught Foundation of the University of Toronto is greatly appreciated. We are also indebted to Drs. Edith Flanigen (Union Carbide) and Paul Kasai (IBM) for various ultrahigh purity zeolites and to Kate Helwig for her invaluable technical assistance with the preparation of zeolite samples.

Identification of Proton NMR Signals from the Metal Ligands in Cadmium-Substituted Plastocyanin via Two-Dimensional Multiple-Quantum Detection in the Absence of Explicitly Resolved ^1H–^{113}Cd Coupling

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Abstract: Two-dimensional (2D) proton-detected heteronuclear $^1H\{^{113}Cd\}$ multiple-quantum (HMQ) NMR spectroscopy of ^{113}Cd-substituted plastocyanin from spinach (*Spinacia oleracea*) yielded signals from the four amino acid side chains ligated to the metal. HMQ coherences over four bonds were observed with the metal ligands histidine-37 and histidine-87. Assignments of signals from the metal ligands, cysteine-84 C_β–$^1H_2$ and methionine-92 C_ϵ–$^1H_3$, are proposed. HMQ signals were observed in the absence of resolved ^1H–^{113}Cd coupling. The 2D HMQ method should be useful for identifying ^1H NMR peaks from metal ligands in metalloproteins.

Identification of resonances from the metal ligand residues represents an essential step in characterizing metalloproteins by NMR spectroscopy. ^1H NMR assignments for resonances from the two histidine and single methionine ligands in the "blue copper" protein plastocyanin have been reported.[1-3] These assignments in the Cu(I) form of the protein have been based on comparisons with spectra of the Cu(II) protein in which signals from groups close to the metal exhibit characteristic paramagnetic broadening.

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This assignment strategy will not work for metalloproteins containing diamagnetic metal ions or metal ions whose oxidation states are not amenable to manipulation. Substitution of the native copper ion in spinach (*Spinacia oleracea*) plastocyanin by $^{113}Cd(II)$[4] has presented such an assignment problem, which is compounded because structural differences between ^{113}Cd-substituted plastocyanin and the native Cu plastocyanin result in shifts of several ^1H NMR signals by as much as 0.4 ppm.[5] The proton-detected two-dimensional (2D) heteronuclear multiple-quantum[6,7] (HMQ) $^1H\{^{113}Cd\}$ NMR experiment[8] has allowed us to identify and assign unambiguously the peaks from the histidine, cysteine, and methionine ligands in $^{113}Cd(II)$ plastocyanin in the absence of resolved ^{113}Cd–^1H spin–spin coupling.

Experimental Procedures

Plastocyanin from spinach (*Spinacia oleracea*) was purified as previously described.[1] Substitution of ^{113}Cd into plastocyanin was performed by Engeseth.[4] The two-dimensional Fourier transform NMR spectra (Figure 1) were obtained using the pulse sequence[6] $90^0_x(^1H)$–$\Delta1$–$90^0_\phi(^{113}Cd)$–t_1–$90^0_x(^{113}Cd)$–$\Delta2$–$t_{2,\phi}(^1H)$. Two delay times ($\Delta1$) were used. $\Delta1$ = 50 ms provides maximal signal for 10-Hz ^1H–^{113}Cd coupling neglecting relaxation effects; $\Delta1$ = 9 ms is a compromise value to alleviate the problem of intensities being lost by T_2 relaxation during the preparation period. The rf carrier zero frequencies were set at 432.3 ppm from $Cd(ClO_4)_2$ for ^{113}Cd (at 66.58 MHz) and 4.96 ppm from TSP for ^1H (at 300.07 MHz). Frequency offsets, $\Delta\nu_{^1H} - \Delta\nu_{^{113}Cd}$, on the double-quantum axis (labeled H–Cd in Figure 1) are relative to the radio frequencies of these nuclei. This axis represents the ^1H frequency offset minus the ^{113}Cd frequency offset. The spectrometer used has been described previously.[8,9]

Results and Discussion

The 2D HMQ spectra of cadmium-substituted spinach plastocyanin is shown in Figure 1. Attributions of ^1H NMR resonances at 8.20, 8.06, 7.46, and 7.41 ppm are readily made to the histidine ring protons. Precise assignments to each of the two ligand residues[10] (histidine-37 and histidine-87) or atom types[11] (C_δ–^1H and C_ϵ–^1H) are obtainable by two-dimensional heteronuclear correlated (HETCOR) $^{13}C\{^1H\}$ spectroscopy.[11] The above assignments corroborate assignments made by one-dimensional ^1H NMR studies[5] for the three resonances farthest downfield. The fourth resonance could not be identified as arising from a metal-ligated histidine without the HMQ results presented here. When the contribution of the ^1H offset to the double-quantum frequency is taken into account, all the cross peaks can be shown to correlate with a single ^{113}Cd frequency. Such a data set can also be digitally converted into a direct ^1H–^{113}Cd shift correlation plot by the procedure of Bax et al.[6]

None of the four histidine resonances in the HMQ spectrum show resolved coupling. The ^1H–^{113}Cd coupling is also unresolved in the one-dimensional ^1H NMR spectrum at 470 MHz.[5] Thus, the HMQ method is applicable not only to systems where coupling is resolvable,[8] but also to systems with unresolved coupling. Since the line widths of these peaks in the projected HMQ spectrum are ~10 Hz, the coupling constants are ≤5–7 Hz. If Cd(II) binds to the N_δ atom of each of the two imidazoles, as in copper plastocyanin,[12] then two of the histidine contours, those from C_δ–^1H resonances, represent four-bond couplings. Although unresolved, these coupling constants are probably larger than

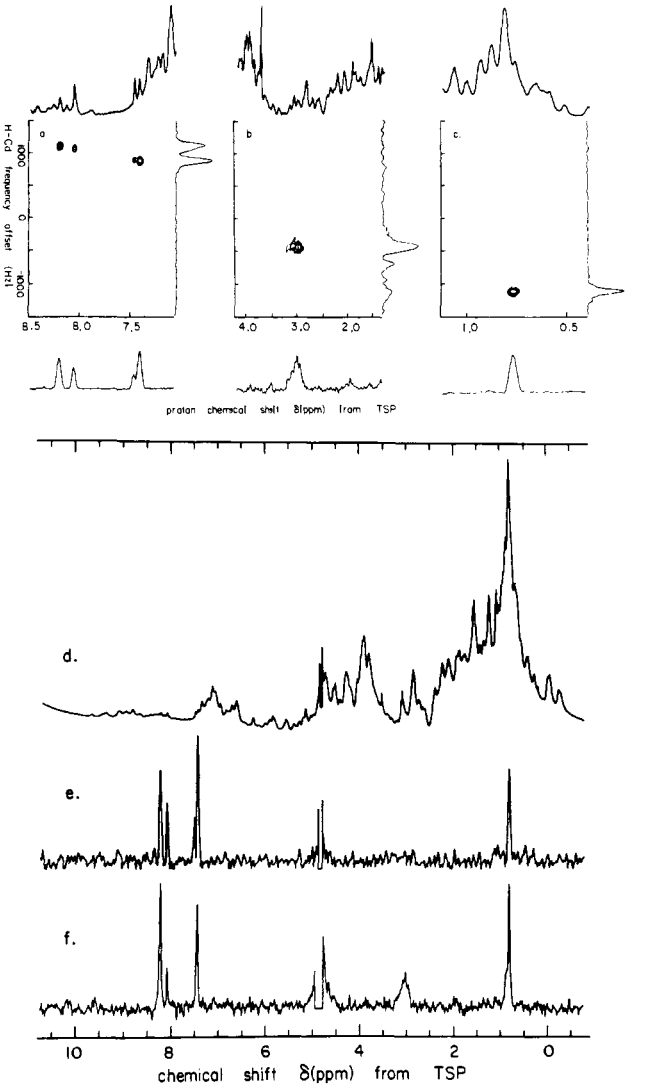

Figure 1. (Top) Proton-detected (^1H–^{113}Cd) double-quantum coherence in cadmium-substituted plastocyanin from spinach. The contour plots are of two-dimensional Fourier transform NMR spectra obtained as described under Experimental Procedures. Projections of the two-dimensional surface are shown at the bottom and side of each spectral region displayed. Corresponding one-dimensional ^1H NMR spectra obtained at 470 MHz[5] are shown for reference at the top of each region. A preparation delay $\Delta1$ = 50 ms was used for (a) and (c), while $\Delta1$ = 9 ms was used for (b). For the $\Delta1$ = 50 ms case, 60 t_1 blocks were accumulated; for the $\Delta1$ = 9 ms case, 32 t_1 blocks were obtained. Total acquisition times were about 11 h each. The sample contained 10 mM protein with 27 mM phosphate buffer at pH* 6.6 in ^2H_2O. Assignments of ^1H–^{113}Cd coherences to metal ligands: (a) histidine ring protons, (b) cysteine methylene protons, (c) methionine methyl protons. (Bottom) Comparison of (d) the one-dimensional ^1H NMR spectrum at 300 MHz of ^{113}Cd plastocyanin with the full ^1H projection of the two-dimensional spectra (e) and (f). Projection (e) was derived from the two-dimensional spectrum shown above in contour plots (a) and c); projection (f) was derived from the data that yielded contour plot (b). Each projection provides a subspectrum containing ^1H NMR peaks only from amino acid side chains ligated to the metal. In general, more than one preparation delay ($\Delta1$) may be required in order to resolve signals from all the metal ligands. The one-dimensional ^1H NMR spectrum is the result of averaging 32 scans using rapid-scan inversion recovery to reduce the HDO signal (1.1-s delay between 180° and 90° pulses; 90° pulse = 8 μs). The sharp intensities near 5 ppm are artifacts from the zeroed HDO signal.

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normal because of the "W" geometry of the interconnecting bonds.[13]

Assignment of the methionine-92 C_ϵ–$^1H_3$ resonance is easily made to a peak at 0.77 ppm. Preliminary HETCOR $^{13}C\{^1H\}$ results had suggested an assignment near 0.7 ppm;[5] however, the signal-to-noise ratio (S/N) was too low to obtain an accurate chemical shift value. The increased sensitivity of the proton-detected HMQ $^1H\{^{113}Cd\}$ experiment[8,9] provides sufficient S/N to obtain a very accurate chemical shift value for this assignment.

The contours near 3 ppm (^1H) could arise from the cysteine-84 C_β–$^1H_2$ signals or methionine-92 C_γ–$^1H_2$ signals. Model peptide studies show the cysteine C_β–$^1H_2$ signals at 2.96 and 3.28 ppm.[14] The nonequivalence of the two cysteine C_β–$^1H_2$ signals, which are coupled to the C_α–^1H methine, gives rise to a homonuclear ABX spin system with strong coupling between spins A and B. This strong coupling would result in reduced intensity in the HMQ spectrum,[15,16] as is observed in the contours at 3 ppm (^1H). On the other hand, the methionine C_γ–$^1H_2$ resonances in model studies are at 2.63 ppm,[14] and ring-current predictions would move them ~0.17 ppm farther upfield. Based on these arguments, we confidently assign the ^1H NMR peaks at 2.93, 2.98, 3.03, and 3.08 ppm to cysteine-84 C_β–$^1H_2$ signals. The peak with low intensity at 3.15 ppm may be part of this system.

The absence of a signal attributable to the methionine-92 C_γ–$^1H_2$ might arise from restricted rotation associated with metal ligation. This leads to magnetic inequivalence of the C_γ–$^1H_2$ protons, as has been illustrated in cytochromes.[17] The coupling to the C_β–$^1H_2$ protons, which are also nonequivalent, yields a homonuclear ABMN spin system. The lack of an observed multiple-quantum coherence signal from coupling between the ^{113}Cd and the methionine-92 C_γ–$^1H_2$ spins could result from decreased intensity caused by strong homonuclear coupling. It is also possible that the ^{113}Cd–(methionine C_γ–$^1H_2$) coupling constant may be too small to produce an observable multiple-quantum coherence.

Conclusion

$^1H\{^{113}Cd\}$ 2D HMQ NMR provides a means of resolving a subspectrum containing ^1H NMR peaks from protein groups ligated to the metal (Figure 1). The present results clearly demonstrate that these resonances need not show resolved coupling to the metal in order to be correlated to the metal.[18] With an uncharacterized metalloprotein this provides the potential to identify, based on the characteristics of the assigned resonances, the amino acid side chains that ligate the metal. Since the method is applicable to a variety of amino acid side chains, it may prove useful in establishing the nature of ligand exchange steps in mechanisms of enzyme catalysis. Multiple cadmium sites are distinguishable in the 2D HMQ spectra provided that ligand exchange is slow.[8] In agreement with earlier results[4] the ^{113}Cd plastocyanin 2D HMQ resonances appear at a single ^{113}Cd shift, indicating a single Cd(II) site, without metal exchange between environments on the time scale of detection. With present instrumentation, useful $^1H\{^{113}Cd\}$ 2D HMQ NMR spectra of a small soluble metalloprotein can be accumulated in a period of a few hours.

Acknowledgments. We thank Dr. Helen R. Engeseth for substituting $^{113}Cd(II)$ into spinach plastocyanin and Drs. William Milo Westler and M. Albert Thomas for helpful discussions. A fellowship from the Allied Corporation (C.L.K.) is gratefully acknowledged. Support also was provided by the U.S. Department of Agriculture Competitive Research Grants Office, Cooperative State Research Service, Science and Education (82-CRCR-1-1045; J.L.M.), and the National Institutes of Health (AM 20357).

Registry No. ^{113}Cd, 14336-66-4; Cd, 7440-43-9.

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