LIVER ALCOHOL DEHYDROGENASE: EVIDENCE FOR A NEW COBALT/ZINC HYBRID

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Summary Two of the four zinc atoms of liver alcohol dehydrogenase have been replaced by cobalt under conditions that gave a fully-active Co_2Zn_2-hybrid. Substitution occurred at one catalytic and one non-catalytic site as judged by three criteria: i) the spectrum of the hybrid is perturbed by NADH; ii) the fluorescence of NADH bound to the hybrid is 50% quenched due to dipole-dipole interactions between the reduced nicotinamide moiety and cobalt substituted at the active site; iii) titration of the hybrid with 1,10-phenanthroline resulted in a stoichiometric decrease in the absorbance of the hybrid at 650 nm which reached an end-point when the concentration of chelator was half the total cobalt of the hybrid. This spectral change was accompanied by an irreversible loss of approximately half the enzymic activity.

Introduction X-ray studies on LADH* indicate that each of the two identical subunits contains one zinc atom at the active site and a second zinc atom bound at a site 20 Å from the catalytic center (1,2). The four zinc atoms of the enzyme can be partially or completely substituted by cobaltous ions (3,4) although there is conflicting data as to the site of metal replacement in the cobalt/zinc hybrid enzymes. For example, the paramagnetic effects of enzyme-bound cobalt on substrates or inhibitors indicate that

*Abbreviations: LADH, crystalline horse liver alcohol dehydrogenase; OP, 1,10-phenanthroline.

the first two equivalents of cobalt are located at the active site (5,6). In contrast, certain kinetic and spectroscopic properties of the cobalt/zinc hybrid enzymes show that cobalt initially substitutes for zinc at the non-catalytic binding site (4,8-10). This communication examines the site of metal substitution in a Co_2Zn_2-LADH hybrid using criteria which differentitate cobalt substitution at the active sites from metal replacement at non-catalytic sites. The data establish an unusual distribution of metal in which one cobalt is at a catalytic site and one cobalt is at a non-catalytic site.

Materials and Methods Crystalline horse liver alcohol dehydrogenase (Boehringer Mannheim Corp.), 10 mg/ml, was dialyzed against 0.2 M sodium phosphate, pH 8.0 and then against two changes of 0.1 M Na_2SO_4, pH 7.0. Cobalt-substituted enzymes were prepared by a modification of the method of Shore and Santiago (11); Zn_4-LADH was dialyzed against 0.2 M $CoCl_2$-0.1 M Na_2SO_4-0.1 M NaAc, pH 5.5. Unbound metal was removed by dialysis against 0.025 M Hepes-0.1 M KCl, pH 7.0. All dialysis steps were carried out in 600 ml air-tight cylinders on rapid-dialysis racks (12) at room temperature. Cobalt and Hepes-KCl buffers were degassed under vacuum before use and saturated with nitrogen at the start of dialysis.

LADH activity was measured in the direction of NADH formation at pH 8.8 in a Cary 118C spectrophotometer in 16.6 mM sodium pyrophosphate and 17 mM ethanol (13). The molar concentration of LADH was determined by titration of the enzyme in the presence of isobutyramide (14). The specific activity (13), ΔA 340 nm$(min)^{-1}$ $(mg\ protein)^{-1}$, of the hybrid enzymes varied from 14 to 12 as the cobalt content increased from zero to 3.8 gram atoms per enzyme. Cobalt chloride, grade I, was purchased from Johnson Matthey Chemicals Ltd.; all other chemicals and methods have been previously described (7,8).

Results: Spectrum of the enzyme It has been previously shown that cobalt/zinc hybrids substituted exclusively at non-catalytic sites show nearly equal absorption at 650 nm and 740 nm (8-10). In contrast, the spectrum of Co_2Zn_2-LADH described here (Fig. 1) shows an absorbance ratio 650 nm/740 nm similar to that seen with Co_4-LADH (3,11). Further, the binding of NADH to Co_2Zn_2-

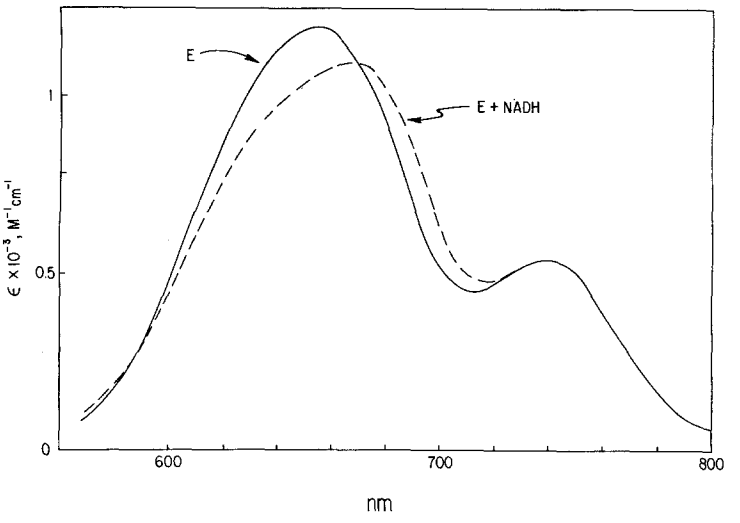

Fig. 1 Absorption spectrum of Co_2Zn_2-LADH in absence (——) and presence (----) of 1 x 10^{-5} M NADH-0.1 M isobutyramide. Extinction coefficients based on molarity of dimeric enzyme.

LADH in the presence of isobutyramide causes a shift in the 650 nm absorption peak of the enzyme, a perturbation observed with Co_4-LADH (3) but not with enzymes containing cobalt exclusively at non-catalytic sites (8). Thus, the spectral properties of Co_2Zn_2-LADH do not correspond to substitution at two non-catalytic sites, but rather suggest a metal distribution similar to Co_4-LADH in which cobalt is necessarily partitioned equally between catalytic and non-catalytic sites.

Energy transfer from NADH to cobalt-substituted-LADH Paramagnetic coupling or non-radiative energy transfer from the reduced nicotinamide moiety of NADH to cobalt substituted at the active site is possible because of the favorable spectral properties and close juxtaposition (4 Å) of the donor-acceptor pair (2,7,15). In contrast, quenching of NADH fluorescence by cobalt located

TABLE I

Enzyme	Quantum yield of enzyme-bound NADH[a]
Zn_4-LADH	0.057
Co_2Zn_2-LADH	0.026
Co_4-LADH	0.005

[a] Fluorescence of enzyme-bound NADH measured in the presence of 0.10 M isobutyramide; excitation 340 nm, emission 460 nm.

22 Å away at the non-catalytic site in the same subunit would be predicted as less than 20% even assuming the most favorable dipole-dipole interaction. Cobalt at either the catalytic or non-catalytic site of the opposing subunit is 40 Å away, and, hence, would not participate in either paramagnetic coupling or Förster energy transfer (15). Table I shows that in the presence of isobutyramide the fluorescence emission of NADH stoichiometrically bound to Co_2Zn_2-LADH is less than half that observed upon binding to Zn_4-LADH where no energy transfer from NADH to metal is possible. NADH bound to Co_4-LADH, in which the two active sites necessarily contain cobalt, shows greater than 90% quenching. This strong quenching indicates that the orientation of the donor (NADH) and acceptor (catalytic cobalt) chromophores is favorable for energy transfer. The partial quenching of the NADH fluorescence observed with Co_2Zn_2-LADH thus does not result from an unusually small value of the dipole-dipole orientation factor (15), but most likely reflects the composite effect of NADH bound at

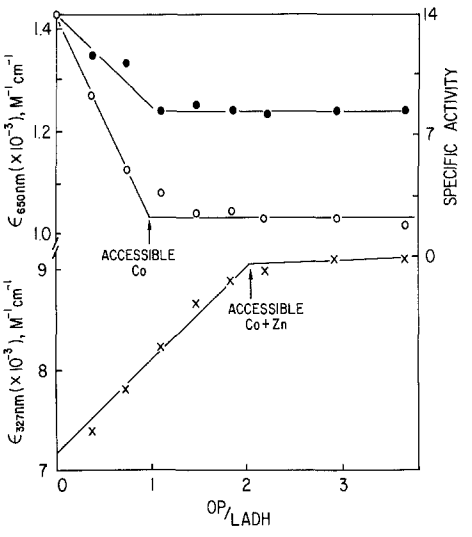

Fig. 2 Titration with 1,10-phenanthroline. Aliquots of 1,10-phenanthroline (10 mM) were added to Co_2Zn_2-LADH (47 uM) in 0.025 M Hepes-0.1 M KCl, pH 7.0. (O), ε_{650} nm; (X) ε_{327} nm; (●), specific activity of LADH.

catalytic sites containing cobalt (nucleotide fluorescence quenched) and NADH bound at active sites containing zinc (nucleotide fluorescence not quenched).

Titration of hybrids with 1,10-phenanthroline The d-d electronic transitions of cobalt are known to be sensitive to the nature of the metal ligands (16). Hence, it would be expected that 1,10-phenanthroline, a ligand which binds specifically to the metal ions at the active sites of LADH (2), would alter the visible absorption of cobalt located at the catalytic site. Fig. 2 shows that the addition of OP to Co_2Zn_2-LADH results in a stoichiometric decrease in the absorbance of the enzyme at 650 nm and a concomitant, irreversible loss of almost half the enzymic activity (Fig. 2). These changes, which reflect the high affinity of OP for cobalt reach a well-defined endpoint at OP/LADH = 1. Thus, only

one of the cobalt atoms in the Co_2Zn_2-LADH hybrid is accessible at the active site for chelation. The absorption at 327 nm which responds to OP binding to either cobalt or zinc at the active site shows an inflection when a total of two OP are bound per LADH (Fig. 2). This is in agreement with the stoichiometry for this chelator established for the native Zn_4-LADH (2).

Discussion The fluorescence characteristics of NADH bound to the Co_2Zn_2-LADH as well as the spectral and kinetic changes induced by OP binding all indicate that one cobalt is located at a catalytic site and one cobalt is at a non-catalytic site. This pattern of substitution is different from that proposed by Sloan et al., (6) (two cobalts initially substituting at catalytic sites) and from that suggested by Vallee and Sytkowski (9) (two cobalts initially substituting at non-catalytic sites). The earlier criteria may not have been stringent enough to differentiate the sites of metal substitution. For example, prolonged dialysis against cobalt buffer using the method described here yields Co_4-LADH which has an enzymic activity essentially equal to that of native Zn_4-LADH. This indicates that the decrease in enzymic activity of Co_4-LADH relative to Co_2Zn_2-LADH reported by Sytkowski and Vallee (9,10) is not diagnostic for cobalt substitution at the active site, but may reflect partial denaturation of the enzyme. Alternatively, the cobalt/zinc hybrids described here and in the literature may each show a unique pattern of cobalt substitution, presumably because of subtle but important differences in the conditions of metal substitution used by the various authors. In either case, the present data define the location of cobalt in a well-characterized hybrid.

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