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^{113}Cd Nuclear Magnetic Resonance Studies of Cabbage Histidinol Dehydrogenase

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ABSTRACT: Histidinol dehydrogenase (HDH), a dimeric protein, catalyzes two sequential oxidation reactions to yield L-histidine from L-histidinol *via* L-histidinal. HDH contains 1 mol of Zn(II) per mol of subunit, and removal of this metal abolishes the enzymatic activity. On substitution of Zn(II) with ^{113}Cd(II), the enzyme ([^{113}Cd]HDH) showed similar catalytic activity. The ^{113}Cd NMR spectra of [^{113}Cd]HDH were measured under various conditions. The ^{113}Cd NMR spectrum of [^{113}Cd]HDH showed a resonance at 110 ppm, which indicates that the metal ion is bound to the protein by a combination of nitrogen and oxygen ligands. ^{113}Cd NMR spectra of [^{113}Cd]HDH were measured as complexes with two substrates (L-histidinol and DL-histidinal) and four inhibitors (imidazole, histamine, L-histidine, and DL-4-(4-imidazolyl)-3-amino-2-butanone) in the absence and presence of NAD^+. Significant shifts of [^{113}Cd]-HDH resonance in the presence of the ligand indicate that the metal ion is located in the catalytic site of HDH and that substrates and inhibitors interact with the metal ion. The role of the metal ion in the HDH reaction is discussed.

Histidinol dehydrogenase (HDH)[1] [L-histidinol:NAD oxidoreductase (EC 1.1.1.23)] is an NAD^+-dependent enzyme that catalyzes the two final reaction steps in histidine biosynthesis, from L-histidinol *via* the intermediate L-histidinal to L-histidine (Figure 1) (Adams, 1955).

The active enzyme is a homodimer with a molecular weight of about 100 000. Each subunit contains one Zn(II) ion. The removal of the metal ion from HDH abolishes the enzymatic activity (Lee & Grubmeyer, 1987). To date, limited information about the state of the metal ion has been available. It is even unknown whether the metal ion is actually involved in the catalytic reaction of HDH or not. Recently, site-specific mutagenesis has been developed for studying the role of conserved cysteine residues in HDH from *Salmonella typhimurium* (Teng et al., 1993) and Cabbage (Nagai et al., 1993). Both studies indicate that the conserved cysteine residues in HDH are not liganded to the metal ion, and that HDH does not use a cysteine-based thiohemiacetal as a catalytic intermediate. X-ray crystallographic study on HDH has not been reported yet.

Cd(II) can often be substituted for Zn(II) at the active site of the metalloenzymes with retention of activity (Chlebowski & Coleman, 1976; Omburo et al., 1993). Hence metal ions in several zinc metalloenzymes have been explored by ^{113}Cd NMR spectroscopy (Summers, 1988). The chemical shift and line width of ^{113}Cd are sensitive to the number, type, geometry, and dissociation constant of its ligands. For proteins whose X-ray structural data are not available, ^{113}Cd NMR is used to provide a first approximation of the ligands at the metal binding sites. In many cases, ^{113}Cd NMR spectroscopy has provided new insights into protein−substrate interactions, conformational changes, and metal displacement reactions.

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[1] Abbreviations: HDH, histidinol dehydrogenase; [Zn]HDH, wild type HDH containing Zn; [^{113}Cd]HDH, ^{113}Cd-substituted histidinol dehydrogenase; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; LADH, horse liver alcohol dehydrogenase; T_1, spin-lattice relaxation time; HMQC, heteronuclear multiple-quantum coherence.

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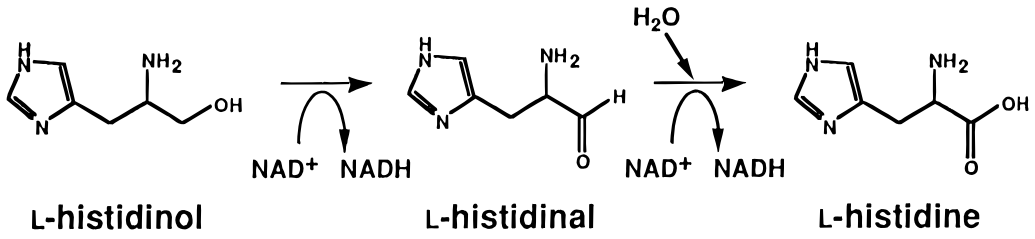

FIGURE 1: HDH reaction scheme.

In this study, HDH apoenzyme (Nagai et al., 1992; Nagai & Ohta, 1994) was reconstituted by adding 1 mol of cadmium per mol of subunit. The ^{113}Cd-substituted HDH ([^{113}Cd]HDH) exhibited the same catalytic activity as [Zn]-HDH. In order to elucidate the role of the metal ion and the reaction mechanism of HDH, ^{113}Cd NMR spectra of [^{113}Cd]HDH have been recorded in the complexes with the two substrates and four inhibitors [imidazole, histamine, L-histidine, and DL-4-(4-imidazolyl)-3-amino-2-butanone] in the absence and presence of NAD^+.

MATERIALS AND METHODS

Materials. ^{113}Cd metal (95 atom %) was obtained from ISOTEC Inc. (Miamisburg, OH). [^{113}Cd]SO_4 was prepared by the following procedure. ^{113}Cd metal (46.5 mg) was dissolved in 80 μL of concentrated nitric acid. After the metal was completely dissolved, 2 M ammonium carbonate was added. The resulting precipitate of cadmium carbonate was filtered off, washed by water, and dried. Diluted sulfuric acid was added to the powder of cadmium carbonate until the evolution of carbon dioxide stopped. The solution of cadmium sulfate was stored at the concentration of 100 mM.

NAD^+ and L-histidinol were purchased from Sigma (St. Louis, MO). Imidazole was obtained from Wako Pure Chemical Industries, Ltd. (Osaka), and L-histidine and histamine derived from Tokyo Kasei Kogyo Co., Ltd. (Tokyo).

DL-Histidinal and DL-4-(4-imidazolyl)-3-amino-2-butanone were prepared in the following manner. To a suspension of DL-histidine in MeOH was added $SOCl_2$ at −20 °C, and the mixture was stirred for 18 h at room temperature to give DL-histidine methyl ester HCl. Treatment of the methyl ester with BOC anhydride in acetone−H_2O (1:1) in the presence of Et_3N gave N_α,N_{im}-di-BOC-DL-histidine methyl ester. Reduction of the protected methyl ester with diisobutylaluminum hydride in THF afforded *N*-BOC-2-amino-3-(1-BOC-imidazol-4-yl)propanol, which was oxidized by activated dimethylsulfoxide in CH_2Cl_2 to *N*-BOC-2-amino-3-(1-BOC-imidazol-4-yl)propionaldehyde (di-BOC-DL-histidinal) (Mancuso et al., 1978). The aldehyde was deprotected with 4 N HCl in dioxane (or 30% HBr in CH_3COOH) to give DL-histidinal [2-amino-3-(1*H*-imidazol-4-yl)propionaldehyde] HCl (or HBr) salt. *N*-BOC-2-amino-3-(1-BOC-imidazol-4-yl)-1-methylpropanol, obtained by reaction of di-BOC-DL-histidinal with methylmagnesium chloride in THF, was oxidized to *N*-BOC-3-amino-4-(1-BOC-imidazol-4-yl)-2-butanone by treatment with periodinane (Dess & Martin, 1983). The deprotection reaction was performed in the same manner with di-BOC-DL-histidinal and gave DL-4-(4-imidazolyl)-3-amino-2-butanone HCl (or HBr) salt (Smissman & Weis, 1971). All compounds exhibited ^{1}H NMR and infrared spectra in agreement with the expected structure.

Enzyme Preparation. HDH used in this study was purified from a nuclear polyhedrosis virus genome expression system containing the plasmid pVL1393 (Summers & Smith, 1987; Nagai et al., 1992). The purification protocol of HDH is the same as that previously described (Nagai et al., 1993).

Preparation and Reconstitution of Apoenzyme. The apoenzyme of HDH was prepared as previously described (Nagai & Ohta, 1994). A solution of HDH (8 mg/mL) was incubated in 200 mM Gly-NaOH buffer (pH 9.2) containing 20 mM ethylenediaminetetraacetic acid (EDTA) at 30 °C for 1 h, followed by a Pharmacia gel filtration NAP-10 column (Pharmacia LKB) equilibrated in 20 mM tris-(hydroxymethyl)aminomethane (Tris) sulfate buffer (pH 7.2). The residual metal−EDTA complex and EDTA were removed by diafiltration with an Amicon ultrafiltration device Centriprep-10 (Amicon Inc., MA) against the Tris-SO_4 buffer solution. It was confirmed that the enzymatic activity dropped to less than 1% of the initial value. As reported in the previous paper (Nagai & Ohta, 1994), Zn analysis by inductivity coupled radiofrequency plasma atomic emission spectrometry showed that the apo-HDH did not contain any detectable amounts of Zn ion. Reconstitution of the apoenzyme was achieved by adding a [^{113}Cd]SO_4 solution to the apo-HDH solution with monitoring the enzymatic activity. About 10% molar excess [^{113}Cd]SO_4 to the subunit concentration of the apoenzyme was added, followed by dialysis against the Tris-SO_4 buffer in order to remove the excess metal salt. The enzymatic activity of reconstituted [^{113}Cd]-HDH was not decreased by dialysis.

Enzyme Assays. The enzyme concentration was determined spectroscopically by using an $A^{1\%}$ at 280 nm = 7.98 (Nagai et al., 1991). The enzymatic activity was routinely determined by monitoring the change in absorbance at 340 nm due to the conversion of NAD^+ to NADH. The reaction mixture contained 25 mM Bis-Tris-propane (pH 7.2), 0.5 mM NAD^+, 0.25 mM L-histidinol, and 1−2 milliunits of enzyme sample in a total volume of 2.0 mL. The reaction was performed at 30 °C and started by addition of L-histidinol (Nagai et al., 1993). We used a Hitachi U-3120 spectrophotometer.

NMR Spectroscopy. ^{113}Cd NMR measurements were mainly performed on a Bruker AMX600 spectrometer (133.1 MHz for ^{113}Cd) with a 10-mm tunable broad-band probe. Spin−lattice relaxation times (T_1) were estimated by using an inversion recovery method. In order to study the ^{113}Cd relaxation mechanism, several experiments were performed on a Bruker ARX400 spectrometer (88.8 MHz for ^{113}Cd). Chemical shifts were referred to the resonance position of 0.1 M $Cd(ClO_4)_2$. Typical acquisition parameters for AMX600 were 36 000 Hz sweep width and 16 μs pulse width (60 degree pulse) without proton decoupling. Samples were 1.8−2.0 mL (10% D_2O) of about 0.5 mM [^{113}Cd]HDH (dimer concentration) in the Tris-SO_4 solution in order to

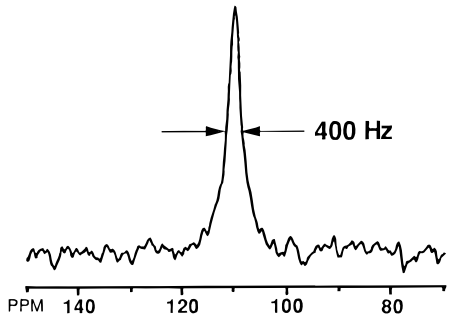

FIGURE 2: ^{113}Cd NMR spectrum of [^{113}Cd]HDH in 20 mM Tris-SO_4, 20 °C. The number of scans was 30 000, and the relaxation delay was 4 s. A 100 Hz line broadening was applied.

avoid the influence of halide ions on the ^{113}Cd chemical shift (Summers, 1988).

RESULTS

Reconstitution of apo-HDH and Activity of [^{113}Cd]HDH. Apoenzyme with an activity of less than 1% of that of [Zn]-HDH was reactivated with increasing amounts of [^{113}Cd]-SO_4. The titration demonstrated that the activity increased linearly until the cadmium concentration is equal to the subunit concentration. The activity of [^{113}Cd]HDH, reconstituted by 1 mol of ^{113}Cd(II) per mol of subunit, was 0.77 units/mg at pH 7.2, an activity which was equal to that of [Zn]HDH (data not shown).

^{113}Cd NMR of [^{113}Cd]HDH. Figure 2 shows the ^{113}Cd NMR spectrum of [^{113}Cd]HDH measured at 20 °C, pH 7.2. A single resonance was observed at 110 ppm. On adding excess ^{113}Cd(II) ion (1 mM) to 0.5 mM [^{113}Cd]HDH solution (dimer concentration), three other peaks with weak intensities were observed at −2, 22, and 83 ppm in addition to the peak at 110 ppm. After dialysis these three peaks disappeared (data not shown). This result indicates that Cd(II) ions bind predominantly to the major site (110 ppm) of the enzyme when Cd(II) ions are added in a 1:1 molar ratio to the subunit. Above this ratio, the excess Cd(II) ions bind to other sites of the enzyme.

The line widths of [^{113}Cd]HDH were about 400 Hz at 133.1 MHz and 280 Hz at 88.8 MHz. Within the temperature range from 4 to 20 °C, the chemical shift and line width of the [^{113}Cd]HDH resonance were identical while the intensity slightly increased with an increase in temperature (data not shown). This indicates that the resonance is not perturbed by a chemical exchange between the bound and free states of the metal ion. The T_1 value at 133.1 MHz was estimated to be 3.5 ± 0.7 s. The line width decreased only slightly, by about 20 Hz under broad-band proton decoupling.

The effect of pH on the chemical shift and line width of [^{113}Cd]HDH was examined. In the pH range 6−7.5, no significant change was observed in the ^{113}Cd spectra, but in the pH range 8−9 where the enzyme shows the highest activity (peak activity is at pH 9.2) (Nagai & Scheidegger, 1991), the ^{113}Cd resonance broadened and disappeared (Figure 3), and a slight downfield shift (<1 ppm) was observed with an increase of pH.

The ^{113}Cd NMR signal generally exhibits downfield shifts with an increase of chloride ion concentration if the external medium is accessible to the metal ion, (Summers, 1988). However, the chemical shift and line width of [^{113}Cd]HDH were unaffected by the presence of chloride ion (0−300 mM) at pH 7.2, indicating that the metal binding site of HDH is not solvent accessible (data not shown).

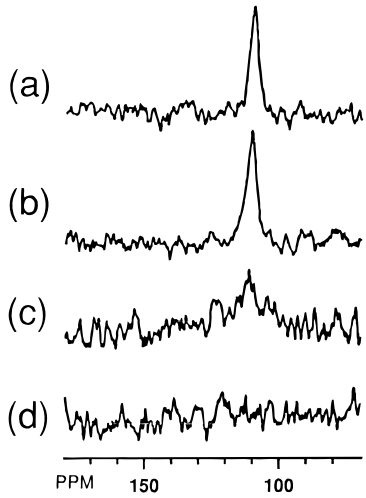

FIGURE 3: ^{113}Cd NMR spectra of [^{113}Cd]HDH at various pH values. The number of scans is given in the parentheses. The relaxation delays were 4 s for all spectra. A 100 Hz line broadening was applied. (a) pH 6.2 (15 000). (b) pH 7.2 (15 000). (c) pH 8.3 (90 000). (d) pH 8.8 (20 000).

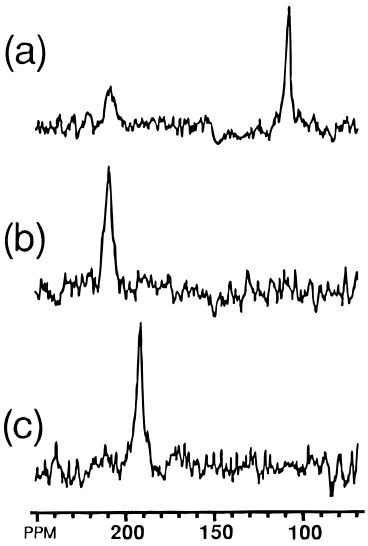

FIGURE 4: ^{113}Cd NMR spectra of the [^{113}Cd]HDH−substrate complexes in 20 mM Tris-SO_4, 20 °C. The number of scans is given in the parentheses. The relaxation delays were 4 s for a and 2.5 s for b and c. A 100 Hz line broadening was applied. (a) [^{113}Cd]HDH plus 0.5 equiv of L-histidinol per subunit (30 000). (b) [^{113}Cd]HDH plus 2 equiv of L-histidinol per subunit (120 000). (c) [^{113}Cd]HDH plus 2 equiv of DL-histidinal per subunit (80 000).

^{113}Cd NMR of Binary [^{113}Cd]HDH−Substrate Complexes. The product inhibition patterns of HDH conformed to a Bi Uni Uni Bi Ping Pong mechanism (Bürger & Görisch, 1981; Grubmeyer et al., 1987; Kheirolomoom et al., 1994). The reaction scheme is ordered with the binding of L-histidinol first and NAD^+ second and L-histidine as the last product to be released. K_m values for L-histidinol and L-histidinal reported for [Zn]HDH are 10 and 2.7 μM, respectively (Grubmeyer et al., 1987; Nagai et al., 1992; Nagai & Ohta, 1994).

Figure 4a,b shows the ^{113}Cd NMR spectra obtained upon titration of L-histidinol into [^{113}Cd]HDH. The resonance of [^{113}Cd]HDH at 110 ppm decreased upon addition of L-

Table 1: ^{113}Cd Chemical Shift of [^{113}Cd]HDH in the Presence of Ligands

ligand	δ (ppm)	line width (Hz)[a]
no	110	400 (280)[b]
NAD^+	108	400
substrate		
L-histidinol	210	580 (350)[b]
DL-histidinal	192	480 (350)[b]
inhibitor		
imidazole	142	550
+NAD^+	100	400
histamine[c]	152	430
L-histidine[c]	190	480
DL-4-(4-imidazolyl)-3-amino-2-butanone[c]	220	450

[a] Values are obtained at 133.1 MHz. [b] Values in parentheses are values of the line width at 88.8 MHz. [c] There was no significant difference in the presence of NAD^+.

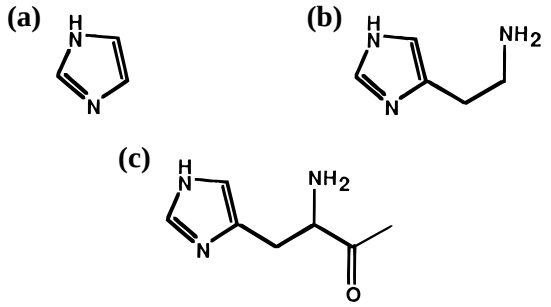

FIGURE 5: HDH inhibitors (a) imidazole, (b) histamine, and (c) DL-4-(4-imidazolyl)-3-amino-2-butanone.

histidinol. This decrease was correlated with the appearance of a new resonance of the [^{113}Cd]HDH−histidinol complex at 210 ppm. This chemical shift did not depend on the concentration of L-histidinol (0.25−5 times concentration of the subunit). The peak intensity of the [^{113}Cd]HDH−histidinol complex slightly increased with an increase of temperature like that of [^{113}Cd]HDH. On the other hand, the [^{113}Cd]HDH−histidinal complex showed a signal at 192 ppm whose line width is 480 Hz at 133.1 MHz (Figure 4c). The line widths of the [^{113}Cd]HDH and the two [^{113}Cd]-HDH−substrate complexes measured at 133.1 and 88.8 MHz are summarized in Table 1. The line widths of the complexes became significantly broader than that of [^{113}Cd]HDH in the absence of the ligand. Taking account of the temperature dependence of ^{113}Cd NMR spectra of the complexes, this line broadening could not be attributed to the chemical exchange between bound and unbound states, but to the conformational change of the metal binding site by the binding of the substrates. The line widths were the same for both complexes when measured at 88.8 MHz, although they are different at 133.1 MHz (Table 1).

In the absence of substrates, the addition of NAD^+ to [^{113}Cd]HDH made the resonance of [^{113}Cd]HDH shift slightly to upfield (108 ppm) without changing the line width (spectrum not shown).

^{113}Cd NMR of Binary [^{113}Cd]HDH−Inhibitor and Ternary [^{113}Cd]HDH−NAD^+−Inhibitor Complexes. The formation of binary and ternary complexes of [^{113}Cd]HDH with inhibitors was examined with following compounds; imidazole, histamine, L-histidine, and DL-4-(4-imidazolyl)-3-amino-2-butanone (Figure 5). These compounds are known as competitive inhibitors for [Zn]HDH. The K_i values for imidazole and L-histidine are about 1 mM, and those for

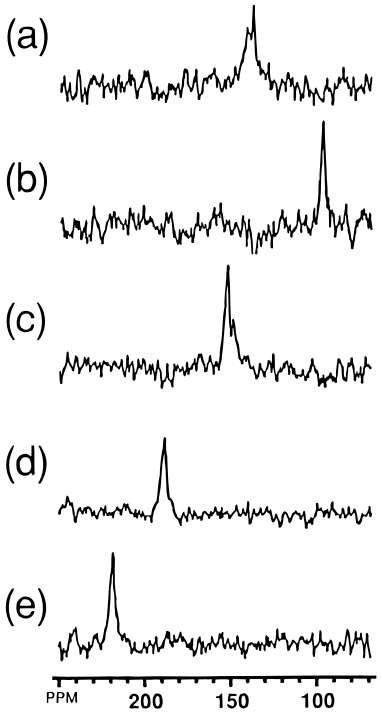

FIGURE 6: ^{113}Cd NMR spectra of the [^{113}Cd]HDH−inhibitor complexes in 20 mM Tris-SO_4, 20 °C. The number of scans is given in the parentheses. The relaxation delays were 2.5 s for all spectra. A 100 Hz line broadening was applied. (a) [^{113}Cd]HDH plus 10 equiv of imidazole per subunit (30 000). (b) As for a but a plus 2 equiv of NAD^+ per subunit. (c) [^{113}Cd]HDH plus 2 equiv of histamine per subunit (50 000). (d) [^{113}Cd]HDH plus 10 equiv of L-histidine per subunit (60 000). (e) [^{113}Cd]HDH plus 4 equiv of DL-4-(4-imidazolyl)-3-amino-2-butanone per subunit (50 000). The signal at 140 ppm is an artifact in a and b.

histamine and DL-4-(4-imidazolyl)-3-amino-2-butanone are 35 and 5 μM, respectively (Grubmeyer et al., 1989). Figure 6 shows ^{113}Cd NMR spectra of [^{113}Cd]HDH in the complex with the inhibitor in the absence and presence of NAD^+. The K_m value for NAD^+ is 50 μM (Nagai et al., 1992).

The ^{113}Cd NMR results for the [^{113}Cd]HDH−inhibitor complexes are summarized in Table 1. It was confirmed that these shifts were unchanged when the NMR measurements were carried out at higher ratios of ligand to enzyme (up to 10-fold molar excess to the subunit).

^{113}Cd NMR resonance of the binary [^{113}Cd]HDH−imidazole complex was observed at 142 ppm (Figure 6a). Addition of NAD^+ to the binary complex caused the peak to shift back to 100 ppm (Figure 6b).

As for the other three inhibitors [histamine, L-histidine, and DL-4-(4-imidazolyl)-3-amino-2-butanone], the binary complex showed resonances at 153, 190, and 220 ppm (Figure 6c,d, and e, respectively). The ternary complex of the inhibitors, however, gave an identical resonance to the corresponding binary complex (spectra not shown). Consequently, the addition of NAD^+ changed only the spectra of the binary complex of [^{113}Cd]HDH−imidazole and not of the other inhibitors.

DISCUSSION

For small metalloenzymes, ^{1}H−^{113}Cd heteronuclear multiple-quantum coherence (HMQC) experiments provide unambiguous evidence for ligand binding to the metal ion [see Summers (1988)]. However, HMQC spectra of [^{113}Cd]HDH with various delay periods did not show any peaks (data not

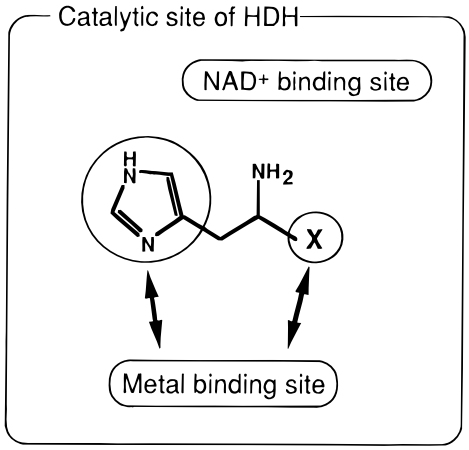

FIGURE 7: Schematic drawing of HDH catalytic site with a ligand. Arrows indicate the interaction between the metal binding site and the functional group of the ligand. X = CH_2OH, CHO, COOH, and $COCH_3$.

shown). An alternative way would be to utilize empirical information based on ^{113}Cd chemical shifts. The significant shifts of [^{113}Cd]HDH resonance in the presence of the ligand suggest that the substrates or inhibitors, except NAD^+, interact with the metal ion, and that the metal binding site of HDH is located in the catalytic site.

It is empirically known that metal binding sites comprising exclusively O-donor ligands give ^{113}Cd signals in the range +40 to −180 ppm, sites with one to three N donors giving shifts in the range 40 to ca. 300 ppm, and sites with S donors give signals with shifts from ca. 400 to 800 ppm (Summers, 1988). Thus, the ^{113}Cd chemical shifts of [^{113}Cd]HDH in the presence and absence of the substrates and inhibitors provide us with indirect information about coordinated ligands.

Downfield shifts of the binary [^{113}Cd]HDH−imidazole and −histamine complexes from [^{113}Cd]HDH were 32 and 42 ppm, respectively. The calculated downfield shift of the coordination of imidazole to ^{113}Cd is 42 ppm, and that of primary amino group is 73 ppm (Summers, 1988). The coordination of imidazole exhibited a 36 ppm downfield shift in a ^{113}Cd NMR study of horse liver alcohol dehydrogenase (LADH) (Bobsein & Myers, 1980). Our results suggest that the imidazole portion of the HDH ligands is coordinated to the metal ion, but the amino group is not. HDH shows a high degree of substrate specificity that arises from specific binding interactions. It was reported that the imidazole portion of the binding site contributes considerably to the overall binding energy and is highly specific and that most of the side-chain binding interaction occurs at the amino group (Grubmeyer et al., 1989). Figure 7 is a schematic drawing of the metal binding site of HDH in the complex with the ligand derived from the ^{113}Cd NMR data. Since the amino group of the ligands is known as a critical binding portion for HDH (Grubmeyer et al., 1989), it may interact with another part of the protein.

The other complexes [L-histidinol, DL-histidinal, L-histidine, and DL-4-(4-imidazolyl)-3-amino-2-butanone] showed diverse downfield shifts (38−65 ppm) from the [^{113}Cd]-HDH−histamine complex. It suggests that the environment around the metal ion is sensitive to difference in the chemical structure of functional groups (X in Figure 7). Furthermore, this suggests that, in addition to the imidazole portion of the ligands, an oxygen atom of the functional group X also interacts with the metal ion. It is possible that the metal ion plays a similar role to that of a Lewis acid, like the Zn(II) in LADH (Zeppezauer, 1986), inducing polarization of the carbonyl group to increase the susceptibility to nucleophile attack for the aldehyde oxidation. The differences in electronegativities and location of the oxygen atoms may cause the diverse downfield shifts. When the coordination of the imidazole portion is taken into account, the coordination number in the transition state of HDH reaction may be 5.

Table 2: ^{113}Cd Chemical Shift of ^{113}Cd-Substituted Proteins Whose Ligand Set Is Composed of Nitrogen and Oxygen[a]

protein	δ (ppm)	ligand set[b]		
alkaline phosphatase	52	N	2COO	W
concanavalin A	32−46	N	3COO	2W
carboxypeptidase A	120	2N	COO	W
insulin	165	3N	3W	
carbonic anhydrase	210−220	3N	W	

[a] From Summers (1988). [b] N = imidazole, COO = carboxyl, and W = water molecule.

The chemical shift (110 ppm) of [^{113}Cd]HDH in the absence of the substrates and inhibitors excludes the possibility of sulfur ligation and an all-oxygen ligand set of the protein residues. This result is consistent with the site-specific mutagenesis studies which found that the conserved cysteine residues are not liganded to the metal ion (Teng et al., 1993; Nagai et al., 1993). Thus, the metal ion in [^{113}Cd]HDH is coordinated to the protein by a combination of nitrogen and oxygen ligands. Examples of such a metal site are listed in Table 2 by a tabulation of the ^{113}Cd chemical shifts available for ^{113}Cd-substituted protein whose ligand set was determined by X-ray crystallography. These data suggest that the metal binding site of [^{113}Cd]HDH contains two nitrogens, most probably His, and oxygen ligands such as Asp and Glu. These results are in accord with a general ligand rule for the catalytic zinc ion, in which His is by far the most common ligand while Cys is not (Vallee & Auld, 1990a,b). LADH is known as an exception of the general ligand rule. One of two metal ions in LADH is located in the catalytic site, where one H_2O, one His, and two Cys are coordinated to the metal ion (Eklund et al., 1976).

With a few exceptions (e.g., LADH), a systematic spacing in the sequence of the metal ligands is observed for the catalytic zinc binding site of the zinc enzymes (Vallee & Auld, 1990a,b): The first two ligands are separated by a "short spacer" consisting of one to three amino acids, and these ligands are separated from the third ligand by a "long spacer" of ∼20 to ∼120 amino acids. Unlike LADH, HDH follows the general ligand rule of the catalytic zinc site like usual zinc enzymes. Assuming that the metal ligand set of HDH (His and Asp/Glu) follows the systematic spacing, only the pair of His^{261}−Asp^{264} satisfies the condition of the short spacer in all the pairs of conserved His-Asp, His-Glu, and His-His (Nagai et al., 1991). Recently, it was reported that His^{261} is essential for the ligation of the zinc of cabbage HDH (Nagai & Ohta, 1994). Therefore, Asp^{264} is also probably a metal ligand of HDH. The positions of the other conserved His residues, His^{327} and His^{367}, satisfy the condition of the long spacer. Since it was confirmed that His^{367} is not essential for biological activity of HDH (Nagai & Ohta, 1994), His^{327} might be a critical residue of the metal ligands.

One water molecule is generally coordinated to the catalytically active Zn(II) ions of zinc metalloenzymes and is critical for the activity of the enzyme (Vallee & Auld, 1990a,b). If, as our data suggest, the metal ion is located in the catalytic site, it is expected that H_2O is coordinated to the metal ion of HDH. The fact that H_2O is involved in the catalytic reaction (Figure 1) also suggests the coordination of H_2O is required. The coordinated water molecule would not readily be exchangeable with solvent water molecules by taking into account that the chemical shift of [^{113}Cd]HDH is not affected by chloride ion concentration.

The ^{113}Cd chemical shift of the ternary complex of [^{113}Cd]HDH−inhibitor−NAD^+, except for the complex with imidazole, is identical to that of the corresponding binary complex. This means that the enzyme structure around the metal binding site of the binary [^{113}Cd]HDH−inhibitor complex remains unchanged by the binding of NAD^+. ^{113}Cd NMR studies on LADH showed that the NAD^+ binding to the catalytic site of LADH caused an upfield shift of the ^{113}Cd resonance due to the conformational change (Bobsein & Myers, 1981). There is no remarkable structural similarity between HDH and LADH, and the reaction order of the alcohol oxidation by HDH differs from that by LADH (Eklund et al., 1986; Zeppezauer, 1986; Coleman, 1992). This suggests that the relative location of the NAD^+ and metal binding sites may be different between HDH and LADH. The exceptional upfield shift of the [^{113}Cd]HDH−imidazole−NAD^+ complex from its binary complex indicates the dissociation of the coordination bond. The lack of the amino group may destabilize the binary complex.

The resonance of [^{113}Cd]HDH broadened with an increase of pH (Figure 3). Such a broadening feature can be explained by a small population of other Cd(II) species (Gettins, 1986). The signal broadening of [^{113}Cd]HDH with an increase of pH could be attributed to an intermediate exchange process between at least two environments around the metal ion. The enzymatic activity of HDH is enhanced with increasing pH and becomes the highest at pH 9.2 where all the catalytic parameters (e.g., K_m, k_{cat}) were determined (Nagai & Scheidegger, 1991). The facts that the metal ion of HDH is readily removed by EDTA at pH 9.2 but not at neutral pH (Nagai & Ohta, 1994) suggest that conformational fluctuations occur around the catalytic site at higher pH. These fluctuations may allow the substrate or EDTA molecules easy access to the metal ion.

The role of the metal ion in the catalytic reaction of HDH was elucidated by the present study. ^{113}Cd NMR results of ^{113}Cd-substituted HDH reveal that the metal ion is located in the catalytic site. Although the conformational change induced by ligand binding may contribute to the chemical shift changes of the complexes to some extent, the chemical shift values of the complexes suggest that the imidazole portion and oxygen atom of the ligand interact with the metal ion. The involvement of the metal ion in the catalytic reactions was demonstrated by the chemical shift changes of [^{113}Cd]HDH−ligand complexes.

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