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Influence of Anions and pH on the Conformational Change of Horse Liver Alcohol Dehydrogenase Induced by Binding of Oxidized Nicotinamide Adenine Dinucleotide: Binding of Chloride to the Catalytic Metal Ion†

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ABSTRACT: The conformational change of horse liver alcohol dehydrogenase induced by binding of NAD^+ was studied by electronic absorption spectroscopy using cobalt as a spectroscopic probe in the active site. The complex of the enzyme with NAD^+ exists in an acidic and an alkaline form. The transition between the two forms proceeds through several intermediates and is controlled by an apparent pK_a of 6.9. Only at pH values below this pK_a can a complex between enzyme, NAD^+, and Cl^- be formed. The spectral changes indicate that chloride displaces the cobalt-bound water molecule in a tetracoordinate structure. We conclude that a negative charge at the active site is necessary to stabilize the closed conformation of the enzyme in the presence of NAD^+. Spectral correlations are given which strongly support the postulation of a metal-bound alkoxide in the closed structure of the enzyme as an essential feature of the catalytic mechanism of horse liver alcohol dehydrogenase.

It is becoming apparent that the catalytic zinc ion in horse liver alcohol dehydrogenase (HLADH,[1] EC 1.1.1.1) plays a multifunctional role (Zeppezauer et al., 1984). The metal ion activates the substrate by direct coordinative bonding, thereby functioning as a Lewis Acid. In addition, it has recently been noticed that the metal ion influences the dynamics of coenzyme binding although direct bonding interactions between the coenzyme molecule and the catalytic metal ion do not exist. In spite of these insights, much more information is needed to delineate the molecular details of the catalytic cycle. In particular, a keystone to the mechanism is the number and chemical nature of intermediates formed during ternary complex interconversion. Such information has recently become

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[1] Abbreviations: HLADH, horse liver alcohol dehydrogenase; Zn-HLADH, native zinc enzyme; Co-HLADH, active-site-specific Co^{2+}-reconstituted enzyme; Bis-Tris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane.

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available by the use of rapid-scanning stopped-flow spectrophotometry, which until now has led to the detection of at least three intermediates during ternary complex interconversion (Gerber et al., 1983). A controversial question of great concern has been whether the alcohol forms a zinc-bound alkoxide during the reaction. It is therefore of general importance to investigate the interactions of anions with the catalytic metal ion and to distinguish it from interactions at other anion binding sites on this protein. An important tool in this kind of investigation has been the use of alcohol dehydrogenases, in which the catalytic zinc ions have been replaced by various other metal ions (Maret et al., 1979; Zeppezauer et al., 1984).

The binding of chemically different anions such as hydrogen sulfide, azide, or acetate consistently causes the same characteristic changes in the spectra of the catalytic cobalt ion in free Co-HLADH, namely, the disappearance of the absorption band at 520 nm with the concomitant appearance of a band in the range of 575 nm (Maret, 1980). Similarly, in ternary complexes containing NAD^+, e.g., Co-HLADH/NAD^+/acetate and Co-HLADH/NAD^+/2,2,2-trifluoroethanol, the same spectral changes indicating the binding of anions have been observed (Maret, 1980). So far, it was not definitely established whether the anion coordinates directly to the metal ion or whether local or gross conformational changes are responsible for the observed spectral effects.

A recent ^{13}C NMR study (Bertini et al., 1985) has given definite proof of direct coordination of acetate to the catalytic metal ion. Much effort has been devoted to probe halogen anion binding to Zn-HLADH by means of halogen NMR quadrupole relaxation spectroscopy. By ^{35}Cl and ^{79}Br NMR spectroscopy, it was demonstrated that chloride and bromide bind to anion binding sites in the coenzyme binding domain of native and chemically modified HLADH (Andersson et al., 1979; Zeppezauer et al., 1969). In these HLADH species, binding of halogen anions to the catalytic metal ion could not be demonstrated.

In this study, it is demonstrated that NAD^+ enables the coordination of chloride ions to the catalytic cobalt ion in Co-HLADH at low pH. It is concluded that the mutual interaction of a negatively charged group in the immediate vicinity of the metal ion and NAD^+ is a prerequisite for the stabilization of the closed protein conformation.

Experimental Procedures

Materials. Horse liver alcohol dehydrogenase (EC 1.1.1.1) and the coenzyme NAD^+ (Li salt) were purchased from Boehringer-Mannheim, FRG. Bis-Tris/propane was purchased from Sigma GmbH München, FRG. The crystalline zinc enzyme was converted to the cobalt enzyme (active-site-specific substituted Co-HLADH) and handled as described (Maret et al., 1979).

Methods. Sodium or potassium chloride is usually used to dissolve the crystalline enzyme. In this study, 0.15 M sodium sulfate was used for this purpose. Sulfuric acid was used for adjusting the pH of the buffer solutions. Because of the limited solubility of the Li salt of NAD^+, the electronic absorption studies were carried out as follows. Solid NAD^+ was weighed into a stoppered optical cell with 1-cm path length, followed by the addition of buffer, coenzyme, and enzyme solutions. The solution was strictly kept under nitrogen and stirred until a clear solution was obtained with a small magnetic bar fitting into the cell. Then the pH was measured at 20 °C in the cell with a Radiometer PHM 62 pH meter. Absorption spectra were recorded on Varian CARY 16 and 17D spectrophotometers at 20 °C. To check for saturating concentrations of NAD^+ in the experiments, the coenzyme concentration was

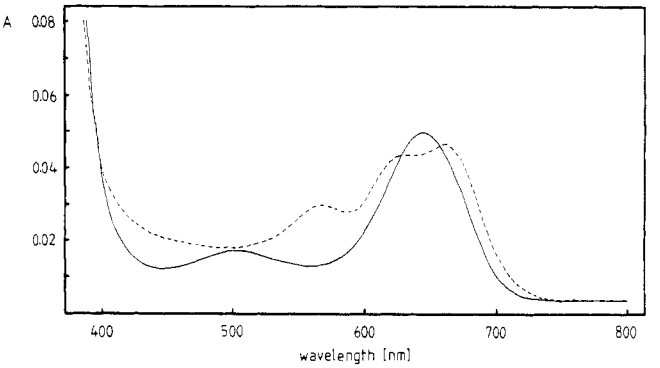

FIGURE 1: Electronic absorption spectra of the binary complex Co-HLADH/NAD^+ at pH 5.2 (solid line) and at pH 9.5 (dashed line). Concentration of Co-HLADH, 7.8×10^{-5} M; concentration of NAD^+, 1.9×10^{-2} M; concentration of buffer, 9×10^{-2} M Bis-Tris/propane.

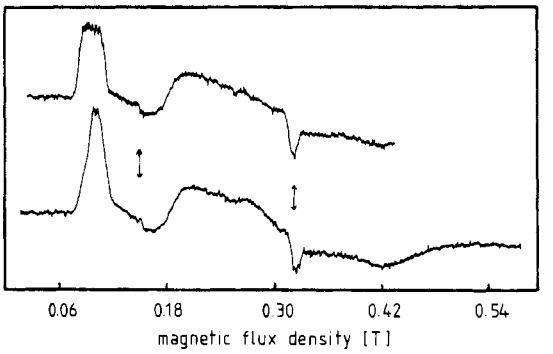

FIGURE 2: EPR spectra of Co-HLADH (upper spectrum) and Co-HLADH/NAD^+ (lower spectrum) in 6.5×10^{-2} M Bis-Tris/propane buffer, pH 5.5. Concentration of Co-HLADH, 8.4×10^{-4} M; concentration of NAD^+, 2.8×10^{-2} M. The arrows in the figure indicate absorptions due to impurities in the quartz sample tubes. The spectra were obtained at a frequency of 9.4593 GHz with a microwave power of 21.7 mW, a modulation frequency of 100 kHz, and a modulation amplitude of 10 G_{pp}.

doubled at low (pH 6) and high pH (pH 10). No spectral changes were observed, indicating that the enzyme is saturated with NAD^+. The binding constant of chloride to the complex Co-HLADH/NAD^+ was determined by following the increase in absorption at 712 nm upon addition of chloride. At this wavelength, the largest difference in absorption between the binary and the ternary complex is observed. The binding constant was evaluated according to Klotz (1946) with an iterative least-squares fit. EPR spectra at 5 K were recorded with a Bruker ER 420 X-band spectrometer equipped with an Oxford ESR 9 cryostat.

Results

NAD^+ Binding to Co-HLADH in the Absence of Chloride. In Figure 1, the electronic absorption spectra of the complex Co-HLADH/NAD^+ are shown at low and high pH, indicating two forms of the enzyme. Compared to free Co-HLADH, there are only minor changes in the spectra at low pH. However, comparison of the EPR spectra of Co-HLADH and Co-HLADH/NAD^+ in Figure 2 shows that this technique is sensitive for NAD^+ binding at pH 5.5 and, hence, that NAD^+ is indeed bound to the enzyme at low pH. On the basis of the EPR spectra, a distinction between a low- and high-pH form of the complex Co-HLADH/NAD^+ is not possible (EPR data for the high-pH form not shown). In contrast, significant spectral changes are observed in the optical absorption spectra of the binary complex at high pH. A new band is present at 570 nm, and the main absorption band centered at 650 nm in the free enzyme is now split into two components with

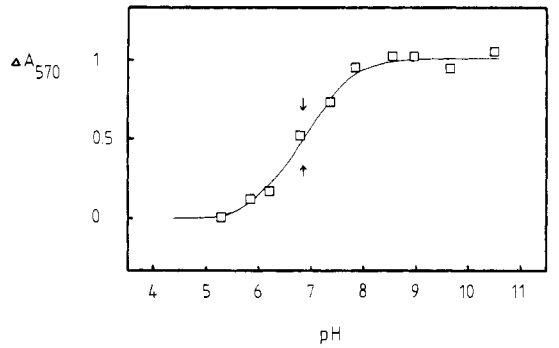

FIGURE 3: Electronic absorption changes of the complex Co-HLADH/NAD^+ at 570 nm as a function of pH. The concentrations of enzyme, coenzyme, and buffer are given in Figure 1. The arrows indicate a pK_a of 6.9 obtained after the data points were visually fitted to a theoretical titration curve.

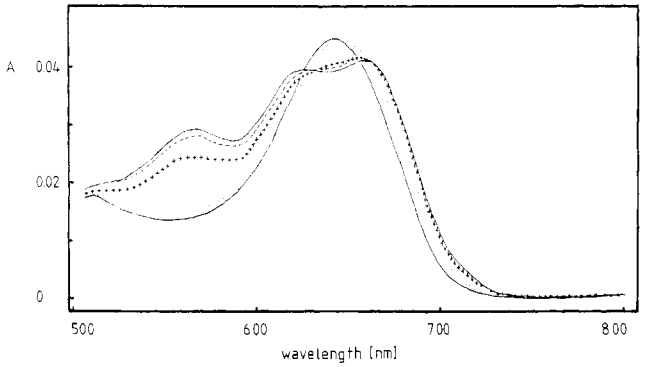

FIGURE 4: Electronic absorption spectra of the complex Co-HLADH/NAD^+ as a function of pH. The pH values are (from bottom to top at 575 nm) 5.3 (solid line), 6.1 (dotted line), 7.5 (crossed line), 8.5 (dashed line), and 9.5 (solid line). The concentrations of enzyme, coenzyme, and buffer are given in Figure 1. In contrast to Figures 1 and 5, the spectra in this figure are not base-line corrected.

maxima at 662 and 627 nm. Plotting the intensity increase of the absorption at 570 nm vs. pH produces a titration curve with an apparent pK_a of 6.9 (Figure 3). This pK_a value is more than 1 unit lower than the pK_a of 8.2 observed when the pH dependence of NAD^+ binding was followed with a different buffer system (Dietrich & Zeppezauer, 1982). To check the reversibility of the complex formation, a sample of the binary complex Co-HLADH/NAD^+ was converted from the acidic form to the alkaline form and back by successive dialyses and pH adjustments. At each pH, the absorption spectrum was measured, and the ratio of the absorbancies at 515 and 575 nm was determined. The absorbancy ratios were 1.28 (pH 5.8), 0.67 (pH 7.8), 0.85 (pH 6.5), 1.0 (pH 6.2), and 1.18 (pH 5.1), demonstrating that the optical characteristics of the acidic species are restored upon reversion of the alkaline to the acidic species. The alkaline form of the complex Co-HLADH/NAD^+ does not show changes in absorption intensity in the range 1150–750 nm when compared to free Co-HLADH. As shown in Figure 4, the spectral changes of the complex Co-HLADH/NAD^+ as a function of pH are due to more than two interconverting forms, because of the lack of common isosbestic points.

NAD^+ Binding to Co-HLADH in the Presence of Chloride. Only at low pH does the binding of chloride result in a change of the optical absorption spectra of Co-HLADH in the presence of NAD^+ (Figure 5). There is a large red shift and increase in intensity of the main absorption band which is now split into three components (668, 686, and 704 nm). A new absorption band appears at 575 nm with a shoulder at higher energies. There are no major absorption changes in the

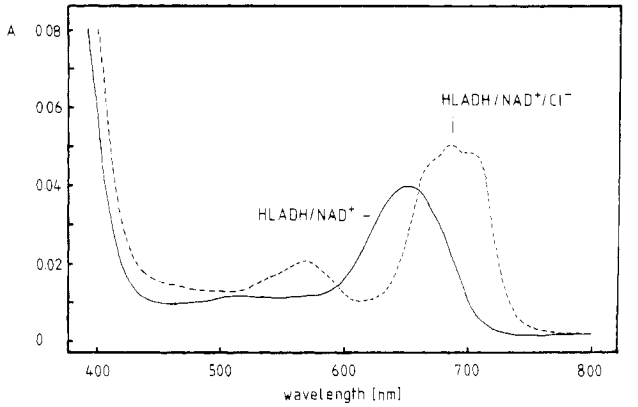

FIGURE 5: Electronic absorption spectra of the binary complex Co-HLADH/NAD^+ (solid line) and of the ternary complex Co-HLADH/NAD^+/Cl^- (dashed line) at pH 6.2. The concentrations of enzyme, coenzyme, and buffer are given in Figure 1. The concentration of chloride is 1 M.

wavelength range 1150–750 nm as compared to the complex Co-HLADH/NAD^+ at low pH. At pH 7, comparable concentrations of chloride do not affect the optical absorption spectra of Co-HLADH or Co-HLADH/NADH. The dissociation constant of chloride to the complex Co-HLADH/NAD^+ was determined as 0.1 M at pH 7. Addition of pyrazole to the ternary complex Co-HLADH/NAD^+/Cl^- produces a spectrum which is typical for the ternary complex Co-HLADH/NAD^+/pyrazole.

Discussion

Anion Binding to the Catalytic Metal Ion. In the absence of coenzyme, there are two well-characterized anion binding sites in HLADH. It has been shown that anions such as hydrogen sulfide, azide, or acetate bind to the catalytic cobalt ion in Co-HLADH and create a new absorption band at 575 nm (Maret, 1980; Bertini et al., 1985). In contrast, many other anions bind to the general anion binding sites which are located at arginine-47 in proximity to the catalytic metal ion and presumably at arginine-271 (Andersson et al., 1979; Oldén & Pettersson, 1982). Binding of anions to the free zinc enzyme is controlled by a pK_a value of 8.8–9.1 (Reynolds & McKinley-McKee, 1969). Binding constants for chloride were given as 23 mM at pH 7.4 (Reynolds & McKinley-McKee, 1969) and 42 mM at pH 7 (Oldén & Pettersson, 1982) with decreasing affinity above the pK_a of about 9.

Upon binding of coenzyme, the general anion binding site is no longer available, since the pyrophosphate group of the coenzyme interacts with this site. Arginine-271 is in van der Waals contact with the adenine ring of the coenzyme (Eklund et al., 1984). However, additional anion binding sites have been inferred from transient kinetic experiments, showing that a quaternary enzyme/NADH/chloride/ethanol complex can be formed (Kamlay & Shore, 1983). The binding site for chloride in this complex is not known. Evidence for extensive binding of chloride ions to Zn-HLADH in the presence of NADH and inhibitors is derived from ^{35}Cl NMR quadrupole relaxation experiments. In these ternary complexes, the coenzyme binding and substrate binding sites are inaccessible, and therefore, possible chloride binding sites are lysine and arginine side chains on the surface of the protein (Andersson et al., 1979). As shown in the present report, a new binding site for chloride with an affinity comparable to that of the general anion binding site in the free zinc enzyme is created in the presence of NAD^+ bound to Co-HLADH. It has also been noted that azide is bound to the catalytic zinc ion in the complexes of NAD^+ and Zn_4-HLADH or hybrid HLADH

Scheme I

$$Cl^- + H^+ \cdot HLADH/NAD^+ \longrightarrow H^+ \cdot HLADH/NAD^+/Cl^-$$
$$\downarrow\uparrow$$
$$H^+ + HLADH/NAD^+$$

containing catalytic zinc ions and noncatalytic cobalt ions (Young & Wang, 1971).

Similarly, binding of acetate and thiocyanate to the catalytic metal ion was postulated from kinetic studies (Andersson et al., 1984). Certain strongly nucleophilic anions also form an adduct with the C-4 position of the nicotinamide moiety of the coenzyme [for a review, see Kellog (1984)].

It is important to recall that the optical changes in the region 525–575 nm are identical in the complexes of Co-HLADH with acetate and the complexes of Co-HLADH/NAD^+ with acetate, 2,2,2-trifluoroethanol, or chloride. Since acetate coordinates directly to the catalytic cobalt ion as shown from our ^{13}C NMR study of acetate binding (Bertini et al., 1985), we must therefore conclude that the spectral effects observed upon chloride binding originate from direct coordination to the catalytic metal ion as well. This conclusion is corroborated by the observation that chloride in the complex Co-HLADH/NAD^+/Cl^- is displaced by pyrazole. Furthermore, it was shown that chloride is a competitive inhibitor toward both NAD^+ and ethanol binding to Zn-HLADH (Coleman & Weiner, 1973). The reported inhibition constant, K_I = 100 mM (toward ethanol at pH 7.0), is in excellent agreement with our value of K_D = 100 mM for binding of chloride to the complex Co-HLADH/NAD^+. Since chloride binds only to the acidic form of the complex Co-HLADH/NAD^+ (Scheme I), it is concluded that chloride binding is governed by the pK_a value of 6.9, which controls the binding of NAD^+.

Interestingly, the optical absorption band at 575 nm, typical for metal-bound anions, is observed in the complex Co-HLADH/NAD^+/chloride as well as in the alkaline form of the complex Co-HLADH/NAD^+. It seems plausible that a negatively charged group is present at the active site above the pH value of 6.9 in the complex Co-HLADH/NAD^+ which precludes chloride binding.

Metal-Bound Water Molecule. Comparison of the electronic absorption spectra in Figure 1 or 5 reveals only minor changes in intensity. Intensity changes have been regarded as an important criterion to distinguish between different coordination numbers in high-spin Co(II) complexes (Bertini et al., 1982). On the basis of this observation, we conclude that the coordination number of the catalytic metal ion does not change with pH and remains 4 in the binary complex Co-HLADH/NAD^+ or upon binding of chloride to the acidic form of this complex. The main absorption band of Co(II) in the complex Co-HLADH/NAD^+/chloride shows a splitting with an energy separation of 390 and 370 cm^{-1} which is typical for spin–orbit coupling in tetrahedral Co(II) complexes. Also, the slight increase in intensity is in agreement with a less distorted tetrahedral environment of Co^{2+} in this complex as compared to Co^{2+} in the free enzyme or in the complexes with NAD^+. The data therefore indicate that chloride displaces the cobalt-bound water molecule at pH values below the pK_a value of 6.9.

The origin of the spectral change from the acidic to the alkaline form of the complex Co-HLADH/NAD^+ is not easily explained. As pointed out by Bertini (Bertini et al., 1982), water and hydroxide are close in the spectrochemical and nephelauxetic series and the ionization of metal-bound water itself is not expected to produce such a significant spectral change. It is therefore more likely that the observed effect

Scheme II

$$H^+ \cdot HLADH/NAD^+ \rightleftharpoons H^+ \cdot HLADH^*/NAD^+$$
$$\downarrow\uparrow \qquad\qquad \downarrow\uparrow$$
$$H^+ + HLADH/NAD^+ \rightleftharpoons H^+ + HLADH^*/NAD^+$$

of pH rather reflects a conformational change of the protein affecting the catalytic metal ion. The pK_a of 6.9 given in Figure 3 is an apparent pK_a since the optical spectra of the complex Co-HLADH/NAD^+ as a function of pH indicate more than two interconverting species (Figure 4). Therefore, the pK_a must include additional equilibria as illustrated in Scheme II. Here, the asterisk indicates a different conformational state of the protein.

Although it is tempting to invoke the ionization of metal-bound water in these processes, for which a pK_a of 7.6 was postulated in the binary complex Zn-HLADH/NAD^+ (Evans & Shore, 1980; Kvassman et al., 1981), our data cannot definitely assign the ionization of metal-bound water.

Conformational Changes upon Binding of NAD^+ and Anions. X-ray crystallographic studies have shown that HLADH changes from the open to the closed structure upon binding of NADH (Eklund & Brändén, 1983). This process is visualized in the electronic absorption spectra of Co-HLADH by a shift of the main absorption band in the visible region from 650 to 680 nm (Maret, 1980). It has recently been shown that crystalline complexes of Zn-HLADH with coenzyme can exist both in the closed and in the open conformation (Cedergren, 1984). No spectroscopic corollaries of the conformational transition induced by the oxidized coenzyme (NAD^+) have been available until now. This is mainly due to the fact that structural data on true complexes with NAD^+ are still lacking. The most frequently studied complex, i.e., Zn-HLADH/NAD^+/pyrazole, chemically resembles the complex with NADH due to the 1,4-dihydropyridine structure of the covalent adduct between pyrazole and the nicotinamide moiety (Eklund et al., 1984). The X-ray structures of the ternary complexes Zn-HLADH/NAD^+/fatty acid and Zn-HLADH/NAD^+/2,2,2-trifluoroethanol have not been reported. The ternary complex crystallized from an equilibrium mixture of Zn-HLADH, NAD^+, and *p*-bromobenzyl alcohol contains predominantly NADH (Bignetti et al., 1979). Therefore, the crystal structure reported for this complex (Eklund et al., 1982) does not describe a complex with oxidized coenzyme. In this regard, the complex Co-HLADH/NAD^+/chloride, which so far has been obtained in solution only, is a genuine ternary complex with NAD^+. The observation of a large red shift of the main absorption band at 650 nm in this ternary complex is consistent with a closed conformation of the protein. It is interesting to note that a similar shift is observed in the alkaline form of the complex Co-HLADH/NAD^+.

We therefore conclude that a negative charge at the catalytic site is necessary to stabilize the closed conformation in the presence of NAD^+. Our data show that at low pH a complex of Co-HLADH with NAD^+ in the open conformation is formed, since a red shift of the main absorption band does not occur. Hence, only at pH values above pH 8 is the HLADH/NAD^+ complex expected to exist predominantly in the closed conformation. Also, the complexity of the binding data for NAD^+ indicates strongly that coenzyme binding and the conformational change are coupled by a series of events.

As mentioned above, in the absence of NAD^+ the binding of certain anions to Co-HLADH creates a transition at 575 nm which is indicative of an effect of an anionic species on

the metal coordination, since the effect is independent of the chemical nature of the anion. This change in the electronic absorption spectrum is due to a conformational change, which is localized to the vicinity of the metal ion and reflects the rearrangement of charged groups as a consequence of the charge compensation which has to accompany anion binding.

Implications for the Mechanism of Action of Horse Liver Alcohol Dehydrogenase. It is informative to compare the electronic absorption spectra of the transients of the Co-HLADH reaction detected so far by rapid-scanning stopped-flow spectrophotometry (Gerber et al., 1983) with those of the NAD^+ complexes described above. In the reaction between Co-HLADH, NAD^+, and benzyl alcohol, it was found that an intermediate is formed with electronic absorption bands at 575 and 680 nm, which preceded the burst appearance of NADH. A similar, but stable complex is formed from Co-HLADH, NAD^+, and 2,2,2-trifluoroethanol.

It is immediately apparent that the spectral properties of this intermediate closely resemble those of the complex Co-HLADH/NAD^+/Cl^- as far as the wavelength region from 500 to 600 nm is concerned, in particular, the novel band at 575 nm. Also, the band at 650 nm is shifted similarly in both the transient intermediate and the stable Co-HLADH/NAD^+/Cl^- complex. Both the transient and the latter stable complex exhibit the spectral features of the metal-bound negative ligand and of the closed protein conformation. It is therefore reasonable to conclude that during ternary complex interconversion the alcoholic substrate does appear as a metal-bound alkoxide in the closed protein conformation. If this were not to be the case, the participation of another ionizable group in the reaction has to be postulated.

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Registry No. HLADH, 9031-72-5; NAD, 53-84-9.

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