The Role of Metal in Liver Alcohol Dehydrogenase Catalysis

SPECTRAL AND KINETIC STUDIES WITH COBALT-SUBSTITUTED ENZYME*

(Received for publication, January 28, 1974)

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SUMMARY

The kinetic and spectral properties of native and totally cobalt-substituted liver alcohol dehydrogenase have been compared. Based on titrimetric determinations of enzyme active site concentration, the turnover number at pH 7.0 for cobalt enzyme was the same as for native enzyme. At pH 10, however, the turnover number was slower for cobalt-substituted enzyme, 3.14 s⁻¹ as compared with 4.05 s⁻¹ for native enzyme. A comparison between native and totally cobalt-substituted enzyme showed a blue-shifted enzyme-NADH double difference spectrum and a splitting and red-shifted enzyme-NAD⁺-pyrazole double difference spectrum in the near-ultraviolet. The 655-nm peak of the cobalt-substituted enzyme was perturbed by the formation of enzyme-NADH binary complex, enzyme-NAD⁺-pyrazole ternary complex, and enzyme-NAD⁺-trifluoroethanol ternary complex, but not by enzyme-NAD⁺ binary complex formation. At pH 7.0, the only observable step in the reaction sequence with a significantly different rate constant for cobalt enzyme was the catalytic hydrogen-transferring step. The rate constant for this step is 92 s⁻¹ for totally cobalt-substituted enzyme as compared with 138 s⁻¹ for native liver alcohol dehydrogenase. The results of this study indicate that zinc is involved in catalysis and in binding interactions with alcohol and NADH.

Using the spectral properties of binary complexes of chelating agents with enzyme, it was possible to show that oxidized and reduced coenzyme and substrates displaced the chelating agents while ADP-ribose did not (8-10).

Initial attempts to determine the functional role of zinc were based on the inhibitory effects of various chelating agents. 1,10-Phenanthroline and 2,2'-bipyridyl were competitive with oxidized and reduced coenzyme, partially competitive against ethanol, and showed complex behavior with acetaldelyde as varied substrate (2, 11, 12). It was shown (13) that these patterns could be predicted for a Theorell-Chance mechanism in which the inhibitor was competitive with coenzyme and that it was not possible to establish conclusively whether the catalyzing agents were competitive with substrate. Since the catalyzing agents are large enough to sterically occlude other molecules, it has not been possible to ascertain the role of zinc in binding either coenzymes or substrates.

Several studies of exchange of enzyme-bound zinc ions have been reported. Under certain conditions the zinc ions present in liver alcohol dehydrogenase exchange with 65Zn²⁺ ions with two distinct first order rates of exchange (5, 14, 15). These exchange studies stimulated studies of the properties of liver alcohol dehydrogenase in which the zinc ions are replaced by other metal ions. Totally cobalt- and cadmium-substituted enzyme (16) and a hybrid cobalt-zinc enzyme (17) in which the readily exchangeable zinc was replaced, have been prepared. Binding of NADH to the totally cobalt-substituted enzyme shifted the 655 nm peak to 670 nm (16).

Despite many investigations regarding the function of the metal ion in the catalytic mechanism of liver alcohol dehydrogenase, its actual role is still somewhat uncertain. The above literature review is inconclusive regarding which steps in the liver alcohol dehydrogenase reaction mechanism, if any, involve zinc. The demonstration that zinc ion efficiently catalyzes the reduction of an aldehyde by an NADH analog in a nonenzymic model system supports the catalytic function of the metal ion (18). It has been proposed that coenzyme is bound to the enzyme by attachment of the adenine moiety as a bidentate ligand to the zinc ion (19), by attachment of the phosphate groups to the zinc ion (20), or by interaction of the nicotinamide moiety with the zinc ion (21, 22); and that pyrazole, a substrate competitive inhibitor, is bound to the zinc ion in the pyrazole-enzyme-NAD⁺ ternary complex (23).

In the present study the properties of totally cobalt-substituted...
liver alcohol dehydrogenase were compared with those of native enzyme to determine whether the metal ion is involved in catalysis, and to delineate its role. The spectral and kinetic properties of complexes of native and totally cobalt-substituted enzyme with coenzymes, substrates, and substrate analogs were studied. Steady state kinetic studies in conjunction with the study of transients and partial reactions were carried out with the three enzyme forms to provide information regarding which individual steps, if any, in the reaction sequence are affected by replacement of the zinc with cobalt.

**MATERIALS AND METHODS**

Crystalline alcohol dehydrogenase was prepared from frozen horse livers by the method of Theorell et al. (24). The enzyme assay method of Dalziel (25) was used at pH 10 in conjunction with the NADH titration of enzyme in the presence of isobutyramide (26) and the NAD+ titration of enzyme in the presence of pyrazole (23). All three methods yielded the same value for concentration of native enzyme to within 0.5%. Coenzymes were obtained from Sigma Chemical Co., and NAD+ was further purified by dioxane sulfuric acid elution from a Dowex 1 chromatography column by the method of Stinson and Holbrook (27). Pyrazole and isobutyramide were purchased from Eastman Chemical Co. All other chemicals were reagent grade.

Totally cobalt-substituted liver alcohol dehydrogenase was prepared by a modification of the method of Young and Wang (17). Crystalline enzyme was dissolved in 0.1 M pH 7.0 sodium phosphate buffer, dialyzed against 1 liter of buffer to remove alcohol, and dialyzed against two 1-liter changes of 0.1 M Na2SO4, pH 7.0, for 8 to 12 hours. The dialyses were carried out at 4°C, and the enzyme concentration was 100 μM at the start of the exchange dialysis. Exchange was carried out at room temperature with nitrogen vigorously bubbled through the flask to maintain anaerobic conditions. Totally cobalt-substituted enzyme was prepared by dialysis against 1 liter of 0.1 M pH 5.5 acetate buffer containing 0.1 M CoSO4 and 0.1 M NaCl for 4 days, with two changes of dialyzing solution. Subsequent to dialysis against cobalt solution, the cobalt-substituted enzyme was dialyzed against four 1-liter changes of 0.2 M Tris-acetate buffer, pH 7.0, during a 24- to 30-hour period at 4°C. Finally, the enzyme solutions were dialyzed against three 1-liter changes of 0.1 M pH 7.0 sodium phosphate buffer during a 24- to 30-hour period. All steps were carried out under nitrogen, with the final enzyme solutions stored under nitrogen at 4°C and stable for approximately 1 week.

The cobalt derivative formed in this way contained a small flocculent precipitate, which was removed by centrifugation. The concentration of derivative enzyme was determined by dialysis against 1 liter of 0.5 M acetate buffer containing 0.1 M CoSO4 and 0.1 M NaCl for 48 hours, with two changes of dialyzing solution. Enzyme concentration was 100 μM at the start of the exchange dialysis. Exchange was carried out at room temperature with nitrogen vigorously bubbled through the flask to maintain anaerobic conditions.

**RESULTS**

**Activity of Cobalt-substituted Enzymes**—The turnover number of totally cobalt-substituted enzyme was determined at pH 7.0 in 0.1 M phosphate buffer and at pH 10 in 0.1 M glycine buffer, and compared with native enzyme. At pH 7.0, cobalt enzyme had the same turnover number as native liver alcohol dehydrogenase, 3.1 ± 0.2 s⁻¹ based on titrimetrically determined active site concentration. At pH 10, the turnover number was 4.1 ± 0.2 s⁻¹ for native enzyme while the turnover number for totally cobalt-substituted enzyme was somewhat lower, 3.1 ± 0.2 s⁻¹. Since the turnover at neutral pH is controlled by the isomerization and dissociation of binary enzyme-NADH complex (32, 33), the first order rate constants for NADH dissociation were determined for native and totally cobalt-substituted enzymes by a stopped flow technique using displacement by ADP-ribose (32). At pH 7.0 in 0.1 M phosphate buffer the rate constants for this partial reaction were similar for both native and totally cobalt-substituted enzyme; 3.4 ± 0.2 and 3.7 ± 0.2 s⁻¹, respectively.

**Spectral Properties of Binary and Ternary Complexes**—The spectral properties of complexes involving native and totally cobalt-substituted liver alcohol dehydrogenase were investigated. There is a blue shift of 5 nm in the trough of the enzyme-NADII binary complex double difference spectrum when native enzyme is replaced by totally cobalt-substituted enzyme for complex formation (Fig. 1). Native enzyme exhibited a trough at 355 nm while for totally cobalt-substituted enzyme it was at 350 nm. The same difference was observed in the double difference spec-

![Fig. 1. Double difference spectra of binary complexes consisting of 30 μM enzyme and 36 μM NADH: native liver alcohol dehydrogenase (+), totally cobalt-substituted liver alcohol dehydrogenase (−). Reference cuvettes contained enzyme and NADH, respectively.](http://www.jbc.org/)
FIG. 2. Spectral characteristics of totally cobalt-substituted liver alcohol dehydrogenase and its enzyme-NADH and enzyme-NADH-isobutyramide complexes: 116 μM enzyme (---); 114 μM enzyme, 236 μM NADH (--); 112 μM enzyme, 225 μM NADH, and 9 mM isobutyramide (---).

FIG. 3. Double difference spectra of ternary complexes consisting of 28 μM enzyme, 200 μM pyrazole, and 100 μM NAD+: native liver alcohol dehydrogenase (---), totally cobalt-substituted liver alcohol dehydrogenase (---). Reference cuvettes contained enzyme plus pyrazole and NAD+, respectively.

FIG. 4. Spectral properties of totally cobalt-substituted liver alcohol dehydrogenase and enzyme-NAD+-pyrazole ternary complex: (a) 110 μM enzyme; (b) 112 μM enzyme, 2.5 mm NAD+, and 370 μM pyrazole.

FIG. 5. Spectral characteristics of totally cobalt-substituted liver alcohol dehydrogenase and ternary complex with NAD+ and trifluoroethanol: 96 μM enzyme (---); 94 μM enzyme, 2.1 mm NAD+, and 0.4 mm trifluoroethanol (---).

A large spectral difference between native and totally cobalt-substituted enzyme occurs in the case of ternary pyrazole-enzyme-NAD+ complex (Fig. 3). When native enzyme is used for ternary complex formation the double difference spectrum shows a flat peak from 284 to 295 nm. The totally cobalt-substituted liver alcohol dehydrogenase-NAD+-pyrazole ternary complex double difference spectrum shows two distinct peaks at 283 and 298 nm. Steady state kinetic studies indicated that the $K_r$ value for pyrazole was similar for native and totally cobalt-substituted enzyme, with values of 0.31 and 0.29 μM, respectively. Binding of NAD+ to totally cobalt-substituted liver alcohol dehydrogenase caused no perturbation of the 655 nm peak of the cobalt enzyme. However, there is a 15-nm red shift and a splitting of the 655 nm peak due to enzyme-NAD+-trifluoroethanol ternary complex formation (Fig. 4). A similar spectral shift occurred with the enzyme-NAD+-trifluoroethanol ternary complex (Fig. 5).

Transient Kinetics of Cobalt-substituted Enzyme—The study of transients in the liver alcohol dehydrogenase catalytic sequence was undertaken to characterize further the involvement of the...
liver alcohol dehydrogenase (b). The reactions were carried out in 0.1 M phosphate, pH 7.0 buffer at 25°C. The metal ion in catalysis. If saturating NAD⁺ and a high ethanol concentration are added to liver alcohol dehydrogenase in a stopped flow instrument with the reaction measured at the isosbestic point for free and bound NADH, a rapid burst of enzyme-bound NADH formation can be observed (34). The rate constant for enzyme-bound NADH formation was measured for native and cobalt-substituted enzyme to determine the rate difference due to replacement of zinc ions by cobalt ions. Fig. 6 shows the burst reaction for native and totally cobalt-substituted enzyme. The first order rate constant for the initial burst of enzyme-bound NADH formation is 138 s⁻¹ for native enzyme, whereas totally cobalt-substituted enzyme has a lower value of 92 s⁻¹.

DISCUSSION

Characterization of the cobalt-substituted liver alcohol dehydrogenase is of substantial importance. The values obtained from cobalt and zinc analysis of the totally cobalt-substituted enzyme, and the similarity of spectral properties to those previously reported (16) for this derivative, indicate that greater than 90% of the zinc has been replaced by cobalt. The small population of native enzyme remaining would not significantly affect the spectral and kinetic differences found between the totally cobalt-substituted derivative and native enzyme. Furthermore, the differences would be even greater if we were able to achieve 100% substitution.

It has been reported (15) that only 2 of the zinc atoms per mol of enzyme exchange with ⁶⁵Zn in 0.1 M acetate at pH 5.5, the conditions we used. However, the same authors used 0.2 M acetate at pH 5.5 to prepare totally cobalt-substituted enzyme (16). It is possible that the higher ionic strength facilitated exchange of the slowly exchanging zinc. Furthermore, our procedure was carried out in the presence of 0.1 M NaCl, which may assist the exchange. It is conceivable that chloride was also present in the previously reported preparation (16) since metallic cobalt dissolved in HCl was used for exchange.

The comparison of the turnover numbers and first order rate constants for NADH dissociation indicated that they were the same for native and totally cobalt-substituted enzyme at pH 7.0. In addition, these results show that at pH 7.0 turnover of the cobalt enzyme is still primarily controlled by NADH dissociation, i.e. that no other step has become rate-limiting. At pH 10 in glycine buffer, the turnover number of totally cobalt-substituted enzyme was 77% of the value obtained for native enzyme. This reflects a specific effect of pH, or buffer composition, or both, on the rate-limiting step of the totally cobalt-substituted enzyme, which does not occur with native enzyme. The identical turnover numbers at pH 7.0 indicate that the cobalt enzyme is fully active. A previous report (16) of a lower specific activity for cobalt enzyme was most probably due to the higher pH used for assay.

It has been shown (32, 34) that the burst of enzyme-bound NADH formation due to adding saturating concentrations of NAD⁺ and ethanol to liver alcohol dehydrogenase showed a substantial deuterium isotope effect, and was therefore a measure of the rate of the catalytic hydrogen-transferring step. The present work shows that at pH 7.0 the rate constant for this step is 138 s⁻¹ for native enzyme and 92 s⁻¹ for cobalt-substituted enzyme. This relatively modest but significant difference is not unexpected considering the similarities between cobalt and zinc. The demonstration of these rate constant differences makes it probable that the metal is involved in the catalytic hydrogen-transferring step of the liver alcohol dehydrogenase mechanism. The involvement of zinc in catalysis requires interaction with substrates, or coenzymes, or both, in the ternary complexes. Since the addition of saturating concentrations of NAD⁺ did not affect the 655 nm peak of the totally cobalt-substituted enzyme, an interaction of oxidized coenzyme with the metal seems improbable. On the other hand, ternary complex formation indicates definite interactions. The broad absorbance band from 285 to 295 nm in the enzyme-NAD⁺-pyrazole double difference spectrum shows splitting and a red-shifted component when the totally cobalt-substituted enzyme is used. The spectrum of the totally cobalt-substituted enzyme shows a splitting and red shift of the 655 nm peak due to ternary enzyme-NAD⁺-pyrazole complex formation, further substantiating an interaction between pyrazole and the metal. This result supports the proposal of Theorell and Yonetani (23) that pyrazole is bound to the zinc in liver alcohol dehydrogenase, and since pyrazole is competitive with alcohol, it infers that the alcohol is bound to the metal. A more directly relevant result was obtained with trifluoroethanol, which is structurally similar to ethanol. The red shift and splitting of the 655 nm peak of the totally cobalt-substituted enzyme due to formation of ternary enzyme-NAD⁺-trifluoroethanol complex indicates interaction of either the alcoholic group with the metal or of the coenzyme with the metal in ternary complexes.

Although our findings indicate that oxidized coenzyme does not interact with the metal at the active center in binary complexes there is evidence that the reduced coenzyme does. A previous study (18) reported a 15 nm shift in the 655 nm peak of totally cobalt-substituted enzyme which our results (Fig. 2) substantiate. Our finding that the double difference spectra of the enzyme-NADH-isobutyramide complexes showed a trough at 350 nm for cobalt enzyme as compared with 355 nm for the native enzyme provides further evidence for interaction between the metal and NADH. Since the totally cobalt-substituted enzyme shows a peak at 343 nm, the small blue shift in the difference spectrum trough probably results from perturbation of the spectrum of the cobalt enzyme. The failure of ternary complex formation with isobutyramide to cause further perturbation of the 670 nm peak of the totally cobalt-substituted enzyme-NADH binary complex makes it plausible that aldehydes do not interact with the metal. It is difficult to reconcile the spectral evidence for interaction between the metal and NADH with the report (35) that zinc-free liver alcohol dehydrogenase binds reduced coenzyme with the same affinity as holoenzyme. Either the interaction between the metal and NADH is not a major contributor to the strength of binding or the changes in the
655 nm peak due to binary complex formation are the result of a change in enzyme conformation (36).

It is possible that the spectral shifts observed due to binary and ternary complex formation with cobalt-substituted liver alcohol dehydrogenase result from conformational changes of the enzyme, with one or both of the 2 metal ions per eq acting as a spectral probe. If this were the case, the spectral shift observed in the 655 nm peak would be caused by interaction with functional groups of the enzyme. Since the red shifts observed due to binding NADH or NAD+ in the presence of either pyrazole or trifluoroethanol were similar, the conformational changes due to formation of all three complexes would have to be similar. A more probable explanation, substantiated by the slower hydride transfer rate of the cobalt-substituted enzyme, is that the alcohol substrate and inhibitors competitive with it are directly bound to the metal at the active center. Since NADH binding also perturbs the 655 nm peak, it is difficult to ascertain whether aldehydes or isobutyramide also interact with the metal. High resolution NMR studies with the cobalt enzyme, currently in progress, should clarify the nature of the interactions between substrates, coenzymes, and enzyme-bound metal.

Acknowledgments—The technical assistance of Mr. Otto Urschel and Dr. Pura Santiago is gratefully acknowledged.

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The role of metal in liver alcohol dehydrogenase catalysis. Spectral and kinetic studies with cobalt-substituted enzyme.

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*J. Biol. Chem.* 1975, 250:2008-2012.

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