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Effect of excess cadmium ion on the metal binding site of cabbage histidinol dehydrogenase studied by ^{113}Cd-NMR spectroscopy

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Abstract **The enzymatic reaction of histidinol dehydrogenase (HDH) was stimulated by about maximally 75% on the addition of Cd^{2+} ion to the reaction mixture. ^{113}Cd-substituted HDH in the presence of excess Cd^{2+} has been studied by ^{113}Cd-NMR. $^{113}Cd^{2+}$ less than 1 equiv. per subunit preferentially binds to the catalytic metal binding site of the apoenzyme. Further addition of the metal ions causes the structural change of the enzyme including the catalytic metal binding site. HDH takes at least three discernible states, which may correspond to the more or less active forms of the enzyme induced by metal ions.**

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Key words: Histidine biosynthesis; L-Histidinol; Histidinol dehydrogenase; Zinc enzyme; ^{113}Cd-NMR

1. Introduction

Histidinol dehydrogenase (HDH) [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 over L-histidinal to L-histidine [1]. HDH is a dimer of two identical subunits whose molecular weight is about 50 000. Each subunit binds one zinc ion as a cofactor, and the removal of the metal ion from wild-type HDH ([Zn]HDH) abolishes the enzymatic activity [2]. However, the role of the metal ion has not been elucidated. Recently, we studied ^{113}Cd-NMR of ^{113}Cd-substituted HDH ([^{113}Cd]HDH) which is catalytically as active as [Zn]HDH, and demonstrated that the metal ion is located in the catalytic site and that the substrate or inhibitor interacts with the metal ion [3]. ^{113}Cd-NMR is widely exploited for studies on metalloenzymes [4] to which ^{1}H-detected heteronuclear multiple quantum coherence spectroscopy was not applicable, e.g. in the case of HDH [3] and is, in particular, useful for the studies of kinetics of metal displacement reactions [5–10] which cannot be investigated by X-ray crystallography.

It was previously reported that Mn^{2+} in the reaction mixture stimulates the HDH reaction [11], whereas other divalent cations such as Zn^{2+}, Mg^{2+}, Ni^{2+}, Co^{2+}, Cu^{2+}, etc. depress it [12,13]. Although Zn^{2+} ion of [Zn]HDH is known to be replaced by Mn^{2+} under alkaline conditions [13], the stimulatory mechanism of Mn^{2+} has not been elucidated.

In the present study, we found that Cd^{2+}, like Mn^{2+}, also enhanced the enzymatic activity by maximally 75%. Effects of the metal ion on the structural change around the metal binding site were investigated by ^{113}Cd-NMR spectroscopy.

2. Materials and methods

2.1. *Materials*

^{113}Cd metal (95 atom %) was obtained from ISOTEC Inc. (Miamisburg, OH). [^{113}Cd]SO_4 was prepared as described previously [3]. NAD$^+$ and L-histidinol were purchased from Sigma (St. Louis, MO). Non-labeled $CdSO_4$ was purchased from Nacalai Tesque (Kyoto, Japan).

2.2. *Enzyme preparation*

HDH used in this study was purified from a nuclear polyhedrosis virus genome expression system containing the plasmid pVL1393 [14,15]. The purification protocol of [Zn]HDH and the preparation and reconstitution of the apoenzyme are the same as that previously described [3,16,17]. Zn analysis of the apoenzyme showed no detectable amounts of Zn ion [17], and reconstituted [^{113}Cd]HDH exhibited the same catalytic activity as [Zn]HDH [3].

2.3. *Enzyme assays*

The enzyme concentration was determined spectroscopically by using an $A^{1\%}$ at 280 nm=7.98. The enzymatic activity of HDH was routinely determined by monitoring the change in absorbance at 340 nm due to the reduction of NAD$^+$. The reaction mixture contained 25 mM *bis*-Tris-propane (pH 7.2), 0.5 mM NAD$^+$, 0.25 mM histidinol, 1–2 mU of enzyme sample ($\approx$1 nM), and various concentrations of Cd^{2+} in a total volume of 2.0 ml. The reaction was started with the addition of histidinol and incubated at 30°C. One unit of enzyme activity was defined in the previous paper [14]. A Hitachi U-3120 spectrophotometer was used and regulated at 30°C.

2.4. *NMR spectroscopy*

^{113}Cd-NMR measurements were performed on a Bruker AM×600 spectrometer (133.1 MHz for ^{113}Cd) with a 10-mm tunable broadband probe. Chemical shifts were referred to the resonance position of 0.1 M $Cd(ClO_4)_2$. Typical acquisition parameters were 36 000 Hz sweep width, 15 000 scans, 4 s relaxation delay, and 16 µs pulse width (60° pulse) without proton decoupling. A 100 Hz line broadening was applied. Samples were 1.8–2.0 ml (10% D_2O) of ~0.5 mM [^{113}Cd]HDH (dimer concentration) in 20 mM Tris-SO_4 (pH 7.2, 20°C) in order to avoid the influence of halide ions on the ^{113}Cd chemical shift [4].

3. Results

3.1. *Catalytic activity of [Zn]HDH in the presence of excess Cd^{2+}*

The enhancement of the catalytic activity of [Zn]HDH ($\approx$1 nM) with increasing amounts of $CdSO_4$ is shown in Fig. 1. The titration demonstrated that the stimulatory effect of Cd^{2+} was saturated between 20 and 200 µM $CdSO_4$, and slowly decreased with a further increase of the Cd^{2+} concen-

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Abbreviations: HDH, histidinol dehydrogenase; [Zn]HDH, wild-type HDH; [^{113}Cd]HDH, ^{113}Cd-substituted histidinol dehydrogenase; EDTA, ethylenediaminetetraacetic acid

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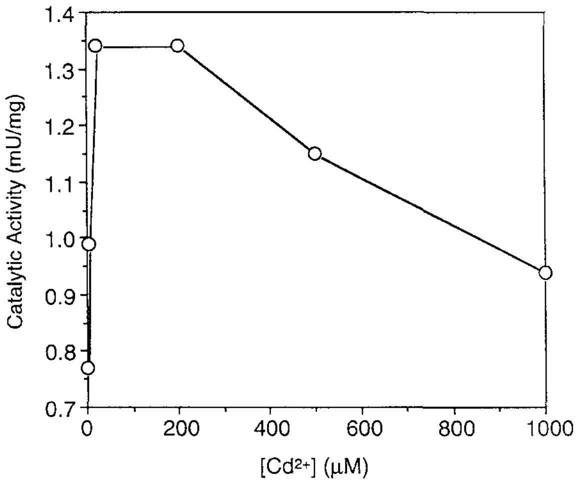

Fig. 1. Enhancement of activity for [Zn]HDH with varying amounts of $CdSO_4$.

tration ($[Cd^{2+}] > 200$ μM). The HDH reaction was stimulated about maximally 75% by the addition of Cd^{2+}. The catalytic activity of $[^{113}Cd]$HDH in the titration of Cd^{2+} showed the same feature as that of [Zn]HDH.

3.2. ^{113}Cd-NMR spectra of [Zn]HDH in the presence of $^{113}Cd^{2+}$

On addition of excess amount of $^{113}Cd^{2+}$ ion to [Zn]HDH, no signal of $^{113}Cd^{2+}$, even free $^{113}Cd^{2+}$ signal was detected at pH 7.2 and in the temperature range of 5–20°C (data not shown). The addition of excess EDTA (5-fold concentration of the protein) to the solution exhibited an EDTA-bound $^{113}Cd^{2+}$ peak at 82 ppm (data not shown). This indicates that $^{113}Cd^{2+}$ actually interacts with the enzyme although the signal is too broad to be observed probably because of chemical exchange broadening [5,6,18]. The enzymatic activity of this NMR sample after the addition of EDTA was completely retained, indicating that Zn^{2+} in the catalytic site is not removed by EDTA under the present conditions. Zn^{2+} in [Zn]HDH was not replaced by $^{113}Cd^{2+}$, which is also the case for Mn^{2+} [13]. Thus, the affinity of Zn^{2+} for the catalytic site would be stronger than those of the other divalent cations such as Cd^{2+} and Mn^{2+}.

3.3. ^{113}Cd-NMR spectra of $[^{113}Cd]$HDH in the presence of $^{113}Cd^{2+}$ or non-labeled Cd^{2+}

A titration of solutions of apo-HDH with $^{113}Cd^{2+}$ ions was performed with monitoring ^{113}Cd spectra. On addition of 0.3 equiv. of $^{113}Cd^{2+}$ per subunit to the apo-HDH, an resonance at 110 ppm was observed. Its line width was equal to that of $[^{113}Cd]$HDH reconstituted with 1 equiv. of $^{113}Cd^{2+}$ per subunit [3], and the intensity and catalytic activity were about 30% of $[^{113}Cd]$HDH reconstituted with 1 equiv. of $^{113}Cd^{2+}$ per subunit. In the concentration range of $^{113}Cd^{2+}$ ions less than 1 equiv. per subunit, only the peak at 110 ppm was observed (Fig. 2a). This result indicates that Cd^{2+} ions preferentially bind to the identical site of each subunit of the apoenzyme dimer. On the other hand, the addition of $^{113}Cd^{2+}$ beyond one equiv. per subunit resulted in a decrease of the signal at 110 ppm, together with a small broadening by 30 Hz, and in appearance of three new peaks at 83, 22, and −2 ppm (Fig. 2b). The intensity of the peak at 110 ppm in Fig. 2b became about 70% of that in Fig. 2a. The intensities of the two peaks at 83 and 22 ppm did not depend on the concentration of $^{113}Cd^{2+}$ while that of the peak at −2 ppm increased gradually with increasing the concentration of $^{113}Cd^{2+}$. In the ^{113}Cd-NMR spectrum with a shorter delay time of 2.5 s, relative intensity ratios among the peaks at 110, 83, and 22 ppm were almost identical to those observed in Fig. 2b within an experimental error, indicating that the relaxation times of these signals are almost the same.

An excess amount of non-labeled Cd^{2+} was added to freshly prepared 1:1 $[^{113}Cd]$HDH complex, in order to investigate the process of the displacement and appearance of the peaks. As shown in Fig. 2c, just after the addition of non-labeled Cd^{2+} (2 mM), the peak at 110 ppm disappeared, and two new peaks at 83 and 22 ppm appeared where the intensities were almost identical to those in Fig. 2b. This means that 70% of $^{113}Cd^{2+}$ at the catalytic binding site was displaced by non-labeled Cd^{2+} and the rest was shifted to 83 and 22 ppm. In this case, the peak at −2 ppm observed in Fig. 2b was not observed for some reason.

Since the chemical shift of $^{113}Cd^{2+}$ resonance is sensitive to the structure around the metal binding site, the results stated

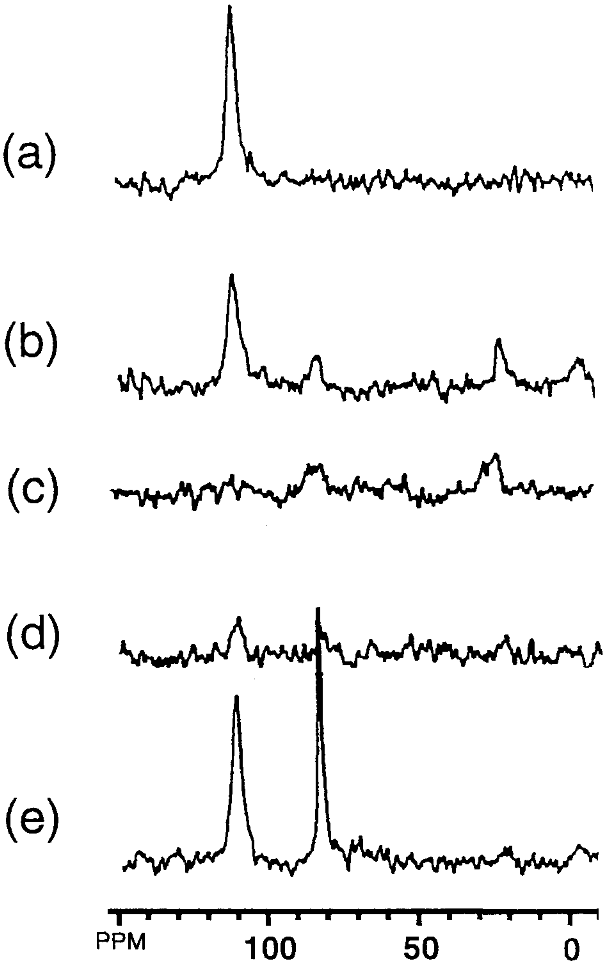

Fig. 2. ^{113}Cd-NMR spectra of the $[^{113}Cd]$HDH in the absence and presence of excess Cd^{2+} ion (protein dimer concentration 0.5 mM, temperature 20°C, pH 7.2). a: $[^{113}Cd]$HDH alone. b: $[^{113}Cd]$HDH plus 2 mM excess $^{113}Cd^{2+}$. c: $[^{113}Cd]$HDH plus 2 mM non-labeled Cd^{2+}. d: 1 day after (c). e: (b) plus 2 mM EDTA. To enable intensity comparisons to be made within this series, all spectra were acquired under identical conditions as described in the text.

above indicate that, by the interaction with the excess amount of Cd^{2+}, the enzyme comes to exist in at least three discernible states in which the binding environments of $^{113}Cd^{2+}$ at the catalytic site, such as the ligation and local conformation, are different. The binding scheme for the broad peak at −2 ppm may be different for the other peaks because of the concentration dependence of the peak intensity in the titration of $^{113}Cd^{2+}$.

One day after addition of $^{113}Cd^{2+}$ (2 mM), the peaks at 83 and 22 ppm became smaller, and a small peak at 110 ppm appeared again (Fig. 2d). No further change was observed, indicating that the equilibrium between Cd^{2+} ions in the free and different binding states was reached.

Hereafter, for convenience, the corresponding metal binding sites at 110, 83, 22, and −2 ppm will be represented as A, A′, A″, and B, respectively. Thus, in the presence of excess Cd^{2+}, Cd^{2+} ions at site A readily exchange with free Cd^{2+} ions faster than those for sites A′ and A″ although, in the absence of excess $^{113}Cd^{2+}$ ion, extensive dialysis by Tris-SO_4 buffer cannot remove $^{113}Cd^{2+}$ ion from [^{113}Cd]HDH [3]. This fact also suggests that the environmental change around site A (peak at 110 ppm in Fig. 2b), induced by interaction with excessive Cd^{2+} ions, is responsible for the reduced binding affinity of the metal ions for the enzyme.

3.4. ^{113}Cd-NMR spectra of [^{113}Cd]HDH in the titration of EDTA

In order to obtain information concerning the relative affinities of Cd^{2+} for sites A, A′, A″, and B, the titration of EDTA was performed for the solution of [^{113}Cd]HDH in the presence of excess amount of $^{113}Cd^{2+}$. With an increase of EDTA concentration, peaks of sites A′, A″, and B became smaller, and a new sharp peak at 82 ppm, which was assigned to a signal of EDTA-bound $^{113}Cd^{2+}$ signal, became larger (Fig. 2e). The intensity and line width of the peak at 110 ppm in Fig. 2e were restored as those in Fig. 2b. The peak at 110 ppm (site A) disappeared on further addition of EDTA in large excess (5-fold concentration of the protein), and only EDTA-bound ^{113}Cd signal was observed at 82 ppm (data not shown). The catalytic activity of the NMR sample was completely lost, and all the enzyme adopts an apo-form. Thus, Cd^{2+} at the catalytic site is removed by EDTA unlike Zn^{2+} of [Zn]HDH.

3.5. ^{113}Cd-NMR spectrum of [^{113}Cd]HDH–histidinol complex in the presence of $^{113}Cd^{2+}$

On addition of a substrate for HDH, L-histidinol, to the solution of [^{113}Cd]HDH in the presence of excess $^{113}Cd^{2+}$ ions, all the peaks (A, A′, A″, and B) disappeared and a new single resonance at 210 ppm was observed. This signal could be readily assigned to a ^{113}Cd signal of the ternary complex of [^{113}Cd]HDH–histidinol by the chemical shift and line width as reported previously [3]. A possible explanation for disappearance of the peaks at sites A′ and A″ is that for A′ and A″ ligation, one ligand, at the catalytic binding site is different from that for A, resulting in the chemical shift difference of ^{113}Cd among A, A′ and A″. On addition of the substrate (L-histidinol) this particular coordination site may be occupied by the substrate, and the binding environment becomes homogeneous, resulting in providing a single resonance of the ternary complex of [^{113}Cd]HDH–histidinol. Thus A, A′, and A″ states are affected by the interaction of HDH with L-histidinol, which may diminish the conformational heterogeneity around the catalytic binding site.

4. Discussion

The stimulatory effect of a metal ion on the HDH enzymatic activity has been known to be specific for Mn^{2+}, but the mechanism has not yet been manifested [13,19]. In this study, we found that, like Mn^{2+}, HDH is also activated by Cd^{2+}. Specific binding of metal ions to the protein at non-catalytic sites would induce structural change of the enzyme, resulting in the conformational heterogeneity around the catalytic metal binding site. At least three discernible states of the enzyme are anticipated, in which the environment of the catalytic metal binding site differs as represented by A, A′, and A″. The removal of $^{113}Cd^{2+}$ at the non-catalytic binding sites by EDTA would make all the protein molecules reversibly take the major or initial HDH conformation in the absence of excess metal ion, represented by the peak at 110 ppm (Fig. 2a). This resulted in simultaneous disappearance of the signals at sites A′ and A″. The affinity of Cd^{2+} for these particular non-catalytic binding sites is lower than that for the catalytic metal binding sites (A, A′, and A″). Relatively fast chemical exchange between the non-catalytic and free site should cause signal broadening to make detection of the signal difficult, which is analogous to Mg^{2+}-specific sites of skeletal troponin C [9,10].

Large chemical shift change observed for A′ and A″ suggests that the conformational change around the catalytic site also includes a change of ligation. Based on chemical shift values (83 and 22 ppm), Cd^{2+} ions in sites A′ and A″ are empirically supposed to be coordinated to nitrogen and oxygen ligands, and for site B it would consist of an all-oxygen ligand set [4]. It is reported that one of the conserved cysteine residues (Cys^{116} for *Salmonella typhimurium*, Cys^{112} for *Cabbage* HDH) may be adjacent to the metal at the metal binding site but not liganded to it [19]. Even after the structural change the cysteine residue would not ligand to the metal ion since ^{113}Cd chemical shifts at sites A′ and A″ (83 and 22 ppm) exclude the possibility for sulfur ligation that should cause a large downfield shift ($>$400 ppm) [4].

With an increase of the Cd^{2+} concentration in solution, the catalytic activity of [Zn]HDH was stimulated first and then gradually inhibited (Fig. 1). This suggests that the enzyme could take more and less active forms depending on the concentration of excess Cd^{2+} ions. The three states represented by A, A′ and A″ might be related to such forms. Cd^{2+} and Mn^{2+} in the reaction mixture can stimulate the HDH reaction [11], whereas other divalent cations such as Zn^{2+}, Mg^{2+}, Ni^{2+}, Co^{2+}, Cu^{2+} depressed it [12,13]. By addition of these divalent metal ions to [^{113}Cd]HDH, the states represented by A′ and A″ are expected to be identified with the more or less active form. Experiments are now proceeding in our laboratory.

Our results demonstrated that Zn^{2+} of [Zn]HDH was not displaced by $^{113}Cd^{2+}$ in solution, and the affinity of Zn^{2+} for the catalytic metal binding site is much higher than that of Cd^{2+} at pH 7. However, at pH 9.2, an optimum pH for the catalytic activity of HDH [12], Zn^{2+} in [Zn]HDH is replaced by Mn^{2+} and Cd^{2+} in the medium [19]. The line width of the ^{113}Cd signal increases with increasing pH ($>$8), which is probably attributed to the change of the chemical exchange rate between Cd^{2+} in the bound state and that in bulk solution [3]. At the higher pH, the enzyme would take a certain conformation in which Zn^{2+} at a catalytic binding site can more readily exchange with metal ions in bulk solution. Thus, enhancement of the enzymatic activity by metal ions and pH can be explained in terms of the conversion to the more active form of the enzyme.

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