Horse Liver Alcohol Dehydrogenase Derivatives Containing Nickel(II) and Cobalt(II) in the Noncatalytic Metal Binding Site

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Abstract

The noncatalytic zinc in horse liver alcohol dehydrogenase was selectively replaced by nickel(II). This novel species, $Zn(c)_2Ni(n)_2$[§] horse liver alcohol dehydrogenase (where c denotes the catalytic and n denotes the noncatalytic site) was compared to $Zn(c)_2Co(n)_2$ horse liver alcohol dehydrogenase with respect to its absorption, circular dichroism and magnetic circular dichroism spectra, as well as its magnetic moment. For $Zn(c)_2Co(n)_2$ horse liver alcohol dehydrogenase (prepared according to refs. 1 and 2) the extinction coefficients were redetermined in the UV, visible and near-infrared region and the molar ellipticities in the range 300–800 nm. The average magnetic moment was determined by the NMR method as 4.5–5.0 B.M. The results confirm a tetrahedral structure in the zinc–cobalt enzyme. In contrast, the spectroscopic data and the zero magnetic moment support a planar geometry for the nickel(II) bound in the noncatalytic site. $Zn(c)_2Ni(n)_2$ horse liver alcohol dehydrogenase is very temperature-sensitive and precipitates after short exposure to room temperature. Stored in the cold it has the same activity as the native enzyme. The results indicate that the protein is flexible in the loop region binding the noncatalytic metal ion and that it may retain catalytic activity even in a partially distorted conformation.

Introduction

Native horse liver alcohol dehydrogenase, HLADH, a dimeric enzyme, contains several metal binding sites:

(1) the catalytic center (c), where three protein ligands (two sulfurs of Cys-46, -176, nitrogen of His-67) and a water molecule are bound to zinc in distorted tetrahedral geometry [3];

(2) the noncatalytic center (n), where zinc is bound to four sulfurs of Cys-97, -100, -103 and -111 in a tetrahedral arrangement [3];

(3) transient and/or peripheral sites, for which the structure and metal binding groups are not known. Two types of such sites for Mn(II) were found by ^{1}H NMRD, one with stronger binding, the other one with weaker binding [4].

Much work has been devoted to the catalytic center of the EE isozyme concerning the kinetics of the catalytic process [5, 6], spectroscopic properties of different metal-substituted derivatives [e.g. 7–10], as well as the different roles of the metal ion in this center. There are some indications that the catalytic metal ion, despite its main catalytic role, can additionally stabilize the structure of the protein [11–13].

Considerably less attention has been paid to the noncatalytic metal center. The four thiol groups which bind the Zn(n) all derive from closely spaced amino acid residues. This loop of the polypeptide chain is in contact with the second subunit, which has been taken as an indication of a possible structural function of this metal. The distance between the catalytic and noncatalytic zinc ions is about 21 Å [14], which is too large for any direct interaction between them. However, it does not exclude the influence of the noncatalytic metal on the catalytic site mediated through the protein structure.

It has been possible to remove all zinc ions from HLADH [15, 16], but the enzyme then exists in a considerably less ordered structure, from which it is

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§Abbreviations: HLADH, horse liver alcohol dehydrogenase (EC 1.1.1.1); DACA, *trans*-4-(*N*,*N*-dimethylamino)-cinnamaldehyde; TRIS, tris-(hydroxymethyl)aminomethane; TES, *N*-tris-(hydroxymethyl)methyl-2-aminoethane; EDTA, ethylenediaminetetraacetic acid. The metal ions are described as catalytic (c) or noncatalytic (n) after the chemical symbol, *i.e.* $Zn(c)_2Co(n)_2$HLADH is the enzyme substituted by cobalt in the noncatalytic site; LMCT, ligand–metal charge transfer; NMRD, nuclear magnetic relaxation dispersion.

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difficult to refold and reactivate it to the native form. The catalytic zinc ion of HLADH can be removed in the crystalline state, and other metals, observable spectroscopically, can then be inserted [e.g. 7–9, 12]. In contrast, the noncatalytic zinc could only be replaced by exchange against another metal applied in excess. In this way two derivatives with cobalt(II) [2, 12] and cadmium [17] in the noncatalytic site have been obtained earlier.

In the present paper we report on the preparation and detailed spectroscopic studies of $Zn(c)_2Ni(n)_2$-HLADH and its comparison with $Zn(c)_2Co(n)_2$-HLADH. Nickel ion was chosen as a substitute for the noncatalytic zinc due to its similar chemical properties: *i.e.* similar ionic radius, high potential affinity for cysteinate sulfur, poor catalytic properties for the oxidation of sulfhydryl groups, and the ability to form tetrahedral complexes. Since Ni(II) adopts tetrahedral geometry in the catalytic site [9], it was of great interest to learn about the coordination chemistry of Ni(II) in the noncatalytic site and its influence on the properties of the enzyme.

Experimental

Horse liver alcohol dehydrogenase, EE isozyme (HLADH, EC 1.1.1.1) was obtained from Boehringer, Mannheim, and further purified by recrystallization from 50 mM TRIS buffer, pH 8, containing 15% (*v/v*) tert-butyl alcohol. β-NAD (grade I) was purchased from Boehringer, Mannheim. *trans*-4-(*N,N*-Dimethylamino)cinnamaldehyde (DACA) was obtained from EGA Chemie, Steinheim and tris-(hydroxymethyl)aminomethane (TRIS) buffer from Fluka, Buchs. All other reagents were of the purest grade available from Merck, Darmstadt.

Double distilled water was used throughout. Glassware was cleaned with conc. HNO_3 and plastic material with 1 M Na_2CO_3 containing 10^{-3} M EDTA to avoid metal contamination. Dialysis tubings (Visking 8/32, Serva, Heidelberg) were washed in a series of solutions (1% acetic acid; double distilled water; 1% Na_2CO_3 with 10^{-3} M EDTA three times, twice at 75 °C for 1 h; four times in double distilled water) and stored in the refrigerator in double distilled water with 0.02% NaN_3. Metal exchange experiments were performed in Schlenk tubes under nitrogen (99.99%). pH adjustments were carried out with suprapure NaOH.

The enzyme activity was determined by measuring the rate of NADH formation ($E_{340} = 6.22$ mM^{-1} cm^{-1}) at 23 °C in 0.1 glycine/HCl buffer, pH 10.0 ($c_{NAD^+} = 0.3$ mg/ml, 0.03% EtOH, $c_{enz} = 1–5$ μg/ml).

The protein concentration was determined spectrophotometrically. The absorption coefficients, A_{280}, were obtained by applying the method of Lowry *et al.* [18], and using native zinc enzyme as a standard with the earlier established value $A_{280} = 0.455$ mg^{-1} cm^2 [19].

The metal analyses were obtained by atomic absorption measured on a Perkin-Elmer 400 atomic absorption spectrophotometer with an HGA 76 graphite furnace. Each measurement was performed three times with three different concentrations in 0.01 M HCl matrix, freshly prepared before the measurement. In this matrix no protein precipitation was observed and a minimal number of pipetting steps was needed, thus decreasing the experimental errors.

All absorption and circular dichroism (CD) spectra were measured at 4–8 °C. The absorption spectra were measured on a Perkin-Elmer Lambda 9 (UV/Vis/NIR) spectrophotometer. Molecular extinction coefficients, ε, are based on the Co(II) or Ni(II) concentration in the enzyme dimer, with units M^{-1} cm^{-1}. Circular dichroism spectra were recorded on a JASCO-J-20 spectropolarimeter. The CD results are expressed in terms of the molar ellipticity, $[\theta]$, with deg cm^2/dmol units. The magnetic circular dichroism (MCD) spectra were obtained on Cary 61 spectropolarimeter with a Varian superconducting solenoid. A magnetic field of 40 kG was applied. The $[\theta]$ values are calculated in units deg cm^2/dmol, normalized to 1 G. The magnetic moment was measured in solution by the NMR method described earlier by Evans [20]. The instrument used was a Bruker AM 400. The following equation was applied for calculation of the molar paramagnetic susceptibility [21]:

$$\chi_M^P = -\frac{3}{4\pi}\frac{\Delta\nu}{\nu}\frac{1000}{c} + \chi_o M - \chi_D \qquad (1)$$

where $\Delta\nu$ is the frequency separation of the marker in Hz, ν is the frequency of the proton resonance spectrometer (400 MHz), χ_o is the mass susceptibility of ^2H_2O, M is the molecular weight of HLADH, χ_D is the diamagnetic susceptibility of HLADH and c is the molarity of the solution. The effective magnetic moment was calculated from eqn. (2):

$$\mu_{eff} = 2.84(\chi_M^P T)^{1/2} \qquad (2)$$

where T is the absolute temperature.

The enzyme samples were lyophilized twice from TRIS buffer in D_2O. The tert-butyl alcohol and HDO signals were used as markers. The measurements were performed at 8 °C.

Results

The 'Green Hybrid', $Zn(c)_2Co(n)_2$ HLADH

Vallee and Sytkowski [1, 2] were the first to obtain HLADH substituted with Co(II) at the non-

catalytic site. The best conditions found by these authors for the exchange of the noncatalytic zinc against Co(II) ions was dialysis over 12 h against 0.2 M $CoCl_2$ in 0.1 M acetate buffer at pH 6.5 or 5.9. Using the same conditions (0.2 M $CoCl_2$ in 0.1 M NaAc buffer, pH 5.9) we have found that the yield of the metal substitution is very much dependent on the quality of the native enzyme. However, the highest range of metal substitution was found when the dialysis with excess cobalt was prolonged to 24 h and when the outer solution was changed two or three times. The best species obtained in this way contains 0.9–1.0 mole Co(II) per subunit, found by atomic absorption. Additional proof that the Co(II) ion is substituted in the noncatalytic site and that Zn(II) remains in the catalytic site is provided by the ternary complex with DACA and NADH. It was found earlier that DACA bound to the catalytic zinc exhibits an absorption band at 464 nm [22], while DACA binding to catalytic cobalt has this band shifted to 478 nm [23, 24]. Free DACA in aqueous solution absorbs at 398 nm. Using these characteristics it was confirmed that in the obtained 'green hybrid' the Zn(II) ions are in the catalytic site. Therefore it is not surprising that the enzymic turnover of this species is the same as for the native enzyme, *i.e.* 3.6–4.5 s^{-1}. For $Zn(c)_2Co(n)_2$HLADH with a stoichiometric degree of Co(II) substitution, the spectroscopic intensity coefficient at 280 nm was obtained as: $A_{280} = 0.56$ mg^{-1} cm^2. This differs considerably from the reported value of $A_{280} = 0.46$ mg^{-1} cm^2 [1]. Based on the new absorption coefficient at 280 nm, the molar extinction coefficients and the molar ellipticities were redetermined for the visible and near-infrared region. In the spectra reported previously [2, 12] the near-infrared region was missing.

The absorption spectra of the 'green hybrid' (Table I, Fig. 1) are typical of tetrahedral Co(II) (visible and near-infrared bands) bound to sulfur atoms (intensive LMCT band at 340 nm). The high

TABLE I. Spectroscopic Data for $Zn(c)_2Co(n)_2$HLADH[a]

Absorption		CD		MCD	
λ (nm)	ϵ (M^{-1} cm^{-1})	λ (nm)	$[\theta] \times 10^{-3}$ (deg cm^2/dmol)	λ (nm)	$[\theta]$ (G)
1240	210 ν_2				
740	640 ν_3	735	−3.30	736	−1.40
660	910 ν_3			670	−1.30
625sh	870 ν_3	620	−3.30	620	+0.20
		387	−15.20	390	+1.20
340	6150	354	+17.80	364	−0.40
		335	−5.00	329	−3.00
		322	+7.60		
278	47000				

[a] ϵ and $[\theta]$ values are based on metal concentration in the enzyme dimer. $\nu_2 = {}^4A_2 - {}^4T_1(F)$; $\nu_3 = {}^4A_2 - {}^4T_1(P)$.

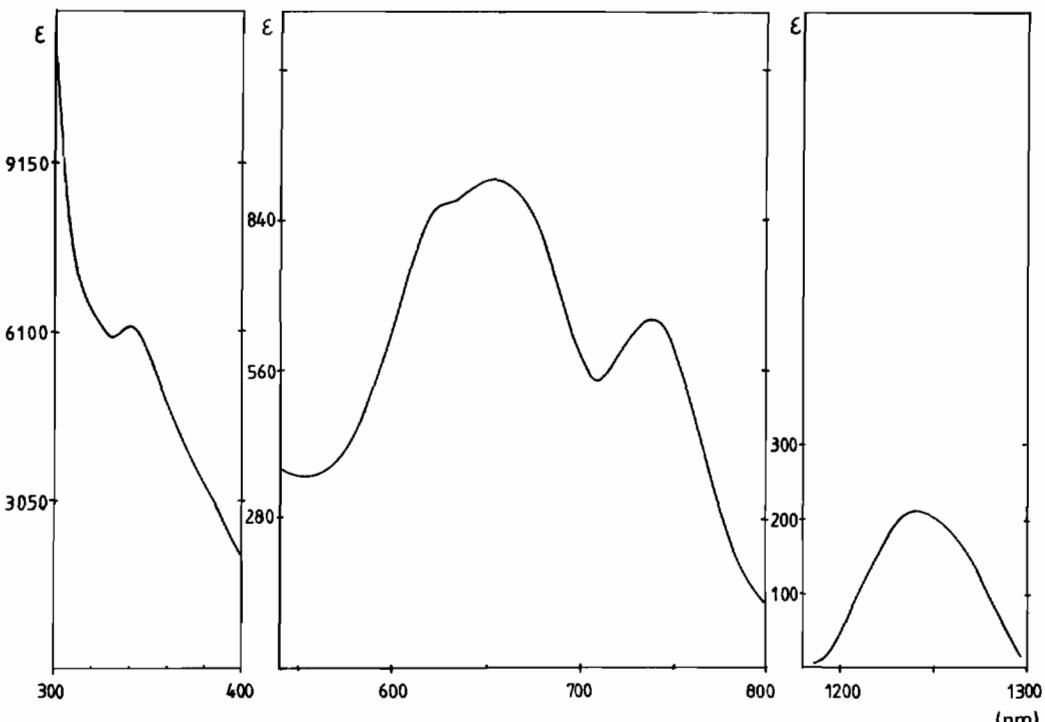

Fig. 1. Absorption spectrum of $Zn(c)_2Co(n)_2$HLADH in 0.2 M TRIS buffer, pH 7.5, at 8 °C.

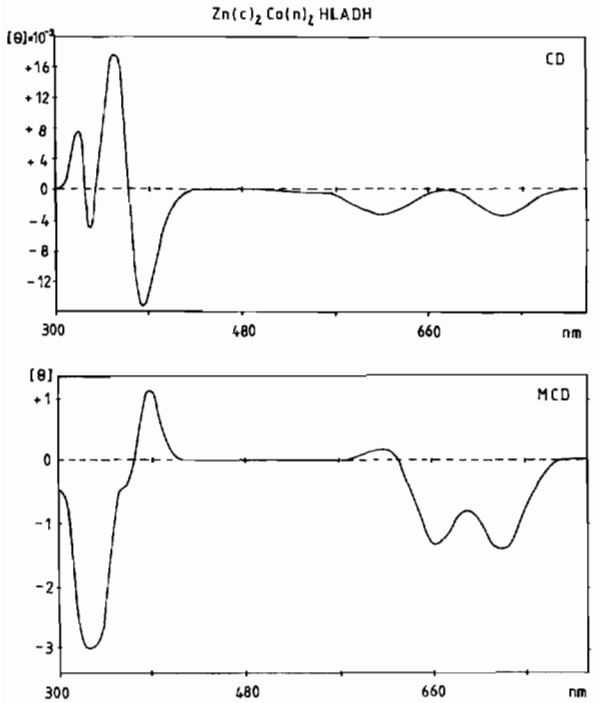

Fig. 2. CD (top) and MCD (bottom) spectra of $Zn(c)_2Co(n)_2$-HLADH in 0.2 M TRIS buffer, pH 7.5, at 6 °C.

intensity of the absorption band at 1240 nm (ϵ = 210 M^{-1} cm^{-1}) must be considered as additional proof of tetrahedral symmetry for the cobalt(II) in the noncatalytic site. Octahedral and five-coordinate complexes would have distinctly lower extinction coefficients of this absorption band [25].

The CD and MCD spectra (Table I, Fig. 2) are similar to Co(II)metallothionein with up to four cobalt ions per metallothionein (*i.e.* before cluster formation) [26], which represent the only metalloproteins with Co(II)–4S tetrahedral chromophores for which CD and MCD spectra have been reported so far.

The 'green hybrid' is paramagnetic with a magnetic moment in the range 4.5–5.0 B.M., which is in fair accordance with tetrahedral Co(II) coordination.

The $Zn(c)_2Co(n)_2$HLADH species could be crystallized applying the same conditions as for the native enzyme [12], *i.e.* 0.1 M TRIS buffer, pH 7.5, with 15–20% (*v*/*v*) tert-butyl alcohol as precipitant, provided that oxygen was thoroughly excluded during all manipulations. The green crystals were morphologically indistinguishable from those of the native enzyme, but no X-ray data have been taken so far.

$Zn(c)_2Ni(n)_2$HLADH

Dialysis of native HLADH (concentration 10–30 mg/ml) against 0.2 M $NiSO_4$ in 0.2 M imidazole buffer, pH 6, over 7 days yielded a novel species which has a yellow color. A higher degree of metal substitution was obtained when the outer solution was changed two or three times. The excess of nickel was then removed by three or four dialyses against 0.2 M TRIS buffer, pH 7.5. The preparation was performed carefully on ice (at 2–6 °C) with all solutions saturated with nitrogen. The obtained HLADH derivative is extremely sensitive to slightly higher temperatures; it denatures even in the pipette after short exposure to room temperature. Therefore all pipettes and cuvets were cooled before use. As long as these conditions are fulfilled, the new species is stable over a long period. It is best stored in liquid nitrogen.

The turnover of the novel species is equal to that of the native enzyme, in our case 4.2–4.5 s^{-1}. The atomic absorption analyses indicate 0.9–1.2 mol of nickel and 0.8–1.2 mol of zinc per subunit. The location of the Ni(II) ion was determined by means of ternary complex formation with NADH and DACA. Ni(II) ion in the catalytic site causes a shift of the maximum of the DACA absorption band to 475 nm [24]. In our preparations the maximum of the DACA band has consistently been observed at 464 nm, which proves that Zn(II) still occupies the catalytic center. The combination of these observations with atomic absorption data leads to the following formula of the new hybrid: $Zn(c)_2Ni(n)_2$-HLADH.

In order to determine the protein concentration photometrically the Lowry test was applied and the absorption coefficient was obtained as: A_{280} = 0.585 mg^{-1} cm^2. The absorption spectra are not very informative. They consist only of three shoulders at about 600, 410 and 310 nm (Fig. 3). Similarly ill-resolved spectra were observed for Ni(II)metallothionein [27]. However, the CD and MCD spectra (Table II, Fig. 4) differ distinctly from those of the nickel–metallothionein. Even at high protein concentrations (50 mg/ml) in D_2O buffer none of the bands in the near-infrared absorption range was observed, which would be expected for tetrahedral Ni(II) coordination. The Ni(II) proteins, Ni(II)-metallothionein [27] and Ni(II)aspartate transcarbamoylase [28] have a Ni(II)–4S chromophore, characteristic of tetrahedral symmetry.

A comparison of the spectral data of $Zn(c)_2$-$Ni(n)_2$HLADH with other Ni(II)–4S systems is presented in Table III. Among low molecular weight complexes with Ni(II)–4S centers, most species have a planar symmetry [29]. One of the rare cases found in the literature of a tetrahedral Ni(II)–4S system is the Ni(II)tetrathiophenolate [30]. Comparison of the data from Table III indicates that the $Zn(c)_2Ni(n)_2$-HLADH species has square-planar symmetry in its Ni(II)–4S site. To furnish additional support for this unexpected conclusion, the magnetic moment of the

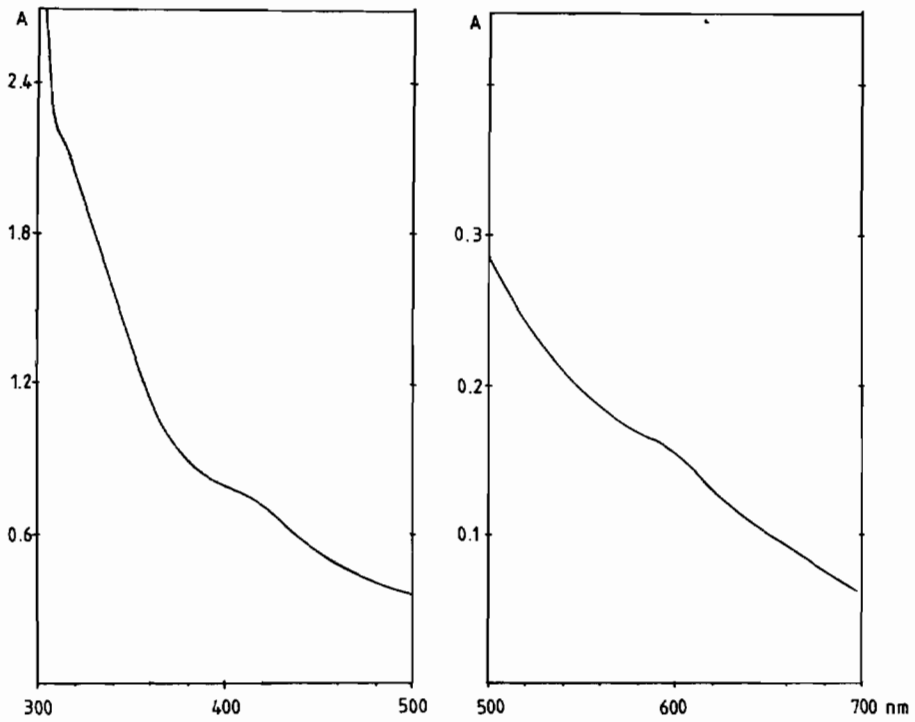

Fig. 3. Absorption spectra of $Zn(c)_2Ni(n)_2$HLADH in 15 mM TES, pH 7.0, at 5 °C; protein concentration was 0.50 mM.

TABLE II. Spectroscopic Data for $Zn(c)_2Ni(n)_2$HLADH[a]

Absorption	CD		MCD	
λ (nm)	λ (nm)	$[\theta] \times 10^{-3}$ (deg cm^2/dmol)	λ (nm)	$[\theta]$ (G)
~600sh	550	−7.70	628	+0.01
~410sh	468	+5.80	467	−0.02
	385	+8.20	374	+0.03
~310sh	330	+12.00	314	−0.32

[a] $[\theta]$ values are based on metal concentration in the HLADH dimer.

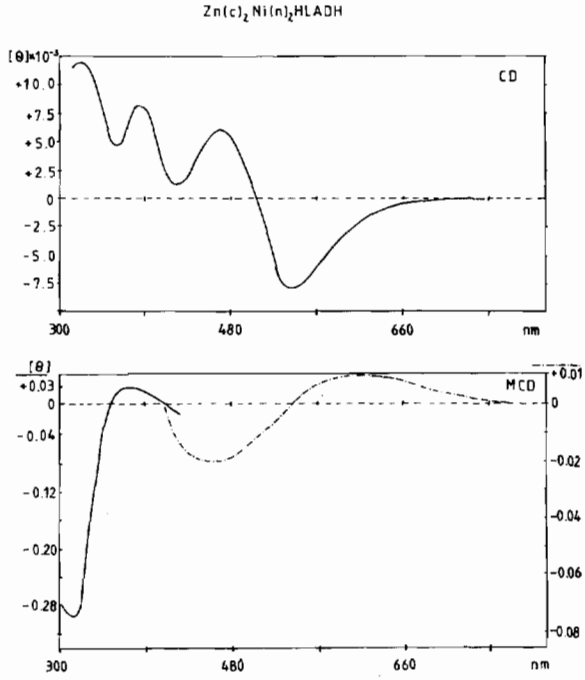

Fig. 4. CD (top) and MCD (bottom) spectra of $Zn(c)_2Ni(n)_2$-HLADH in 0.2 M TRIS buffer, pH 7.5, at 5 °C.

novel species was determined by the NMR method. This experiment showed that $Zn(c)_2Ni(n)_2$HLADH at 8 °C is diamagnetic, its magnetic moment being 0 B.M. To elucidate the real magnetic properties of this derivative, MCD measurements at different temperatures are in progress. Attempts to crystallize the novel species were not successful, when applying the methods used for the crystallization of native HLADH and the 'green hybrid'. The addition of only 2% tert-butyl alcohol caused the enzyme to denature.

Discussion

In native horse liver alcohol dehydrogenase the noncatalytic zinc ion is tetrahedrally coordinated to four cysteine thiol groups [3]. No X-ray structure analysis has been performed for alcohol dehydrogenase with cobalt(II) substituted for zinc at the noncatalytic site. Therefore only spectroscopic data

TABLE III. Comparison of Ni(II)–4S Systems

System	Metal ion symmetry	Absorption spectra (nm)	Reference
Ni(II)metallothionein	T_d	750sh 560sh 430sh 340sh	27
Ni(II)aspartate *trans*-carbamoylase	T_d	720 665 440 360	28
Ni(II)tetrathiophenolate, rare case	T_d	1700 ($^3T_1(F)-{}^3A_2$) 720, 680 ($^3T_1(F)-{}^3T_1(P)$)	30
Ni(II)–4S low molecular weight complexes, e.g. Ni(II)(diethyldithiocarbamate)$_2$	planar	650 ($^1A_{1g}-{}^1A_{2g}$) 590 ($^1A_{1g}-{}^1B_{1g}$) 520, 470 ($^1A_{1g}-{}^1E_g$)	29

can be used for a structural characterization of the Co(II) species substituted specifically at the noncatalytic site. Our data are fully consistent with previous results but are more complete for a spectroscopic characterization of the noncatalytic site. The data given in this paper provide good evidence for a preservation of tetrahedral Co(II)–4S coordination in the noncatalytic site, *i.e.*:

(i) the high intensity of the near-infrared band at 1240 nm;

(ii) the overall shape of the absorption and CD spectra, which could be compared to tetrahedral Co(II)metallothionein;

(iii) the high value of the magnetic moment, which is consistent with the high-spin tetrahedral cobalt(II) system.

These results indicate that cobalt(II) ion bound in the noncatalytic site adopts the geometry of the native zinc enzyme.

Nickel ion, however, behaves in a distinctly different way. It forces the four cysteine sulfurs to arrange into planar symmetry around the metal and, consequently, forces the protein to change its native geometry. At low temperatures the protein does not denature and remains as active as the native enzyme. This indicates that the protein is rather flexible in the region of the noncatalytic center. This surprising result may shed some light on the question about the structural function of the noncatalytic metal ion, since the enzyme is active even if the noncatalytic metal adopts a non-native structure. The fact that the new hybrid has a planar geometry in its Ni(II) site may explain its high instability, as compared to Co(II), Zn(II) and Cd(II) at the same site. The protein around the noncatalytic center provides the tetrahedral environment for the metal but, on the other hand, it is the nickel(II) ion bound to four sulfur ligands which has a strong preference for planar geometry. These two opposing tendencies result in a quasi-stable system, which is preserved only at low temperatures.

It is interesting to compare the noncatalytic center in ADH's from other sources. In rat LADH about 10% of the cysteines in the noncatalytic site are linked in disulfide bridges with each other, so certainly the affinity for zinc in this noncatalytic center is considerably reduced without impact on protein function. Also ADH from yeast is active and stable without Zn(n), which is lost during recrystallization. It was found recently [31] that in certain human liver alcohol dehydrogenase isozymes the noncatalytic zinc can be removed totally and such forms of 'apo-zinc(n)enzymes' are stable and fully active. The empty site can then be substituted by a Co(II) ion, forming a 'green hybrid' similar to that of HLADH. Thus it appears that noncatalytic metal binding sites in various alcohol dehydrogenases differ widely in affinity and reactivity. Metal substitution in these sites has great potential for probing structure–function relationships in this family of enzymes.

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