References and Notes

(1) This work was funded by U.S. Department of Energy, Contract No. DE-AS02-78ER05016.
(2) Lamb, J. D.; Izatt, R. M.; Christensen, J. J.; Eatough, D. J. In "Coordination Chemistry of Macrocyclic Compounds", Melson, G. A., Ed.; Plenum Press: New York, 1979; pp 145–217.
(3) Choy, E. M.; Evans, D. F.; Cussler, E. L. *J. Am. Chem. Soc.* **1974**, *96*, 7085–7090.
(4) Caracciolo, F.; Cussler, E. L.; Evans, D. F. *AIChE J.* **1975**, *21*, 160–167.
(5) Schwind, R. A.; Gilligan, T. J.; Cussler, E. L. In "Synthetic Multidentate Macrocyclic Compounds", Izatt, R. M., Christensen, J. J., Eds.; Academic Press: New York, 1978; pp 289–308.
(6) Wong, K. H.; Yagi, K.. Smid, J. *J. Membrane Biol.* **1974**, *18*, 379–397.
(7) Kobuke, Y.; Hanji, K.; Horiguchi, K.; Asada, M.; Nakayama, Y.; Furukawa, J. *J. Am. Chem. Soc.* **1976**, *98*, 7414–7419.
(8) Reusch, C. F.; Cussler, E. L. *AIChE J.* **1973**, *19*, 736–741.
(9) Kirch, M.; Lehn, J. M. *Angew. Chem., Int. Ed. Engl.* **1975**, *14*, 555–556.
(10) Christensen, J. J.; Lamb, J. D.; Izatt, S. R.; Starr, S. E.; Weed, G. C.; Astin, M. S.; Stitt, B. D.; Izatt, R. M. *J. Am. Chem. Soc.* **1978**, *100*, 3219–3220.
(11) Sugiura, M.; Shinbo, T.; Takizawa, N. *Nippon Nagaku Kaishi* **1979**, 1427–1429.
(12) "The Merck Index", 7th ed.; Stecher, P. G., Ed.; Merck and Co.: Rahway, N.J., 1960; pp 366–367.
(13) Sillen, L. G.; Martell, A. E. "Stability Constants of Metal-Ion Complexes"; The Chemical Society: London, England, 1964; p 402. Sillen, L. G.; Martell, A. E. "Stability Constants of Metal-Ion Complexes", Suppl. No. 1; The Chemical Society: London, England, 1971; p 304.
(14) Li, N. N. *AIChE J.* **1971**, *17*, 459–463. Matulevicius, E. S.; Li, N. N. *Sep. Purif. Methods* **1975**, *4*, 73–96. Kitagawa, T.; Mishikawa, Y.; Frankenfeld, J. W.; Li, N. N. *Environ. Sci. Technol.* **1977**, *11*, 602–605.

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^{113}Cd NMR Spectrum of Substituted Horse Liver Alcohol Dehydrogenase

Sir:

In recent months there have been several reports of the use of ^{113}Cd NMR in metalloproteins. It has been reported for carbonic anhydrase,[1] alkaline phosphotase,[2] carboxypeptidase,[3] and concanavalin A.[4] These reports have shown the large chemical-shift range which is possible for ^{113}Cd even when dealing with simple coordinate bonding in biological systems. In this report we extend even further the range of expected ^{113}Cd chemical shifts to +751 ppm with respect to aqueous Cd^{2+}. This has been observed for Cd(II) bound to four cysteine residues in horse liver alcohol dehydrogenase (LADH). In addition, we have observed a second resonance assigned to the catalytic site of this enzyme. Further studies of this site with bound substrates should prove to be a powerful tool in mechanistic studies of this important enzyme.

LADH is a dimeric protein of 80 000 mol wt.[5] This enzyme, together with its coenzyme (NAD^+), catalyzes the oxidation of a wide variety of alcohols. Each of the two identical subunits normally contains two different Zn(II)'s. The "catalytic" Zn(II) is at the bottom of the active site pocket where both coenzyme and substrate are bound.[6] This Zn(II) is coordinated by two cysteine residues and one histidine. The fourth coordination position is presumably occupied, in the absence of a substrate, by an H_2O or an OH^-. The second Zn(II) in each subunit is a "noncatalytic" or "structural" Zn(II) according to kinetic and chemical evidence,[7] and it is coordinated by four cysteine residues in an approximately tetrahedral array. In 1970 Vallee[8] completely replaced the native Zn(II) by either Co(II) or Cd(II) using competitive equilibrium dialysis at pH 5.5. The metal-substituted enzymes retain catalytic activity, although it is reduced over that for the native enzyme. More recently, two methods have been developed[9,10] to replace selectively both kinds of Zn(II)'s. Our sample of LADH had both sites replaced by ^{113}Cd using a modification of Vallee's most recent method.[9]

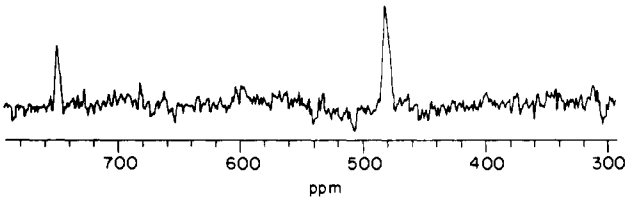

Figure 1. ^{113}Cd NMR spectrum of 90% ^{113}Cd-substituted LADH at 4 °C. The dimeric enzyme concentration was 0.29 mM. The measurement was made on 19 mL of sample with a 25-mm tube in a 180-MHz (^{1}H) magnet. The buffer was 50 mM sodium phosphate, pH* 7.5, with D_2O added to provide an internal lock. The NMR parameters were as follows: acquisition time, 0.2 s; pulse delay, 3 s; pulse angle, 70°; and spectral width, 20 kHz. This spectrum required 24 000 transients. A 45-Hz line broadening was used for sensitivity enhancement. The chemical-shift scale is referenced to 50 mM $CdSO_4$ at 22 °C as 0 ppm.

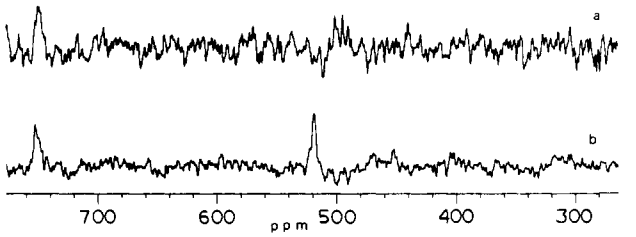

Figure 2. ^{113}Cd NMR spectra of 0.24 mM (dimeric) ^{113}Cd-substituted LADH at 4 °C (the buffer in both cases includes 50 mM sodium phosphate and 10% D_2O): (a) total imidazole concentration 2.4 mM, pH* 7.5; (b) total imidazole concentration 24 mM, pH* 7.8. Part a includes fewer data accumulations than b.

Figure 1 shows the ^{113}Cd NMR spectrum of Cd^{2+}-substituted LADH at pH 7.5. The resonance at 483 ppm is assigned to the catalytic site and the one at 751 ppm to the noncatalytic site. There are two bases for this assignment. The NMR studies of model Cd(II) systems[11,12] show that oxygen-containing ligands are the least deshielding and so aqueous Cd^{2+} is usually used as the 0 ppm reference at the high-field, or really low-frequency, end of the spectrum. Sulfur appears to be the most deshielding of any ligand. Haberkorn et al.[11] observed a deshielding trend for alkylthiolate complexes derived from glutathione and β-hydroxyethanethiol. Deshielding increased sharply with complete sulfur ligation and for $Cd(SR)_2$ it was 318 ppm, for $Cd(SR)_3$ it was 380 ppm, and for $Cd(SR)_4$ it was 520 ppm. Halides and ligands binding through nitrogen have intermediate deshielding effects. Very recently, in metallothionein,[13] chemical shifts in the range of 610–680 ppm have been observed for a coordination which must involve bridging cysteine sulfur. Our assignment appears to be quite certain on the basis of the known coordination of the two sites and the previously observed deshieldings.

An imidazole binding experiment confirms this assignment. It is known that imidazole competes with H_2O for the fourth coordination position of the catalytic Zn(II) in the native enzyme. X-ray crystallography shows imidazole displacing zinc-bound water.[14] In solution it removes the pH dependence of the binding of NAD^+ by apparently blocking the fourth position to H_2O and OH^-.[15,16] Figure 2a shows the ^{113}Cd NMR spectrum with the imidazole concentration five times the catalytic site concentration. The binding constant of the native enzyme for imidazole is fairly small at pH 7.5.[15] The 483-ppm peak cannot be observed, and it is presumably broadened by chemical exchange. The large chemical shifts characteristic of ^{113}Cd can lead to considerable NMR broadening if a dynamic equilibrium is involved. Figure 2b shows the NMR spectrum resulting from addition of a large

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excess of imidazole to the protein sample. The 483-ppm peak now reappears at 519 ppm and the 751-ppm peak is still unshifted. Since imidazole is not known[5] to coordinate to the noncatalytic site, this directly identifies the 483-ppm resonance as the catalytic Cd(II).

There has been some discrepancy between solid and solution experiments in regard to first- or second-sphere imidazole coordination to the catalytic metal ion. Crystal studies indicate direct coordination to zinc,[14] while imidazole proton relaxation studies with completely cobalt substituted LADH have been interpreted in terms of second-sphere coordination.[17] Our solution data indicated first-sphere coordination. In a titration of $CdSO_4$ at pH 7.4 with 0.5–10 equiv of imidazole we observe a continuously shifted resonance, and we can calculate the average number of imidazoles bound per cadmium from published equilibrium constants.[18] The positions of these ^{113}Cd NMR resonances yield downfield shifts for one imidazole replacing an H_2O ranging from 41 to 30 ppm. The downfield shift of the catalytic cadmium resonance is within this range at 36 ppm. We do not believe that this shift could be due to conformational changes affecting Cd(II) ligation. Our preliminary coenzyme binding experiments indicate upfield shifts of 42 ppm upon the conformational change with NADH binding. In addition, the X-ray study of the native enzyme–imidazole complex detected no movement in the protein ligands of the catalytic metal ion compared with the native enzyme alone.[14] We believe that the 36-ppm upfield shift upon imidazole binding, assuming insignificant conformational changes, demonstrates direct coordination of imidazole to the catalytic Cd(II).

Further coenzyme and substrate binding studies are under way at various pH values to learn more about the nature of coordination at the catalytic site.

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References and Notes

(1) Armitage, I. M.; Pajer, R. T.; Schoot Uiterkamp, A. J. M.; Chlewbowski, J. F.; Coleman, J. E. *J. Am. Chem. Soc.* **1976**, *98*, 5710–2.
(2) Chlewbowski, J. F.; Armitage, I. M.; Coleman, J. E. *J. Biol. Chem.* **1977**, *255*, 7053–61.
(3) Armitage, I. M.; Schoot Uiterkamp, A. J. M.; Chlewbowski, J. F.; Coleman, J. E. *J. Magn. Reson.* **1978**, *29*, 375–92.
(4) Bailey, D. B.; Ellis, P. D.; Cardin, A. D.; Behnke, W. D. *J. Am. Chem. Soc.* **1978**, *100*, 5236–7.
(5) Brändén, C.-I.; Jörnvall, H., Eklund, H.; Furugren, B. *Enzymes*, **1975**, *11A*, 103–90.
(6) Eklund, H., Nordström, B.; Zeppezauer, E.; Söderlund, G.; Ohlsson, I.; Boiwe, T.; Söderberg, B.-O.; Tapia, O.; Brändén, C.-I. *J. Mol. Biol.* **1976**, *102*, 27–59.
(7) (a) Drum, D. E.; Harrison, J. H.; Li, T.-K.; Bethune, J. L.; Vallee, B. L. *Proc. Natl. Acad. Sci. U.S.A.* **1967**, *57*, 1434–40. (b) Drum, D. E.; Li, T.-K.; Vallee, B. L. *Biochemistry* **1969**, *8*, 3792–7. (c) Drum, D. E.; Vallee, B. L., *Biochemistry* **1970**, *9*, 4078–86.
(8) Drum, D. E.; Vallee, B. L. *Biochem. Biophys. Res. Commun.* **1970**, *41*, 33–9.
(9) Sytkowski, A.; Vallee, B. L. *Biochemistry* **1979**, *18*, 4095–9.
(10) Maret, W.; Andersson, I.; Dietrich, H.; Schneider-Bernlöhr, H.; Einarsson, R.; Zeppezauer, M. *Eur. J. Biochem.* **1979**, *98*, 501–12.
(11) Haberkorn, R. A.; Que, L.; Gillum, W. O.; Holm, R. H.; Liu, C. S.; Lord, R. C. *Inorg. Chem.* **1976**, *15*, 2408–14.
(12) (a) Cardin, A. D.; Ellis, P. D.; Odom, J. D.; Howard, J. W. *J. Am. Chem. Soc.* **1975**, *97*, 1672–9. (b) Kostelnik, R. J.; Bothner-By, A. A. *J. Magn. Reson.* **1974**, *14*, 141–51.
(13) Otvos, J., private communication.
(14) Boiwe, T.; Brändén, C.-I. *Eur. J. Biochem.* **1977**, *77*, 173–9.
(15) Theorell, H.; McKinley-McKee, J. S. *Acta Chem. Scand.* **1961**, *15*, 1811–33.
(16) Taniguchi, S.; Theorell, H.; Åkeson, Å. *Acta Chem. Scand.* **1967**, *21*, 1903–20.
(17) Young, J. M.; Mildvan, A. S. "Alcohol and Aldehyde Metabolizing Systems", Thurman, R. G., Williams, J. R., Drott, H. R., Chance, B., Eds.; Academic Press: New York, 1977, Vol. 2, pp 109–17.
(18) Jensen, J. B. *Acta Chem. Scand., Ser. A* **1975**, *29*, 250–4.

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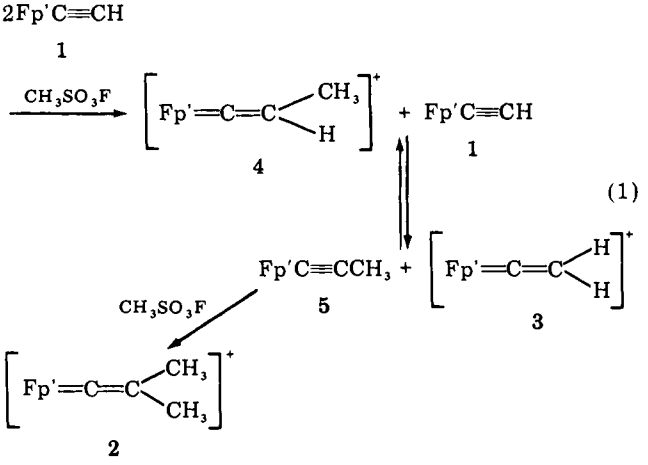
Stepwise Reduction of an Ethynyl–Iron Complex to a Neopentylidene Complex

Sir:

We report here the stepwise reduction of a metal-ethynyl complex to an 18-electron neopentylidene complex. Each intermediate has been isolated and characterized.

The sequence of reactions shown in Scheme I exploits the tendency for monohapto carbon ligands attached to late transition metals to undergo α attack by nucleophiles and β attack by electrophiles.[1] The ethynyl complex **1**[1b] reacts rapidly with methyl fluorosulfonate in benzene to give principally a equimolar mixture of the isobutenylidene complex **2**[1b] and the vinylidene complex **3**,[2] along with a small quantity of the propenylidene complex **4**, as shown by ^1H NMR and infrared spectroscopy. Evidently, the ethynyl complex **1** is more basic than the propynyl complex **5** (pK_a = 7.7).[1b] Thus, equilibrium 1 explains the formation of **2** and **3**.



Complex **2** reacts with the sodium trimethoxyhydridoborate in THF to give an ~4:1 mixture (by ^1H NMR) of the isobutenyl complex **6** and Fp'H.[4] The complex **6** can be isolated in 20% yield by fractional crystallization. Anal. ($C_{35}H_{36}FeP_2$), C, H, P. Mass spectrum: parent ion *m/e* 574. ^1H NMR (60 MHz, C_6D_6): δ 7.60, 7.15 (m, 20 H, Ph), 5.33 (t, $^3J_{PH}$ = 8.5 Hz, 1 H, H_α), 4.30 (t, $^3J_{PH}$ = 1.1 Hz, 5 H, Cp), 2.3–1.7 (br m, 4 H, PCH_2), 2.05 (s, 3 H, CH_3), 1.08 (s, 3 H, CH_3). ^{13}C NMR (15 MHz, C_6D_6): 144.5–131.0 (complex, Ph), 128.6 (br s, C_β), 127.7 (t, $^2J_{PC}$ = 3.9 Hz, C_α), 78.4 (s, Cp), 33.7 (s, CH_3), 27.0 (t, $^1J_{PC}$ = 21.5 Hz, PCH_2), 21.9 ppm (s, CH_3).

$Fp'CH{=}C(CH_3)_2$ (**6**) reacts with trimethyloxonium tetrafluoroborate in dichloromethane to give the neopentylidene complex **7** in ~60% crude yield. Recrystallization of **7** from acetone gave a mixture of black crystals of [Fp'(acetone)]-[BF_4] (ν_{CO} 1700–1710 cm^{-1}; lit.[4b] for PF_6^- salt, ν_{CO} 1710 cm^{-1}) and orange crystals of **7**. Anal. ($C_{36}H_{39}BF_4FeP_2$) C, H, P. ^1H NMR (60 MHz, CD_2Cl_2): δ 13.68[5] (t, $^3J_{PH}$ = 14.0 Hz, 1 H, C_α), 7.6–6.9 (m, 20 H, Ph), 5.13 (br s, 5 H, Cp), 3.18 (d, $^2J_{PH}$ = 11.0 Hz, 4 H, PCH_2), 0.71 (s, 9 H, $(CH_3)_3$). ^{13}C NMR (15 MHz, CD_2Cl_2): 359.5 (t, $^2J_{PC}$ = 26.4 Hz, C_α), 137.0–129.3 (complex, Ph), 88.4 (s, Cp), 63.0 (s, C_β), 28.3 (t,

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