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The influence of anions and inhibitors on the catalytic metal ion in Co(II)-substituted horse liver alcohol dehydrogenase *

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Abstract. ^{1}H-NMR and electronic spectroscopic data are reported for the interaction of the effector molecule imidazole and the inhibitor molecule pyrazole with horse liver alcohol dehydrogenase whose catalytic zinc ions were replaced by Co(II). In addition ^{13}C-NMR and optical data are given for the binding of acetate to this enzyme species. For the binary complex with imidazole an assignment of the protons of the metal-coordinated imidazole has been made and it was found that the rate of exchange of the effector molecule is slow on the NMR time scale. In the presence of NADH which is bound to the open conformation of the binary complex, the most pronounced change is a shift of the β-CH_2 protons of the metal-coordinated cysteine residues which is attributed to hydrogen bonding interactions between the carboxamide group of the nicotinamide moiety with cysteine 46. The ^{1}H-NMR spectra of the binary complex of Co(II)-HLADH with pyrazole show resonances assigned to the protons in the 3- and 4-positions of the bound inhibitor, the NH proton resonance is not detectable. In the ternary complex with pyrazole and NAD^+ only the resonances of the β-CH_2 protons (beyond 150 ppm) are changed whereas the protons of histidine 67 and the bound inhibitor are unchanged. The data demonstrate that the coordination environment of the catalytic metal ion is changed very little when the protein changes from the open to the closed conformation. The only changes observed are the β-CH_2 proton resonances of the metal-coordinating cysteines which are sensitive to local conformational changes within the ternary complex Co(II)-HLADH · Imidazole · NADH in the open conformation or global changes in the ternary complex Co(II)-HLADH · Pyrazole · NAD^+ in the closed conformation. Acetate which can be regarded as a substrate model was shown to induce a similar change in the optical spectra of the Co(II) enzyme as all other anions observed so far. From the optical changes a dissociation constant of acetate at the catalytic metal site of 200 ± 50 mM was calculated and from the changes of the ^{13}C-NMR linewidth of ^{13}C acetate direct bonding of the anion to the catalytic Co(II) ion can be demonstrated to occur under the conditions of rapid exchange. The implications of these data for the assessment of tetracoordination around the catalytic metal ion as well as the chemical nature of intermediates occurring along the catalytic pathway are discussed.

Key words: ^{1}H-NMR, ^{13}C-NMR, electronic spectra; Co(II)-substituted horse liver alcohol dehydrogenase; acetate, imidazole, pyrazole binding

Introduction

Alcohol dehydrogenase from horse liver (HLADH, EC 1.1.1.1) is a homodimeric enzyme with a molecular weight of 80,000. It contains two zinc ions in each subunit located in different sites and different chemical environments. One of these zinc ions is bound tetrahedrally to cysteines 97, 100, 103, 111 and has a structure-stabilizing function, whereas the other is bound in a distorted tetrahedral fashion to cysteines 46 and 174, to histidine 67 and a water molecule in the catalytic site (Brändén et al. 1975, Eklund et al. 1976).

It is now generally believed that the catalytic metal ion acts as a Lewis acid towards the substrate which replaces the water molecule and is activated in the metal-ligated state. There is still discussion about the ionization state of the bound substrate, i.e. whether the alcohol binds as a neutral molecule or whether it is deprotonated and bound as an alkoxide anion. In addition, it has recently become obvious

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that the catalytic metal ion indirectly controls the dynamics of coenzyme binding (Zeppezauer et al. 1984). This step precedes the binding of substrate and is an important step in the catalytic cycle because binding of coenzyme is a prerequisite for the transition from the open to the closed protein conformation.

It has been observed that chemical modification of the metal ligand cysteine 46 or substitution of the metal-bound water by imidazole prevents the transition from the open to the closed protein conformation upon binding of NADH (Cedergren-Zeppezauer 1983, 1986). On the other hand, removal of the catalytic zinc ion does not prevent the conformation change upon binding of NADH (Schneider et al. 1983a). The coenzyme molecule is not a ligand of the catalytic metal ion; rather, the nicotinamide moiety is in Van der Waals contact with the side chains liganding the metal ion. Removal of the catalytic zinc ion or its substitution by other metal ions dramatically influences the rate parameters of coenzyme binding, especially the dissociation rate constant for NADH which limits the turnover rate of the enzyme (Zeppezauer et al. 1984). This shows that the catalytic metal is essential for the dynamics of coenzyme binding, in addition to its function in binding and activating the substrate. These results illustrate the necessity to further characterize the influence of metal ligands such as imidazole on the interaction of protein and coenzyme as well as the interaction with the catalytic metal ion of substrate-analog and/or -competitive molecules e.g. acetate or other fatty acid anions. ^{1}H-NMR studies of cobalt(II)-substituted HLADH have proven particularly useful since they allow the observation of the paramagnetically shifted protons of the metal ligands and their changes upon coenzyme binding (Bertini et al. 1984).

Here, we report on the examination by ^{1}H-NMR and optical spectroscopy of the interaction of the catalytic Co(II) ion with imidazole and pyrazole in the absence and presence of coenzyme. In addition, ^{13}C-NMR data are presented for the binding of acetate which provide evidence for the direct coordination to the catalytic metal ion of this substrate-analog molecule.

Materials and methods

Horse liver alcohol dehydrogenase, NAD^+ and NADH (grade I) were purchased from Boehringer, Mannheim, FRG. Imidazole from Fluka, Buchs, Switzerland, was recrystallized four times from water. ^{13}C-enriched acetate was purchased from Prochem B.O.C., UK, pyrazole from Merck, Darmstadt, FRG. All other reagents were of analytical grade.

Co(II)-LADH was prepared by the method of Maret et al. (1979). Co(II)-LADH was dissolved in 33 m*M* TES-buffer at pH 7.5 containing 0.3 *M* KCl. The final concentration of Co(II) in the samples was between 1 and 1.5 m*M* as determined by measuring the optical absorption at $28.6\times10^3\,cm^{-1}$. The coenzymes and acetate were added as solid powders to the solutions of enzyme. Aliquots of stock solutions of imidazole and pyrazole were added to the enzyme solutions. In no case was the dilution more than 10%. The pH values of the solutions in D_2O are reported as uncorrected readings from the pH-meter.

The ^{1}H-NMR spectra of the enzyme samples in H_2O or in D_2O were run at 5 °C on a 60 MHz instrument based on a Bruker CXP 100 console, equipped with a 1.41 T Varian DA 60 electromagnet and an external lock circuit giving 1 Hz long term stability. All spectra were recorded in quadrature detection mode using a 100 kHz spectral width. The strong signals of the solvent protons and of all the diamagnetic protons were suppressed using the modified DEFT pulse sequence ($90^\circ_x-\tau-180^\circ_x-\tau-90^\circ_x$-acquisition), which was further phase alternated according to the standard Bruker PAPS sequence to reduce the build up of coherent noise. The 90°_x and the 180°_x pulse lengths were adjusted by monitoring the water signal of each sample. Typical 90°_x pulse lengths were around 2 µs; typical τ-values and recycle time values were 30 and 45 ms respectively. Spectra were accumulated for $(1-4)\times10^5$ scans, which, owing to the fast recycle time employed, required approximately from 3 to 15 h. Weighting factors of 20–100 Hz were employed. The chemical shifts were measured from the H_2O or residual HDO signals and have been reported relative to TMS, assuming a 4.8 ppm downfield shift for H_2O. The shift value readings were accurate to 0.4 Hz (i.e. the digital resolution of the spectra), but the uncertainty is larger for the broadest signals.

The ^{13}C-NMR spectra were recorded on the Co(II)-LADH-samples in D_2O (for internal lock) after addition of 90% ^{13}C-enriched acetate ($CH_3{}^{13}COONa$). The measurements were done at 5 °C on a 1.87 T Varian FT 80 A instrument at 20 MHz and a spectral width of 5×10^3 Hz. The spectra were obtained using a 90°_x pulse and $(1-2)\times10^3$ scans. No weighting factor was employed. The paramagnetic contribution to the linewidth was calculated by subtracting the linewidth of a 10^{-2} *M* 90% ^{13}C-enriched acetate solution at the same temperature.

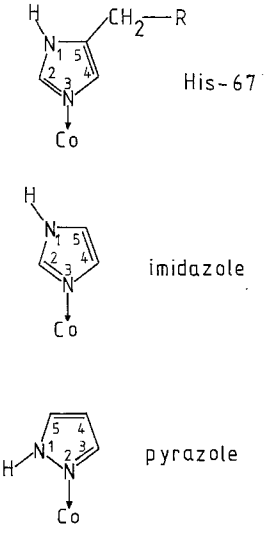
The electronic spectra of the samples were recorded at room temperature on a Cary 17 D spectrophotometer. The electronic absorption spectra of the NMR samples were routinely monitored before and after the NMR experiments.

Results

The imidazole-enyzme system

The ^{1}H-NMR spectrum of Co(II)-LADH in H_2O in the presence of 0.3 *M* KCl and 0.05 *M* imidazole is reported in Fig. 1 B together with the spectrum of free Co(II)-LADH (Fig. 1 A) previously reported (Bertini et al. 1984).

The spectrum of the imidazole derivative (Fig. 1 B) consists of four resonances beyond 150 ppm downfield from TMS which are assigned to the β-CH_2-protons of the metal-liganded cysteine residues. This is in analogy with the assignment of the signals of free Co(II)-LADH (Bertini et al. 1984). Closer to the diamagnetic region two sharper signals are observed at 68 ppm and 42 ppm. The former signal is assigned to the NH of the metal-coordinated *His*-67. The latter signal, which is absent in the spectrum of free Co(II)-LADH is due to the proton in the 5-position of coordinated imidazole (Scheme 1). The observation of this signal indicates that the inhibitor exchange is slow on the NMR time scale. An upper limit for this exchange rate can be set at about 10^3 s^{-1}. Underneath the signal at 68 ppm there is one broad resonance, which is clearly visible when the spectrum is recorded in D_2O because the sharp signal of *His*-67 disappears (inset). Another broad signal is recorded at 105 ppm. These two resonances at 68 and 105 ppm in D_2O are attributed to the protons in the 2- and 4-position of *His*-67 or imidazole (Scheme 1). The spectrum in Fig. 1 B shows also



Scheme 1

a signal at 53 ppm, with intensity smaller than unity, which disappears in D_2O. It is tentatively assigned to the NH of the metal-liganded imidazole. Its low intensity might be due to a partial saturation induced by the proton exchange with the bulk water,

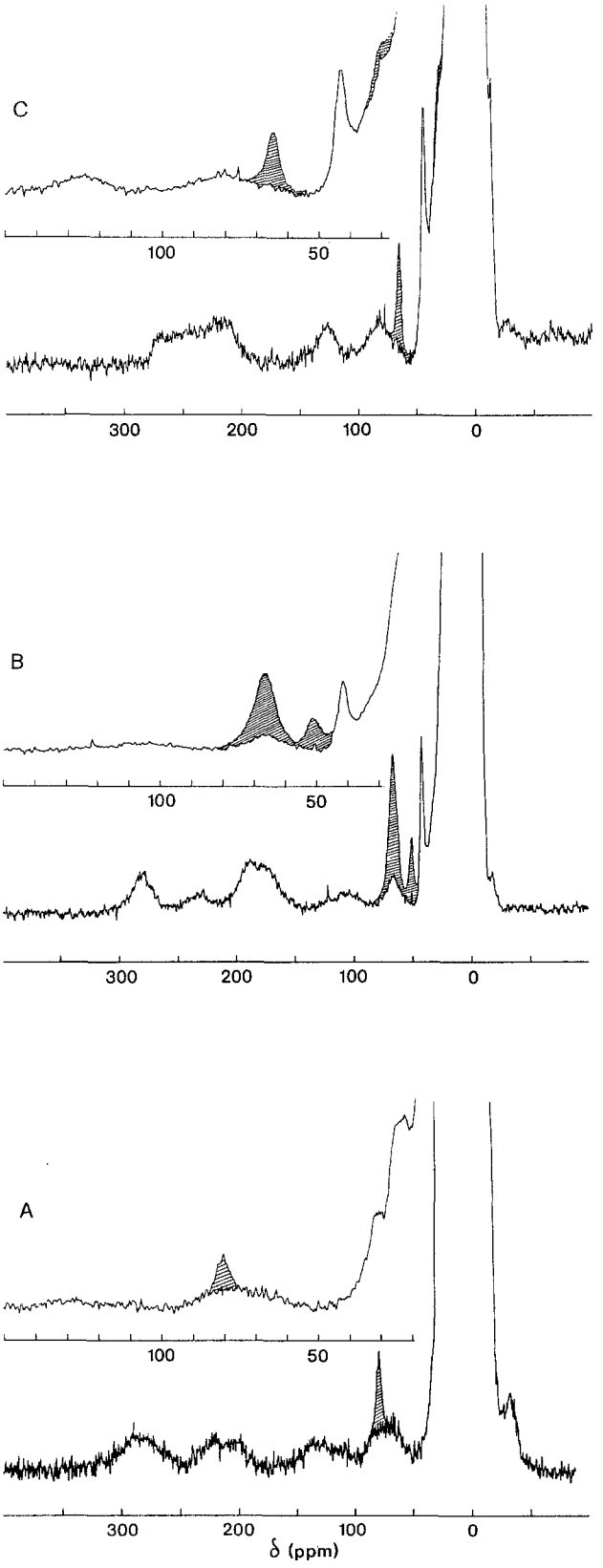


Fig. 1 A–C. 60 MHz ^{1}H-NMR spectra at 5 °C of solutions of Co(II)-LADH (1 m*M*) (**A**), of its binary complex with imidazole (64 m*M*) (**B**) and of its ternary complex with imidazole and NADH (20 m*M*) (**C**), in *TES* buffer (33 m*M*, pH 7.5). The dashed signals disappear in D_2O

which on its turn is heavily saturated under the present conditions.

In the spectrum of the ternary complex Co(II)-LADH · NADH · Imidazole in H_2O (Fig. 1 C) signals are observed at 65 ppm from the NH of *His*-67 and at 43 ppm from the proton in the 5-position of imidazole in analogy to the binary complex (Scheme 1). There is no clear evidence of the imidazole NH signal which is observed at 53 ppm in the binary complex. Slight differences in the spectra of the ternary complexes at about 32 ppm in H_2O, as compared to D_2O, cannot be definitely interpreted. At variance with the spectra of free Co-LADH and of its binary complex with imidazole, as well as with both binary and ternary complexes with pyrazole (see below), the Co(II)-LADH · NADH · Imidazole systems shows a closer grouping of the four resonances belonging to the β-CH_2 protons of the coordinated cysteines.

A ^{1}H-NMR spectrum has also been recorded in the presence of imidazole and NAD^+ at pH 7.5 in H_2O (not shown). The dissociation constant of the inhibitor from the ternary complex Zn_4-LADH · NAD^+ · Imidazole at pH 8 was found to be about 5.9 m*M* (Theorell and McKinley-McKee 1961), so that under these conditions the ternary complex is fully formed. However the spectrum is identical to that of the binary complex of Co(II)-LADH · Imidazole, indicating that the oxidized coenzyme does not affect the metal environment in this derivative.

The electronic absorption spectra of the free enzyme (Maret et al. 1979) as well as those of the binary and ternary complexes are shown in Fig. 2. The influence of the inhibitor can be seen as a redshift of the bands at $28.6 \times 10^3\,cm^{-1}$ ($\varepsilon = 7{,}000\ M^{-1}\,cm^{-1}$) in free enzyme to $27.6 \times 10^3\,cm^{-1}$ in the binary complex. Furthermore, absorption maxima at $18.5 \times 10^3\,cm^{-1}$ and $14.9 \times 10^3\,cm^{-1}$ and a shoulder at $15.7 \times 10^3\,cm^{-1}$ were recorded in the binary complex. A further redshift was observed upon addition of NADH.

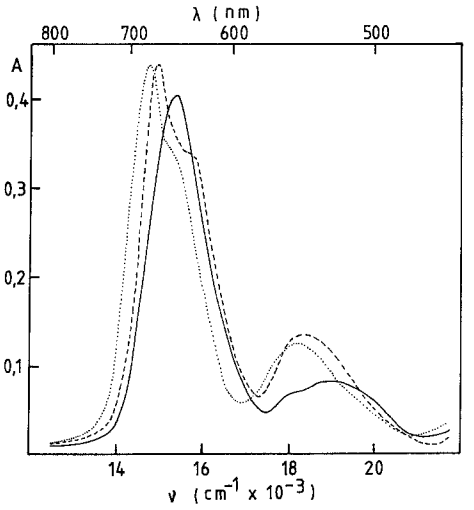


Fig. 2. Electronic absorption spectra at room temperature of Co(II)-LADH (375 μ*M*) (*solid line*), of its binary complex with imidazole (16.4 m*M*) (*dashed line*) and of its ternary complex with imidazole and NADH (1.8 m*M*) (*dotted line*)

The pyrazole-enzyme system

The ^{1}H-NMR spectrum of the binary complex Co(II)-LADH · Pyrazole in H_2O shows the usual signals beyond 150 ppm assigned to the β-CH_2 protons of the metal-coordinated cysteines (Fig. 3 A). Furthermore, there are signals at about 110 ppm and 65 ppm, the last one underneath the sharp NH signal of *His*-67 (69 ppm). An intense signal at 32 ppm could be consistent with the two protons in the 3- and 4-position of pyrazole (Scheme 1). In contrast to the binary complex with imidazole, the pyrazole NH signal is not observed, either because it is too

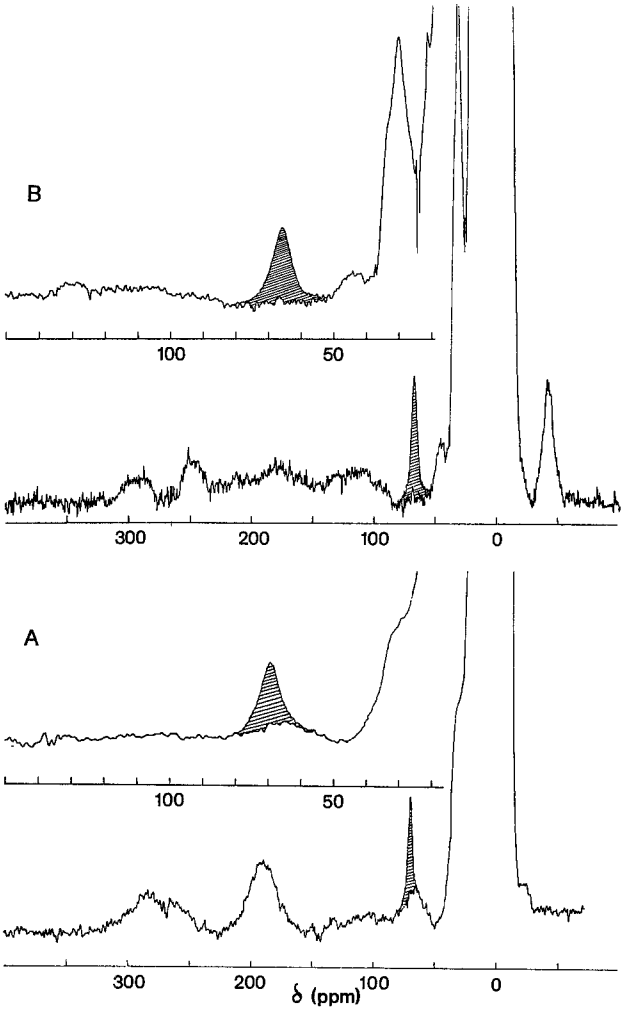


Fig. 3 A and B. 60 MHz ^{1}H-NMR spectra at 5 °C of solutions of the binary complex of Co(II)-LADH (1 m*M*) with pyrazole (**A**) and of the ternary complex with pyrazole and NAD^+ (20 m*M*) (**B**). The dashed signals disappear in D_2O

broad or saturated or absent. The spectrum in D_2O provides evidence for the disappearance of the signal at 69 ppm assigned to the NH signal of *His*-67.

In the presence of NAD^+ (Fig. 3B) there is a change in the shape of the resonance beyond 150 ppm, compared to the spectrum of the binary complex with pyrazole or the imidazole complexes. Also in accordance with the binary complex, the NH-signal of *His*-67 (66 ppm), as well as that of the protons in the 3- and 4-positions of pyrazole (32 ppm), remain almost at the same position (Scheme 1).

The electronic spectra of the binary and ternary complexes are shown in Fig. 4. The binary complex has absorption maxima at $26.8 \times 10^3\,cm^{-1}$, $18.9 \times 10^3\,cm^{-1}$ and $14.9 \times 10^3\,cm^{-1}$ and a shoulder at $15.5 \times 10^3\,cm^{-1}$. In the ternary complex there is a red shift and a splitting of the band at $28.6 \times 10^3\,cm^{-1}$ in free Co(II)-LADH to $25.7 \times 10^3\,cm^{-1}$, as well as a shoulder at $24.1 \times 10^3\,cm^{-1}$.

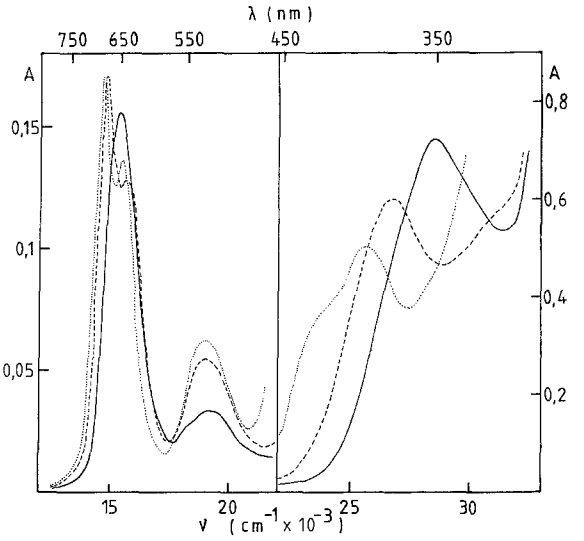


Fig. 4. Electronic absorption spectra at room temperature of Co-LADH (95 μ*M*) (*solid line*), of its binary complex with pyrazole (14 m*M*) (*dashed line*) and of its ternary complex with pyrazole and NAD^+ (1.8 m*M*) (*dotted line*)

The enzyme-acetate system

The interaction of acetate with Co(II)-LADH has been studied by ^{13}C-NMR spectroscopy using carbon 13-enriched acetate and electronic absorption spectroscopy at pH 7.5.

The electronic spectra of the free enzyme show a shift in the band at $19.2 \times 10^3\,cm^{-1}$ to $17.9 \times 10^3\,cm^{-1}$, with a shoulder at $18.6 \times 10^3\,cm^{-1}$ in the binary complex with acetate. The band at $15.4 \times 10^3\,cm^{-1}$ remains unchanged (Fig. 5).

The ^{13}C-NMR spectra show measurable linewidth enhancements (Fig. 5 (inset)). It is apparent that the linewidth decreases outside the experimental error with increasing acetate concentration. From the electronic spectra a dissociation constant of about 200 ± 50 m*M* has been estimated. This dissociation constant is consistent with the observed pattern of linewidth enhancements, as shown by the dashed line through the experimental points in Fig. 5 (inset), which was calculated with the value for the dissociation constant of acetate as taken from the electronic absorption spectra (Fig. 5 inset, solid line). Therefore, one can see that acetate exchanges rapidly on the NMR time scale and that the linewidth is due to the coupling of the ^{13}C nucleus with the unpaired electrons of Co(II). The limiting enhancement of the linewidth would be about 900 Hz, which is consistent with carboxylate coordination to high spin Co(II). In fact, assuming only a dipolar interaction between the unpaired electrons and the ^{13}C nucleus and estimating the electronic relaxation time for Co(II) to be about 2×10^{-11} s from previous ^1H-NMR

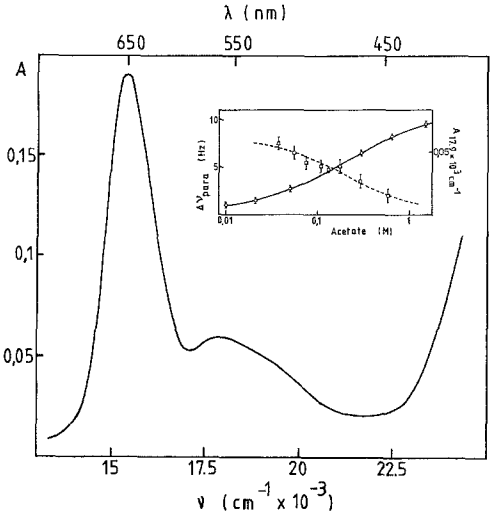


Fig. 5. Electronic absorption spectrum of Co(II)-LADH (200 μ*M*) in the presence of 1.5 *M* acetate ions. The *inset* shows the variation of the absorbance at $17.9 \times 10^3\,cm^{-1}$ (*solid line*) and the paramagnetic contribution to the 20 MHz ^{13}C-NMR linewidth in Co(II)-LADH solutions, 1.0×10^{-3} *M* in cobalt(II) (*dashed line*) as a function of the acetate concentration. The best fit curves are obtained with dissociation constants of 222 m*M* and 208 m*M*, respectively

data (Bertini et al. 1984) a metal-nucleus distance Co–^{13}C of about 2.4 Å is calculated. Such a distance is shorter than expected for the usual bond length. This indicates that ligand-centered dipolar and/or contact contributions are also operative. If the latter contributions are operative a covalent bond between the metal ion and acetate is present.

The need for large concentrations of acetate to observe the signal does not allow one to study acetate

binding to the general anion binding site, which is expected to occur with a higher affinity. The quality of the experimental data does not allow us to detect contributions to the line broadening from the latter binding site, which in any case are expected to be rather small.

Discussion

The imidazole-enzyme system

The NH proton of *His*-67 of free Co(II)-LADH has a chemical shift of 68 ppm downfield from TMS (Bertini et al. 1984). The present investigation has shown that the NH signal of *His*-67 remains in the same position both in the binary complex of Co(II)-LADH with imidazole and in the ternary complex with NADH. The signals of the protons in the 2- and 4-position of *His*-67 appear in the same region between 50 and 110 ppm in the binary and ternary complexes, as observed in free Co(II)-LADH. This is also true for the signals of the protons in the 5-position of imidazole. Only the cysteine β-CH_2 signals, the shifts of which are sensitive to the angles between the H−C−S and the C−S−Co planes, indicate some flexibility.

This implies that the arrangement of the donor groups around the metal ion remains essentially the same in spite of the substitution of the coordinated water molecule by the inhibitor.

These results are in agreement with the X-ray structures of the ternary complex of the native enzyme with imidazole and NADH. In this complex a movement of the whole metal binding site, including the bound inhibitor, toward the substrate binding pocket is observed (Cedergren-Zeppezauer 1983). The position of *His*-67 does not vary with respect to the metal ion. Only a slight movement of this residue in free Co(II)-LADH relative to the position in the zinc enzyme is observed (Schneider et al. 1983b). It is worth mentioning that the ternary complex LADH · NADH · Imidazole is the only species in which a direct bonding interaction occurs between the coenzyme molecule and the ligand sphere of the catalytic metal ion, i.e. the hydrogen bond between the sulphur atom of *Cys*-46 and the carboxamide group of NADH. This interaction seems to be responsible for the shift of the positions of the β-CH_2 protons relative to those of the free enzyme or other binary and ternary complexes.

The NH signal of imidazole, which is tentatively assigned in the binary complex as the one at 53 ppm, is not observed in the ternary complex. This is possibly due to saturation of this signal or to a movement to the diamagnetic region, where it cannot be detected. Unfortunately, no crystal structure analysis of HLADH with bound NAD^+ has been reported so far. Our data demonstrating that NAD^+ does not affect the ^{1}H-NMR spectrum of bound imidazole lead us to suggest that the nicotinamide part of NAD^+ interacts quite differently with the catalytic site as compared to NADH. It is conceivable that the imidazole prevents the hydrated nicotinium moiety from being accommodated in the catalytic site and that NAD^+ adopts a totally unproductive conformation, analogous to that of pyridine adenine dinucleotide in its complex with HLADH and imidazole (Samama et al. 1977).

The pyrazole-enzyme system

From a recent investigation of the pH dependence of pyrazole binding to native LADH, Andersson et al. (1981) concluded that pyrazole exhibits a pK_a value above 10 in the binary complex. At pH 7.5 which was employed in our experiments, a NH signal of pyrazole should have been observed. Nevertheless, we could not observe a NH signal of any complex of pyrazole with Co(II)-LADH, possibly due to a very fast exchange of this proton in the binary complex. The ternary complex with NAD^+ exists in a closed conformation with an A side orientation of the nicotinamide ring (Eklund et al. 1982). This conformation allows a covalent interaction of one of the nitrogens of pyrazole with the C4 atom of the nicotinamide ring (Becker and Roberts 1984; Eklund et al. 1982). This is, of course, possible only after dissociation of the proton from the NH group. Such a tight interaction of coenzyme and inhibitor is not possible in the case of the system enzyme-imidazole-NADH. The latter complex exists in the open conformation with a B side orientation of the nicotinamide ring (Cedergren-Zeppezauer 1983).

As far as the external ligands are concerned, the observation of isotropic shifts of the protons in the 5-position of imidazole and the 3- and 4-positions of pyrazole confirms direct metal-to-nitrogen bonding (Boiwe and Brändén 1977; Eklund et al. 1982). Their position relative to the metal ion is not very sensitive to the presence of coenzyme.

The acetate-enzyme system

In liver alcohol dehydrogenase, the binding of anions occurs at several sites. For the binding of the coenzyme molecule it is important that the pyrophosphate moiety interacts with arginine 47. This anion binding site is large enough to accommodate different anions of considerable size such as acetate,

$Au(CN)_2^-$, and $Pt(CN)_4^{2-}$. A second anion binding site within the coenzyme binding region is found in the adenine binding pocket and contains arginine 271 as a center of positive charge. Both sites have been probed by ^{35}Cl-NMR quadrupole relaxation and shown to interact with larger anions; they have therefore been termed general anion binding sites (Andersson et al. 1979a). The interaction of acetate with the pyrophosphate binding site has been probed by kinetic methods and has been characterized by dissociation constants between 51 m*M* and 84 m*M* (Reynolds and McKinley-McKee 1969; Dahl and McKinley-McKee 1977; Oldén and Pettersson 1982). It is difficult to detect the simultaneous binding of acetate to the catalytic zinc ion in the experimental set-ups cited if the dissociation constant at the metallic site is higher than at the so called general anion binding site. The cobalt(II)-substituted enzyme offers the advantage that anion binding in the immediate vicinity of the catalytic metal ion is accompanied by the creation of an absorption band around 17.5×10^3 cm^{-1} irrespective of the chemical nature of the anion (Maret et al. 1979; Maret and Zeppezauer 1986). Here we have demonstrated the binding of acetate to the catalytic Co(II) ion in the absence of coenzyme and determined a dissociation constant of 200 m*M*. In the presence of NAD^+, the binding of 2,2,2-trifluoroethanol (Andersson et al. 1979b; Dietrich and Zeppezauer (1982), acetate (Gerber et al. 1983; Maret 1980), and chloride (Maret and Zeppezauer 1986) has been reported to create similar spectral features. The spectra of the binary complex Co(II)-LADH·NAD^+ show different features depending on pH. At high pH this binary complex also exhibits the absorption band at 17.4×10^3 cm^{-1} indicating the creation of a charged group close to the catalytic metal ion. Based on these observations, it has been suggested that 2,2,2-trifluoroethanol ionizes in the ternary complex with NAD^+ forming a metal-bound alkoxide anion (Dietrich and Zeppezauer 1982; Gerber et al. 1983) and that in the alkaline form of the binary complex Co-HLADH/NAD^+ the metal-bound water molecule possibly forms a metal-bound hydroxide ion. It is therefore of major importance to reveal the origin of this anion-linked spectral transition. In this respect the ^{13}C-NMR data reported here represented a key experiment since they unequivocally prove the direct coordination of acetate to the catalytic Co(II) ion. This implies that the creation of the transition at 17.4×10^3 cm^{-1} cannot be a spectrochemical effect due to the binding of the anion itself, since the exchange of water by hydroxide or alkoxide is not expected to give rise to such spectral changes. Rather, a local conformational change due to charge compensation upon introduction of an anionic ligand is the probable cause of the observed spectral alterations.

The coordination number of the catalytic metal ion in the complexes with inhibitors and anions

Both the ^1H-NMR relaxation rates of isotropically shifted ligand protons (Bertini et al. 1984) and the intensities of the electronic spectral transitions can be taken as parameters assessing the coordination number of Co(II) ions in high spin Co(II) complexes (Bertini and Luchinat 1984). Indeed, four-coordinated complexes are found to have larger intensity than five-coordinated complexes (Rosenberg et al. 1973). If the intensity of the electronic spectra, as well as the shifts and linewidths, remain the same upon ligand binding, it means that the coordination number remains the same. Furthermore, five-coordinated cobalt(II) complexes are expected to have strong absorptions above 20,000 cm^{-1} (Morassi et al. 1973). It appears from the above data that the binding of imidazole, pyrazole, or acetate does not change the coordination number of the catalytic Co(II) ion in Co(II)-HLADH. The same holds true for the complexes Co(II)-LADH·NAD^+·acetate and Co(II)-LADH·NAD^+ at low and high pH (Maret and Zeppezauer 1986). For the binary complex with NAD^+ a penta-coordinate Co(II) ion has been inferred from zero field splitting data at liquid helium temperature (Makinen and Yim 1981). Since no significant losses of intensity are observed in the electronic spectra of all complexes discussed so far and the proton relaxation rates of the metal ligand protons are virtually unchanged both in the binary complexes and the ternary complexes containing NAD^+ we believe that the coordination number is four in all of the complexes studied so far at room temperature.

Mechanistic implications

The question as to the coordination number of the catalytic metal ion and the ionization state of the metal-bound water or alcohol molecules are intimately linked with each other. The earliest mechanism proposed by Theorell and Chance (1951) which was extended by Brändén et al. (1975) and Pettersson (1986) restricts the catalytic events to four-coordinated intermediates. In this mechanism, the metal-bound water molecule is replaced by the substrate. In the case of alcohols, the substrate ionizes under the influence of enzyme-bound NAD^+; the metal-bound alkoxide is regarded as the molecular species

best suited for hydride transfer between alcohol and NAD^+. The mechanisms proposed by Dworschak and Plapp (1977) and Makinen et al. (1981, 1983) imply five-coordinated states for the catalytic metal ion. In the latter mechanism it is proposed that both water and alcohol coordinate directly to the metal and that neutral, metal-bound water is the base accepting the proton which is liberated in the course of the ternary complex conversion. It is therefore important to note that metal-bound anionic species can be detected by NMR and optical spectroscopy as demonstrated here. Of particular relevance is the observation that transient species detected in the reaction between alcohols and NAD^+ as well as aldehydes and NADH with Co-HLADH by means of rapid scan stopped flow spectroscopy, show the absorption band at $17.6 \times 10^3\ cm^{-1}$ characteristic of metal-bound anions (Gerber et al. 1983; Sartorius et al. 1985; Zeppezauer 1986; Sartorius et al. 1987). It is difficult to avoid the conclusion that the species characterized by the absorption bands at 17.6×10^3, 15.6×10^3 and $14.7 \times 10^3\ cm^{-1}$ represents the ternary complex Co(II)-LADH · NAD^+ · alkoxide. Based on the data presented here even these short-lived, ternary complexes contain the alkoxide in direct coordination with the catalytic metal ion. The latter retains the coordination number four in all steps which hitherto have been amenable to direct observation.

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