HETEROGENEITY IN THE RAPIDLY EXCHANGING METALS OF HORSE LIVER ALCOHOL DEHYDROGENASE

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Summary Substitution of the two rapidly exchanging zinc atoms of liver alcohol dehydrogenase by cobalt is biphasic; replacement by the first cobalt occurs at a rate ($t_{\frac{1}{2}}$ = 15 minutes) approximately ten times faster than substitution by the second cobalt atom. The hybrid enzyme containing one gram atom of cobalt has a characteristic visible absorption spectrum which is not perturbed by NADH or 1,10-phenanthroline. The fluorescence of NADH or ε-NAD bound to the hybrid is not quenched. These data indicate a previously unrecognized heterogeneity in the rapidly exchanging zinc atoms; one of the exchange labile zinc atoms is located at a structural metal binding site rather than an active site.

Introduction Liver alcohol dehydrogenase (LADH) is a metalloenzyme containing two identical polypeptide subunits, two active sites and four gram atoms of zinc (1-4). Two of the zinc atoms exchange readily with free metal and are selectively removed by the chelator diethyldithiocarbamate with a concommitant loss in enzymic activity (5-7). These zinc atoms, defined operationally as rapidly exchanging, are thought to be the two active site zinc atoms shown by X-ray crystallography (8). The two remaining zinc atoms which exchange slowly with free metal are assumed to be identical with the zinc atoms remote from the active site.

The correspondence between metal exchange properties and intramolecular location has been investigated by substitution of the chromophoric and paramagnetic cobaltous ion for the two

rapidly exchanging zinc atoms of LADH (9). Harvey and Takahashi (10) found that in the Co_2Zn_2-LADH hybrid the fluorescence of bound NADH or rose bengal was only partially quenched suggesting that the first two cobalts to be substituted in LADH are not at the active site, a conclusion in agreement with the earlier spectral studies of Young and Wang (9). In contrast, nuclear relaxation (11,12) and electron resonance data (13) on the cobalt-zinc hybrid enzyme have been interpreted to indicate a close proximity of cobalt to the catalytic center of liver alcohol dehydrogenase. These conflicting reports may in part be explained by the present data which indicate that the rapidly exchanging zinc atoms of LADH are heterogeneous; one exchange labile metal appears to be located at a structural metal binding site.

Materials and Methods Twelve different lots of horse liver alcohol dehydrogenase were purchased from Boehringer Mannheim Corp. over a period of two years. These preparations typically had a specific activity of 3.5 nanomoles of substrate consumed per minute per milligram protein (10), 3.4 gram atoms of zinc/LADH and 1.6 active sites LADH, as determined by titration with NADH in the presence of isobutyramide (14). EE isozyme purified from fresh horse livers by repeated chromatography on DEAE (15) and double crystallization from 0.02 M phosphate buffer, pH 8.0 containing 12% ethanol was provided by Dr. R. Pietruszko. The EE isozyme, characterized by electrophoretic mobility on starch gel, had a specific activity of 3.8 nanomoles of substrate consumed per minute per milligram protein, contained 3.9 gram atoms of zinc/LADH and 1.9 active sites/LADH. The concentration of LADH was determined spectrophotometrically using a molecular weight of 80,000 and the following absorbtivities: Zn_4-LADH, 0.43 $mg^{-1}cm^2$; $CoZn_3$-LADH; 0.45 $mg^{-1}cm^2$, Co_4-LADH, 0.49 $mg^{-1}cm^2$. Cobalt chloride was a grade I, spectro-pure metal purchased from Johnson Matthey Chemicals Ltd. Alcohol dehydrogenase substituted with cobalt was prepared according to the procedure of Shore (16). All other chemicals and methods have been described previously (10).

Results and Discussion The rate of cobalt substitution was determined by analysis of LADH dialyzed against cobalt buffer at pH 5.5 for 0.5 to 144 hours. Aliquots were removed and dialyzed exhaustively against Hepes buffer, pH 7 to remove unbound metal

ions. A plot of cobalt incorporation as a function of time (Fig. 1) showed that the complete exchange of cobalt for zinc is apparently biphasic in agreement with the previous results of Young and Wang (9). The faster incorporation observed during the first 10 hours had been attributed to substitution at the catalytic sites, while the subsequent slower phase was thought to be due to cobalt incorporation at structural sites (6). Unexpectedly, the extent of cobalt substitution extrapolated to approximately 0.7 gram atoms of cobalt at zero hours of dialysis, suggesting heterogeneity in the fast exchanging metal binding sites.

The presence of metal binding sites which rapidly incorporate cobalt was confirmed by directly monitoring (in the presence of cobalt buffer) the appearance of absorption bands characteristic of cobalt-substituted LADH (Fig. 2). Absorbance maxima at 655 nm and 730 nm increased rapidly during the first

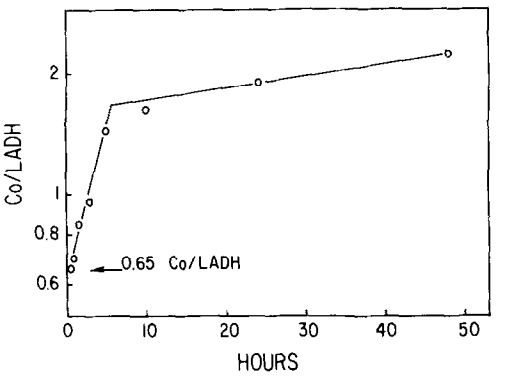

Fig. 1 Time course of cobalt incorporation. LADH (5.0 mg/ml) was dialyzed against 0.2 M $CoCl_2$-0.10 M $NaHSO_4$-0.10 M Na acetate, pH 5.5, at room temperature under an atmosphere of nitrogen. Aliquots of enzyme were removed from cobalt buffer at times indicated and dialyzed exhaustively in 0.025 M Hepes - 0.10 KCl, pH 7. Metal content of the isolated enzyme was determined by atomic absorption spectrophotometry. Co/LADH presented on logarithmic scale.

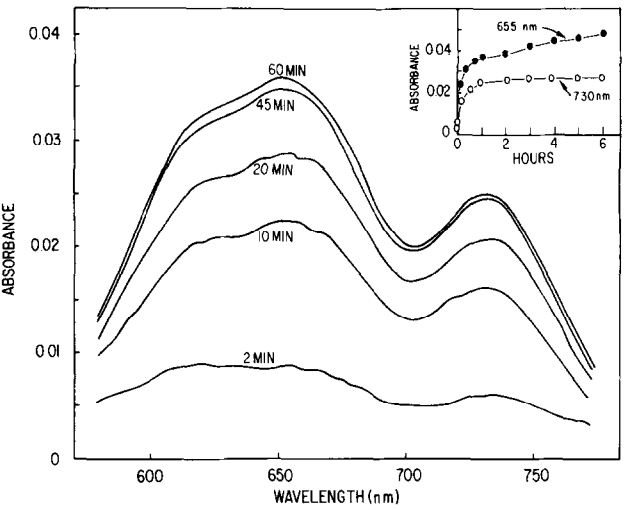

Fig. 2 Spectra of rapidly incorporated cobalt. LADH and cobalt buffer mixed to give following concentrations: LADH, 5.0 mg/ml; 0.2 M ^{57}Co-Cl_2 (2.9 X 10^{11} cpm/mole) - 0.10 M Na_2SO_4-0.10 M Na acetate, pH 5.5. Solution placed in 1.0 cm cuvette and scanned at times indicated at room temperature. Reference cell contained cobalt buffer without enzyme.

60 minutes of incubation. A further gradual increase in the absorbance at 655 nm was observed during the next 6 hours of incubation; however, the absorption at 730 nm remained virtually constant after the first hour of incubation (inset, Fig. 2).

The stoichiometry of metal substitution was determined by incubating ^{65}Zn-LADH (labeled at the rapidly exchanging sites) with cobalt buffer for one hour as described in Fig. 2. The cobalt-zinc hybrid isolated by Sephadex chromatography was found to contain 0.9 gram atom cobalt per mole of enzyme. Analysis of column fractions containing free metal showed the presence of 1.1 gram atom ^{65}Zn released per mole enzyme. Thus, cobalt is not binding at "empty" metal binding sites but is substituting for rapidly exchanging zinc with a 1:1 stoichiometry. The $CoZn_3$-LADH hybrid exhibited absorption maxima in the visible

region (ε_{650nm} = 670 $M^{-1}cm^{-1}$, ε_{730nm} = 500 $M^{-1}cm^{-1}$) which differed quantitatively with those of the completely substituted enzyme (ε_{650nm} = 650 $M^{-1}cm^{-1}$, ε_{730nm} = 250 $M^{-1}cm^{-1}$). These latter values are in reasonable agreement with the molar extinction coefficients reported by Vallee (17) and Shore et al. (16).

While the radioisotope data indicate substitution at a rapidly exchanging site, the cobalt of the hybrid enzyme does not exhibit the spectral properties predicted of metal at the active site. For example, hybrid enzyme containing less than one gram of cobalt did not quench the fluorescence of bound NADH or ε-NAD [an analog of NAD (18)]. The visible spectrum of the hybrid was not perturbed by the presence of NADH or 1,10-phenanthroline, a chelator known from X-ray data (8) to bind to zinc atoms at the active site of the enzyme. In contrast, LADH preparations with greater than one gram atom of cobalt did show spectral changes in the presence of cofactor or chelator and were effective in quenching the fluorescence of bound cofactor.

The rapid incorporation of one gram atom of cobalt per enzyme molecule cannot be explained by preferential substitution of the isozymes known to be minor components of commercial LADH preparations since enzyme containing one gram atom of rapidly incorporated ^{57}Co migrated on starch gel electrophoresis at the same position as "EE" isozyme, the major enzymic component (Fig. 3). Further, the kinetics of cobalt substitution and the spectral properties of the cobalt-zinc hybrid are similar for commercial Boehringer LADH and purified EE isozyme.

The heterogeneity in the rapidly exchanging zinc atoms may reflect conformational changes in the enzyme occurring on binding of the first cobalt. However, the lack of quenching of fluorescence of NADH or ε-NAD bound to the $CoZn_3$-LADH hybrid

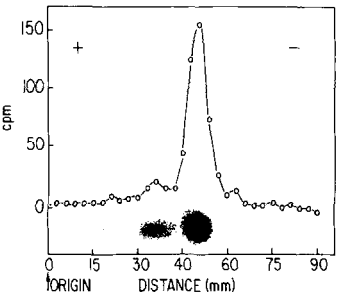

Fig. 3 Starch gel electrophoresis of ^{57}Co-LADH. ^{57}Co-LADH isolated from reaction mixture described in Fig. 2 after 1 hour of incubation. Labeled enzyme subject to electrophoresis in 0.02 M Tris, pH 8.6 for 24 hours at 3°. Enzyme activity as visualized by staining using ethanol, nitroblue tetrazolium, and phenazine methalsulfate shown in lower portion of the figure. After 30 minute incubation, the gel was cut into 3 nn slices which were dissolved in minimal volume of 1 M NaOH and counted in a liquid scintillation counter. The radioactivity of each gel slice is shown in the upper portion of the figure.

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suggests that the first gram atom of cobalt to be incorporated is some distance from the active site. The observation that 1,10-phenanthroline and NADH fail to perturb the visible absorption spectrum of the hybrid also indicates that the cobalt is not at the active site. The presence of polymorphic enzyme forms in the EE isozyme seems unlikely since the diversity of starting material (twelve different lots of commercial LADH) ensures that the enzymes studied were derived from numerous livers. The present data are thus most consistent with cobalt substituting initially for a rapidly exchanging zinc located at the structural metal binding site. Such an assignment will require reevaluation of the widely accepted identity between rapidly exchanging and active site metals.

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