Iron-Activated Alcohol Dehydrogenase from *Zymomonas mobilis*: Isolation of Apoenzyme and Metal Dissociation Constants

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Abstract: The title enzyme contains ferrous iron in its native, active form. Inclusion of Co^{2+} in the isolation buffer leads to ~90% substitution of Fe^{II} for Co^{II}, providing a preparation that is more stable and also active. Treatment of the Co^{II} enzyme with 1,10-phenanthroline leads to apoenzyme (<3 atom % total metal content), which can be completely reactivated upon addition of a single equivalent of Fe^{2+} or Co^{2+}. Zn^{2+} is ineffective under the same conditions. Apparent metal dissociation constants, K_M, were determined for the bivalent metal ions of the first transition series. That for Co^{II} was defined by using nitrilotriacetic acid as metal buffer, while the remainder were determined by competition of two metal ions for the apoenzyme. Complementary experiments in which the total concentration of first one metal ion and then the other was kept constant were employed to maximize precision. The K_M values increase slowly with atomic number and do not follow the classic Irving–Williams series:

M	Mn^{II}	Fe^{II}	Co^{II}	Ni^{II}	Cu^{II}	Zn^{II}
pK_M	7.4	7.5	7.8	8.4	8.5	9.0

The tight binding allows generation of well-defined metalated enzyme species from apoenzyme and M^{2+}.

Two alcohol dehydrogenases (ADH) have been isolated from the bacterium *Zymomonas mobilis*.[1-4] One, designated ZADH-1, is a typical zinc enzyme[5,6] while the second, ZADH-2, contains ferrous iron, Fe^{II}-ADH. This appears to be the initial report of native iron occurring at the active center of an ADH or, in fact, of any NAD^+-linked dehydrogenase.

Fe^{II}-ADH contains neither heme nor iron–sulfur groups. Ferrous centers of this type are detected in a number of enzymes that involve dioxygen redox chemistry. Examples include bacterial superoxide dismutase,[7] soybean lipoxygenase,[8] phenylalanine hydroxylase,[9] catechol 2,3-dioxygenase,[10] and protocatechuate 3,4- and 4,5-dioxygenases.[11,12] The dioxygen carrier hemerythrin[13] with its binuclear site presents another aspect. Both ferrous and ferric states are involved in these catalytic cycles.

The functions and proposed mechanisms of these systems are not obviously related to those of an ADH. This novelty, plus the apparent contrast with the $ZnNS_2O$ site of horse liver ADH,[6] has encouraged us to examine the nature of the ferrous site in ZADH-2 in some detail. The present paper reports generation of the apoenzyme and determination of the apparent dissociation constants for its complexes with the bivalent metals Mn, Fe, Co, Ni, Cu, and Zn. The companion paper[14] examines the various magnetic and spectroscopic properties of those complexes. Preliminary results have been communicated previously.[15]

Experimental Section

Protein concentrations were estimated with Coomassie Brilliant Blue G-250[16] and bovine serum albumin standardized by a dry weight determination on ADH. Quintuplicate assays were routinely taken. ADH activity was monitored at pH 8.5 by following the rate of formation of NADH at 340 nm (ϵ, 62 500 M^{-1} cm^{-1}).

Electrophoresis was carried out on a vertical unit comprising a LKB-2001 cell stand and a Pharmacia EPS 500/400 power supply.

Buffers were made up from deionized water (Millipore) and depleted of adventitious metal by passage through a Chelex-100 column (bed volume, 24 cm^3, 0.8 cm^2 × 30 cm; flow rate, 2.0 cm^3 min^{-1}).[17] The concentration of iron in the treated buffers was 2.4 (1) ng cm^{-3} compared to 98.4 (6) ng cm^{-3} before treatment. The maximum volume of buffer permitted in contact with apoenzyme was calculated to ensure a maximum contamination below 1 g atom %. All containers were acid-washed and copiously rinsed with deionized water. Dialysis tubing was depleted of metal by boiling in a solution of EDTA (0.1 M) and Na_2CO_3 (0.1 M) for 0.5 h followed by copious rinsing with deionized water.

Quantitative estimation of Mn, Fe, Co, Cu, and Zn was carried out on a Varian AA Series 275 atomic absorption spectrometer equipped with a GTA-95 graphite tube atomizer and an automatic sample dispenser. Standard solutions of metals were prepared by dissolving salts (Aldrich Gold Label) in 0.7% HNO_3 (analytical reagent) except for Fe where iron wire (99.9%; Riedel Dehaën AG Sielze-Hanover) was dissolved in concentrated hydrochloric acid before dilution to 0.7% acid. Iron analysis employed the standard addition method to allow for the presence of iron contaminants in the hydrochloric acid.

Generation of Apoenzyme a-ADH. Co^{II}-ADH was isolated from wet cells (70 g) of *Z. mobilis* by the reported method,[3] in which Co^{2+} ions in the buffer displaced the naturally occurring Fe^{2+} ions. Characterization after elution with NAD^+ from the second (blue) dye–ligand column revealed the presence of 105 mg of protein of specific activity 290 IU mg^{-1} in a volume of 200 cm^3. The volume was reduced to ~8 cm^3

(1) Wills, C.; Kratofil, P.; Londo, D.; Martin, T. *Arch. Biochem. Biophys.* **1981**, *210*, 775-785.
(2) Scopes, R. K. *FEBS Lett.* **1983**, *156*, 303-306.
(3) Neale, A. D.; Scopes, R. K.; Kelly, J. M.; Wettenhall, R. E. *Eur. J. Biochem.* **1986**, *154*, 119-124.
(4) Kinoshita, S.; Kakizono, T.; Kadota, K.; Das, K.; Taguchi, H. *Appl. Microbiol. Biotechnol.* **1985**, *22*, 249-254.
(5) Zeppezauer, M. In *The Coordination Chemistry of Metalloenzymes*; Bertini, I., Drago, R. S., Lucinat, C., Eds.; Reidel: Dordrecht, The Netherlands, 1983; pp 99-122.
(6) Brändén, C.-I.; Jörnwall, H.; Eklund, H.; Furugren, B. In *The Enzymes (3rd Ed.)*; Boyer, P. D., Ed.; Academic Press: New York, 1975; Vol. 11, pp 103-190.
(7) Stallings, W. C.; Paltridge, K. A.; Ludwig, M. L. In *Superoxide and Superoxide Dismutase in Chemistry, Biology and Medicine*; Rotilio, G., Ed.; Elsevier: New York, 1986.
(8) Veldink, G. A.; Vliegenthart, J. F. G. *Adv. Inorg. Biochem.* **1984**, *6*, 139-162.
(9) Gottschall, D. W.; Dietrich, R. F.; Benkovic, S. S.; Shiman, R. J. *J. Biol. Chem.* **1982**, *257*, 845-849.
(10) Nozaki, M.; Kagamiyama, H.; Hayaishi, O. *Biochem. Z.* **1963**, *338*, 582-590.
(11) Que, L., Jr.; Heistand, R. H., II; Mayer, R.; Roe, A. L. *Biochemistry* **1980**, *19*, 2588-2593.
(12) Arciero, D. M.; Lipscomb, J. D.; Huynh, B. H.; Kent, T. A.; Münck, E. *J. Biol. Chem.* **1983**, *258*, 14981-14991.
(13) Klotz, I. M.; Kurtz, D. M., Jr. *Acc. Chem. Res.* **1984**, *17*, 16-22.
(14) Bakshi, E.; Tse, P.; Hanson, G. R.; Scopes, R. K.; Murray, K. S.; Wedd, A. G. *J. Am. Chem. Soc.*, following article in this issue.
(15) Tse, P.; Scopes, R. K.; Wedd, A. G.; Bakshi, E.; Murray, K. S. *J. Am. Chem. Soc.* **1988**, *110*, 1295-1297.
(16) Sedmak, J. J.; Gossberg, S. E. *Anal. Biochem.* **1977**, *79*, 544-552.
(17) Riordan, J. F.; Vallee, B. L.; Holmquist, B.; Auld, D. S.; Martin, M. T.; Wagner, W. F. *Methods Enzymol.* **1988**, *158*, 3-32.

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Table I. Metal Content (g Atom Subunit^{-1})[a]

	spec act., IU mg^{-1}	Fe	Co	Zn	Cu
a-ADH	1.5	0.01 (1)	0.02 (1)	<0.01	<0.01
Fe^{II}-ADH	750	0.9 (2)	0.02 (1)	0.02 (1)	*b*
Co^{II}-ADH	300	0.10 (2)	1.04 (7)	0.01 (1)	*b*

[a] Standard deviation of last figure given in parentheses. [b] Not measured.

by ultrafiltration (Amicon YM-10 membrane, cutoff 10 kDa). The ligand 1,10-phenanthroline (10 mM) was added to a final concentration of 0.1 mM and the enzyme applied to a GCl-2000 SF (Amicon) gel filtration column (96 cm^2 × 80 cm; flow rate, 40 cm^3 h^{-1}) equilibrated with buffer [K-Mes (10 mM), KCl (30 mM), $MgCl_2$ (2 mM), 1,10-phenanthroline (0.1 mM)]. Apo-ADH appeared as a single peak at an elution time of 7.5 h followed by NAD^+ and Co^{II}–phenanthroline complex at 14 and 17 h, respectively. The volume was reduced to ~7 cm^3 by ultrafiltration and the sample dialyzed against metal-free K-Mes buffer (50 mM; pH 6.5) at 4 °C. Six buffer changes at 10-h intervals were necessary for complete removal of 1,10-phenanthroline, as monitored by its characteristic absorption in the 290–340-nm range superimposed on the background protein absorption. The yield of 94 mg of apo-ADH was stored as frozen beads at 77 K.

Metal Dissociation Constants. Pure metal salts (Aldrich Gold Label) were employed.

In the Co^{II} system, the apoenzyme a-ADH, 3.3 (2) × 10^{-4} M, was used in a metal buffer system containing 0.10 M K-Mes (pH 6.5) and nitrilotriacetic acid at a concentration of (1.0, 2.5, 5.0, 10.0, or 20.0) × 10^{-3} M. Total Co^{2+} concentration was varied to obtain ADH activities in the range 15–80% of the maximum observable activities. At least four Co^{2+} concentrations were examined for each concentration of nitrilotriacetic acid.

In the competition experiments between the active enzymes, Co^{II}- and Fe^{III}-ADH, and the inactive enzymes, M-ADH (M = Mn^{II}, Ni^{II}, Cu^{II}, Zn^{II}), two independent experiments for each system were carried out, one with a constant concentration of active metal and the other with a constant concentration of inactive metal. This concentration was held close to that of the enzyme concentration while the concentration of the other metal was varied systematically to obtain activities between 15 and 90% of the maximum.

An incubation time of 0.3–0.5 h was sufficient for equilibrium to be reached.

Tables of data for each system, experimental graphs, and details of control experiments are available as supplementary material (Tables S1–S8).

Results

Properties of Apoenzyme, a-ADH. The native enzyme Fe^{II}-ADH (stability $t_{1/2}$, 2.5 h; 4 °C) can be isolated in the presence of a stabilizing excess of ferrous ammonium sulfate and ascorbate.[3] Inclusion of Co^{2+} instead of Fe^{2+} in the isolation buffer leads to the more stable Co^{II}-ADH ($t_{1/2}$, 40 h). a-ADH was generated from Co^{II}-ADH by treatment with 1,10-phenanthroline followed by exhaustive dialysis to remove ligand. The ligands EDTA and α,α'-dipyridyl used in the original inactivation studies[2] were less effective than 1,10-phenanthroline in the present work.

The metal contents of a-ADH, Co^{II}-ADH, and Fe^{II}-ADH as isolated are listed in Table I. The total metal content of a-ADH is 3 atom %. The Co^{II}-ADH is significantly contaminated with Fe. This problem can be circumvented by reconstitution of a-ADH with 1 equiv of pure cobaltous chloride. Such reconstitution leads to Co^{II}-ADH, whose activity is at least that of the original sample and whose stability is markedly improved ($t_{1/2}$, >60 h). Similar observations apply to reconstituted Fe^{II}-ADH ($t_{1/2}$, >5 h). The divalent ions Mn and Ni have no effect on the residual activity of a-ADH while Cu and, importantly, Zn abolish it completely (see also, ref 2). Interestingly, a-ADH essentially retains its ability to be reactivated (>90%) after storage at 77 K for 6 months while reactivated samples of Fe^{II}-ADH and Co^{II}-ADH exhibit markedly lower activities (12 and 78% of original, respectively) upon thawing after the same period of storage.

Maximum reactivation of both Fe^{II}-ADH and Co^{II}-ADH is reached after addition of a single equivalent of metal ion per subunit (Figure 1).

SDS–gel electrophoresis of a-ADH and the reactivated enzymes indicated subunit molar masses of 37 (1) kDa while gel filtration

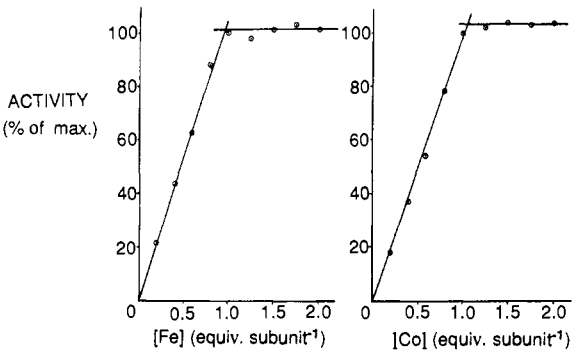

Figure 1. Activation of a-ADH (3.3 × 10^{-4} M).

on Sephacryl S-200 confirmed the presence of tetrameric molecules of molar mass 150 (3) kDa. These properties mirror those originally reported for Fe^{II}-ADH.[2] However, the amino acid sequence as determined from the gene sequence[18] indicates a subunit molar mass of 40 141. Addition of 4 equiv of oxidizing substrate NAD^+ per subunit to Co^{II}-ADH produces a limiting electronic spectrum[14,15] but has no effect on the molar mass, 147 (5) kDa, nor that of Fe^{II}-ADH, 149 (3) kDa. Addition of 2600 equiv of the inhibitor *i*-PrOH alone also has no effect on molar mass. This quantity of *i*-PrOH added to a solution of Co^{II}-ADH:NAD^+ = 1:4 produces a new limiting spectrum[14,15] and appears to dissociate the enzyme into dimers of molar mass 84 (4) kDa. A similar apparent molar mass, 86 (4) kDa, is observed for Fe^{II}-ADH under the same conditions.

Dissociation Constants, K_M. K_{Co} was determined by employing nitrilotriacetic acid, H_3L, as metal buffer[19,20] to establish known concentrations of free Co^{2+}, [Co]. Unlike 1,10-phenanthroline, H_3L did not perturb the ADH assay. Fe^{2+} oxidized rapidly in the presence of H_3L at pH 6.5, preventing the determination of K_{Fe} in that metal buffer. K_Ms for the inactive M^{II}-ADH (M = Mn, Ni, Cu, Zn) were determined via competition of M^{2+} and Co^{2+} for the ligand a-ADH. Then $K_{Fe(II)}$ was estimated via competition between Fe^{2+} and Zn^{2+}.

Cobalt^{II}. The activity of Co^{II}-ADH was not affected by the presence of Co^{2+} and H_3L in 1:1 proportion. In addition, the binding of Co^{2+} to a-ADH in the presence of H_3L was independent of the concentration of K-Mes buffer (pH, 6.5) in the range 10–100 mM.

The association constants K_{CoL} and K_{CoL_2} of the 1:1 and 1:2 complexes of Co^{2+} and H_3L at I = 0.1 M are known (2.51 × 10^{10} and 1.02 × 10^4 M^{-1}) as are the relevant acid association constants for H_3L.[21] The latter indicate that, at pH 6.5, the concentrations of both HL^{2-} and L^{3-} (connected by acid association constant K_1 = 5.13 × 10^9 M^{-1}) must be included in calculations. The concentration of Co^{II}-ADH, [Co-ADH], was estimated from the activity assay:

$$v/V = [\text{Co-ADH}]/[\text{ADH}]_t \quad (1)$$

where v is the measured ADH activity, V is the maximum activity, and $[ADH]_t$ is the total enzyme concentration. Crucially, the presence of NAD^+ in the assay mixture prevents dissociation of metal ions, validating eq 1.

As a starting point, only the 1:1 complexes Co^{II}-ADH and CoL^- were included and the ligands a-ADH and L^{3-} were assumed not to interact:

$$\text{Co-ADH} \rightleftharpoons Co^{2+} + \text{a-ADH} \quad K_{Co} = \frac{[\text{Co}][\text{a-ADH}]}{[\text{Co-ADH}]} \quad (2)$$

$$Co^{2+} + L^{3-} \rightleftharpoons CoL^- \quad K_{CoL} = \frac{[\text{CoL}]}{[\text{Co}][\text{L}]} \quad (3)$$

(18) Conway, T.; Sewell, G. W.; Osman, Y. A.; Ingram, L. O. *J. Bacteriol.* **1987**, *169*, 2591–2597.
(19) Raaflaub, J. *Methods Biochem. Anal.* **1956**, *3*, 301–325.
(20) Baker, J. O. *Methods Enzymol.* **1988**, *158*, 33–55.
(21) Anderegg, G. *Pure Appl. Chem.* **1982**, *54*, 2693–2758.

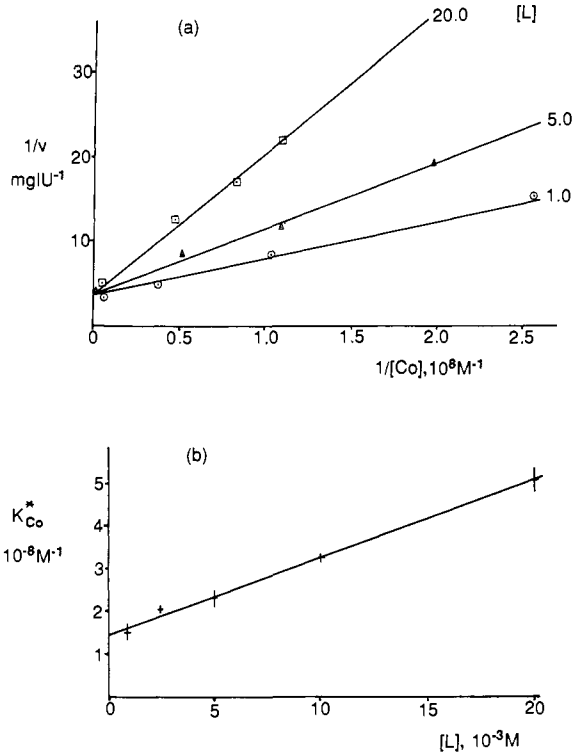

Figure 2. (a) Dependence of Co^{II}-ADH activity on the concentration of free Co^{2+}, assuming the formation of the 1:1 complexes Co^{II}-ADH and CoL^-. $[ADH]_t$ = 3.3 (2) × 10^{-4} M. For clarity, results at $[L]_t$ = 1.0, 5.0, and 20.0 mM only are presented. (b) Dependence of K^*_{Co} on $[L]_t$.

Then, use of eq 1–3 and the mass balance relationships allow derivation of the Lineweaver–Burk equation as well as the required estimate of [Co]:

$$\frac{1}{v} = \frac{1}{V} + \frac{K_{Co}}{V}\frac{1}{[Co]} \tag{4}$$

$$[Co] = \frac{K_1[H](V[Co]_t - v[ADH]_t)}{K_{CoL}(V[L]_t - V[Co]_t + v[ADH]_t)} \tag{5}$$

The latter requires that $[L]_t \gg [Co]$ and $K_{CoL}[L]_t \gg 1 + K_1[H] \gg 1$, and the experiments were designed with this in mind. $[L]_t$ and $[ADH]_t$ were kept constant for each experiment with $[Co]_t$ allowed to vary so that v covered the range 15–80% of the maximum activity V.

The slope of the double reciprocal plot (4) varies with $[L]_t$ (Figure 2a), i.e., it appears as if the metal buffer ligand is a competitive inhibitor preventing binding of Co^{2+} to a-ADH. To account for this effect, equilibrium 6 was included:

$$\text{L-ADH} \rightleftharpoons L^{3-} + \text{a-ADH} \quad K_L = \frac{[L][\text{a-ADH}]}{[\text{L-ADH}]} \tag{6}$$

Equation 4 becomes

$$\frac{1}{v} = \frac{1}{V} + \frac{K^*_{Co}}{V}\frac{1}{[Co]} \tag{7}$$

where

$$K^*_{Co} = K_{Co}\left(1 + \frac{[L]}{K_L}\right) \tag{8}$$

If $K_L \gg K_{Co}$, then $[L]_t$ can substitute for [L] in (8) and a plot of K^*_{Co} versus $[L]_t$ should be linear and permit extraction of K_{Co}. In practical terms, this new assumption requires the condition $[L]_t \gg [ADH]_t$. Furthermore, (5) is still a valid estimate of [Co] as the assumption allows the additional term in [L-ADH] to be eliminated in the derivation of (5).

Figure 2b demonstrates that K^*_{Co} varies linearly with $[L]_t$ with derived values of K_{Co} [1.5 (2) × 10^{-8} M] and K_L [8.1 (3) × 10^{-3} M]. Full numerical details are given in the supplementary material, including tests of the assumptions. All assumptions are valid with the possible exception of $[L]_t \gg [ADH]_t$. Experimentally, $[L]_t/[ADH]_t$ covered the range 3.1–57 and is probably the largest source of error in K_{Co}.

Table II. Apparent Dissociation Constants for Metal Ions Binding to a-ADH

metal ion, M	K_M, M[a]	pK_M
Mn^{2+}	3.6 (6) × 10^{-8}	7.4
Fe^{2+}	3 (1) × 10^{-8}	7.5
Co^{2+}	1.5 (1) × 10^{-8}	7.8
Ni^{2+}	4.4 (8) × 10^{-9}	8.4
Cu^{2+}	3.1 (6) × 10^{-9}	8.5
Zn^{2+}	1.1 (3) × 10^{-9}	9.0

[a] Standard deviation of the last figure is given in parentheses.

Of course, the variation of slope (Figure 2a) may not be due to direct interaction of the two ligands (eq 6) but to neglect of the presence of the 1:2 complex CoL_2^{4-}:K_{CoL_2}, 1.02 × 10^4 M^{-1}.[21] Inclusion of that equilibrium rather than (6) leads to terms in $[L]^2$ in the expression for [Co].[20,22] Use of the latter with eq 4 provides an estimate of K_{Co} indistinguishable from that obtained above.

The Inactive Metals Mn, Ni, Cu, Zn. If M-ADH is inactive, then eq 1 is still valid. Providing that M^{2+} and Co^{2+} do not interact, then the expressions for K_{Co} (eq 2) and K_M and those for mass balance lead to

$$\frac{1}{v} = \frac{1}{V} + \frac{1}{V}\frac{K_{Co}}{[Co]}\left(1 + \frac{[M]}{K_M}\right) \tag{9}$$

However, [M] cannot be substituted by $[M]_t$ in analogy with eq 8 as the metal ions M^{2+} of interest bind strongly to a-ADH. Estimates of both [Co] and [M] must be found. That for [Co] follows from eq 1 and the cobalt mass balance:

$$[Co] = \frac{V[Co]_t - v[ADH]_t}{V} \tag{10}$$

That for [M] follows from eq 2 and 10 and the ADH and M mass balances:

$$[M] = \frac{V[M]_t - [ADH]_t(V - v)}{V} \tag{11}$$

provided that

$$K_{Co}vV \ll (V - v)(V[Co]_t - v[ADH]_t) \tag{12}$$

In practice, this requires v to be rather less than V (supplementary material) and the experiments were designed to this requirement. Equations 9–11 lead to

$$\frac{1}{v} - \frac{K_{Co}}{V[Co]_t - v[ADH]_t} = \frac{1}{V} + \frac{K_{Co}}{VK_M}\left(\frac{V[M]_t - [ADH]_t(V - v)}{V[Co]_t - v[ADH]_t}\right) \tag{13}$$

which is of the form

$$y = \frac{1}{V} + \frac{K_{Co}}{VK_M}x \tag{14}$$

A plot of y vs x will be linear with $1/V$ as intercept and the gradient provides K_M, assuming eq 12 is satisfied.

The form of eq 13 allows two complementary but independent sets of experiments to be designed, one with fixed $[M]_t$ and varying $[Co]_t$ and the other with fixed $[Co]_t$ and varying $[M]_t$.

The assumption that M^{2+} and Co^{2+} do not interact was tested under noncompetitive conditions, i.e., $[M]_t + [Co]_t < [ADH]_t$. Within experimental error, the predicted $[Co^{II}\text{-ADH}]$ activities were observed for M = Mn, Ni, Cu, and Zn (Table S2).

Derived K_M values are listed in Table II. Figure 3 shows the linear dependence of y upon x for the two complementary ex-

(22) Sellin, S.; Mannervik, B. *J. Biol. Chem.* **1984**, *259*, 11426–11429.

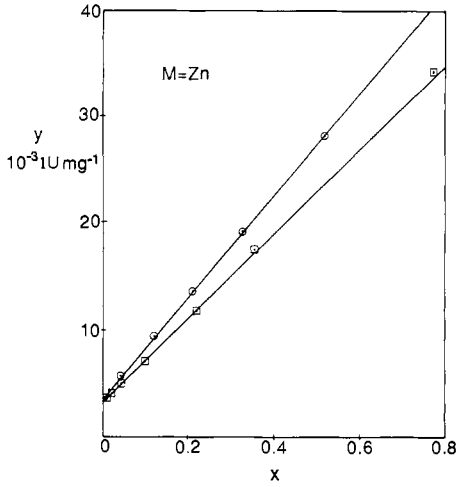

Figure 3. Dependence of y on x for M = Zn. See text for details. (□): $[ADH]_t$, 2.85×10^{-4} M; $[Zn]_t$, 2.87×10^{-4} M; $[Co]_t$, $0.04-4.00 \times 10^{-3}$ M; v, 20.1–180 IU mg^{-1}; V, 310 IU mg^{-1}. (○): $[ADH]_t$, 2.92×10^{-4} M; $[Co]_t$, 3.00×10^{-3} M; $[Zn]_t$, $0.05-2.56 \times 10^{-3}$ M; v, 281–29.2 IU mg^{-1}; V, 301 IU mg^{-1}.

periments performed for M = Zn. The derived estimates for K_{Zn} were 0.99 (9) $\times 10^{-9}$ and 1.10 (8) $\times 10^{-9}$ M, averaged to K_{Zn} of 1.1 (2) $\times 10^{-9}$ M. Further details are provided as supplementary material.

Iron(II). The presence of two active species Co^{II}-ADH and Fe^{II}-ADH in competition experiments involving Co^{2+} and Fe^{2+} complicates the activity assay (see eq 1). For simplicity, $K_{Fe(II)}$ was determined by competition between Fe^{2+} and Zn^{2+} for a-ADH using the theory outlined in the previous section. Zn^{II}-ADH is inactive, the activity assay provides [Fe^{II}-ADH], and Zn^{2+} and Fe^{2+} do not interact (Table S7). Two independent experiments with, respectively, $[Zn]_t$ and then $[Fe]_t$ constant provided an average $K_{Fe(II)}$ of 3 (1) $\times 10^{-8}$ M. Both ferrous ion and Fe^{II}-ADH ($t_{1/2}$, >5 h) are unstable to oxidation in air. However, the result was the same under anaerobic conditions, suggesting that autoxidation is not important on the present experimental time scale (<0.3 h).

Discussion

High-quality a-ADH for the *Z. mobilis* enzyme ZADH-2 is accessible via extraction of metal by 1,10-phenanthroline from the enzyme isolated in the presence of excess Co^{2+}. Metal content is <3 atom %. The preparations have excellent reactivation properties and provide active Fe^{II}-ADH and Co^{II}-ADH samples upon addition of a single equivalent of metal ion. These species are somewhat more stable than those isolated directly, while a-ADH is stable to storage at 77 K for at least 6 months.

The bivalent metal ions of the first transition series Mn^{2+}–Zn^{2+} bind strongly (pK_M, 7.4–9.0; Table II) to a-ADH. The presence of millimolar Fe^{2+} stabilizes Fe^{II}-ADH during isolation,[3] probably by replacing the less strongly bound ferric iron in the Fe^{III}-ADH produced by aerial oxidation.[14] About 90 atom % of Fe^{II} can be substituted by Co^{II} (Table I) by inclusion of millimolar Co^{2+} during isolation. However, the stoichiometric metals do not dissociate easily during dialysis against buffer.

This paper presents unequivocal evidence that active forms of the *Z. mobilis* enzyme ZADH-2 contain 1 equiv of Fe^{II} or Co^{II} at the active site. Since Fe^{II}-ADH is the most active of these and

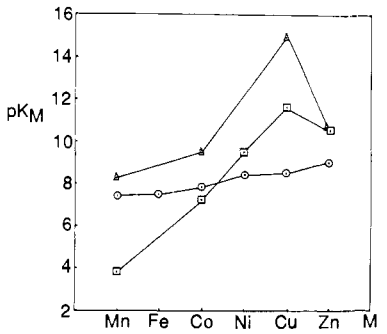

Figure 4. Dependence of metal dissociation constants on atomic number. ADH, (○); carbonic anhydrase,[26] (□); glyoxalase I,[22,27] (△).

Co^{II} is not normally available to organisms in simple growth media in sufficient amounts to saturate the enzyme, there can be little doubt that Fe^{II}-ADH is the naturally occurring form. Recent evidence indicates that the yeast enzyme ADH-IV is highly homologous with ZADH-2, contains Zn, and is inactivated by Fe^{2+}.[23] However, the specific activity of the ADH-IV preparation was only ~3% of that observed here for Fe^{II}-ADH. It should be noted that the first report of the ZADH-2 enzyme[1] indicated that it too contained Zn. Again, the preparation was of low specific activity, less than 1% of its potential Fe^{II}-ADH activity, and exchange of Fe for Zn had presumably occurred during isolation. Further investigation of the metal content of the yeast ADH-IV enzyme seems to be justified.

The observed pK_M values for ZADH-2 increase slowly across the first transition series (Figure 4) and do not follow the Irving–Williams series.[24,25] This contrasts with the behavior of carbonic anhydrase (ligands, three His, one H_2O)[26] and glyoxylase I (ligand atoms, two N, four O?),[22,27] for which reasonably extensive data are also available (Figure 4). Complimentary competition experiments involving Co and Cu confirm the relatively low pK_M for Cu^{II}-ADH in the present system.

However, the degree to which the conformational preferences of the protein ligand influence the metal binding site will produce deviation from the simple Irving–Williams behavior. Certainly, no inferences about the coordination number of and the nature of the ligand atoms at the metal binding site in ZADH-2 can be drawn from the pK_M values. Their value lies in establishing that a well-defined derivative M^{II}-ADH can be generated in pure form from a-ADH and M^{2+}. Spectroscopic and magnetic properties of these species are reported in the following paper.[14]

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Registry No. Fe, 7439-89-6; Co, 7440-48-4; Ni, 7440-02-0; Cu, 7440-50-8; Zn, 7440-66-6; Mn, 7439-96-5.

Supplementary Material Available: Tables of data from competition experiments, details of control experiments, and graphs of eq 14 (9 pages). Ordering information is given on any current masthead page.

(23) Drewke, C.; Ciriacy, M. *Biochim. Biophys. Acta* **1988**, *950*, 54–60.
(24) Irving, H.; Williams, R. J. P. *Nature* **1948**, *162*, 746–747.
(25) Sigel, H.; McCormick, D. B. *Acc. Chem. Res.* **1970**, *3*, 201–208.
(26) Lindskog, S.; Nyman, P. O. *Biochim. Biophys. Acta* **1964**, *85*, 462–474.
(27) Sellin, S.; Eriksson, L. E. G.; Mannervik, B. *Biochemistry* **1987**, *26*, 6779–6784.