LIGAND BINDING TO THE BLUE COPPER CENTER OF HORSE LIVER ALCOHOL DEHYDROGENASE

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1. Introduction

The catalytic zinc ion of horse liver alcohol dehydrogenase (LADH) is liganded by 2 thiol groups (Cys 46 and 174) and one imidazole nitrogen (His 67), its fourth ligand being water [1].

Incorporation of Cu^{2+} into the catalytic zinc binding site of horse liver alcohol dehydrogenase (EC 1.1.1.1) produces a metastable cupric protein with optical and EPR spectra reminiscent of the so-called 'blue' copper proteins [2]. The blue Cu^{2+} center in cupric alcohol dehydrogenase differs however from the known blue protein metal centers since at least one co-ordination site is occupied by a water molecule. This water molecule is in rapid exchange with solvent water [3]. Small ligand molecules such as pyrazole, an ethanol-competitive inhibitor of the zinc enzyme, are also able to coordinate to the copper ion [2]. This makes possible the use of the copper LADH system as a model not only for the study of structural features of blue cupric centers but also of its chemical behaviour, e.g., the redox reaction mechanisms. Here, we describe results of EPR and optical absorption spectroscopy studies of several complexes of Cu-LADH with small anionic ligand molecules, such as cyanide, azide, imidazole and 2-mercaptoethanol. These complexes were found to be metastable and their EPR spectra were indicative of a geometry more tetragonal than in the unliganded cupric enzyme. Binding of NAD^+ and NADH to the cupric enzyme gave rise to EPR spectra similar to those observed in 'blue' copper centers.

2. Materials and methods

Cupric horse liver alcohol dehydrogenase was prepared as in [2]. X-Band EPR spectra were recorded on a Varian V-4502 spectrometer at liquid nitrogen temperature. The optical spectra were measured with a Beckman UV 5230 spectrophotometer. A combined optical/EPR cell was used throughout to handle the preparation under anaerobiosis, to avoid bleaching processes of the cupric enzyme [2] which are accelerated by the presence of oxygen. The chemical nature of these reactions is under investigation. The pathlength of the optical cell was 2 mm. All spectra were taken in 25 mM TES/Na^+ buffer (pH 7.0).

3. Results

Fig.1 reports the visible spectra of solutions of the cupric enzyme (a) in the presence of CN^- (b), N_3^- (c), or imidazole (d). The added ligand causes distinct changes of the 'blue' transition at 620 nm of the cupric enzyme with respect to both intensity and energy. In particular, the absorption band is shifted towards shorter wavelengths and its intensity is decreased when the ligand is added. In all cases, the spectra show the presence of both the free enzyme and of the ligand–enzyme complex characterized by absorption at shorter wavelengths as compared to the maximum of the free enzyme. As already described for the complex of the enzyme with pyrazole [2], an accelerated bleaching of the blue colour is observed in the liganded forms. The most profound influence on the transition at 620 nm is exerted by cyanide (fig.1b)

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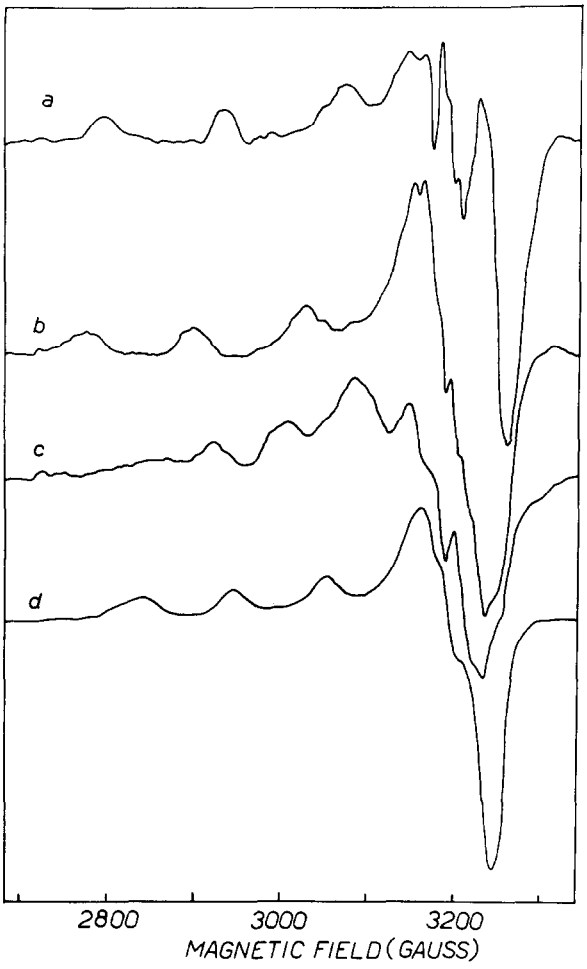

Fig.1. Electronic absorptions spectra of cupric horse liver alcohol dehydrogenase (——) and its complexes with N_3^- (– – –), CN^- (– · – ·) and imidazole (. . .) at room temperature: protein was 190 μM/monomer; copper, 0.7 g-atom Cu/monomer protein; azide, 6.13 × 10^{-3} M; cyanide, 1.9 × 10^{-4} M; imidazole, 1.17 × 10^{-3} M.

Table 1
Low field EPR parameters of complexes of cupric alcohol dehydrogenase

	$A_{\parallel}$ (×10^{-4} cm^{-1})	$g_{\parallel}$
Enzyme + CN^-	133	2.18
+ N_3^-	80	2.14
+ Imidazole	127	2.20
+ Pyrazole[a]	115	2.20
+ 2-Mercaptoethanol	107	2.18
+ NAD^+	74	2.16
+ NADH	<30	2.20

[a] From [2]

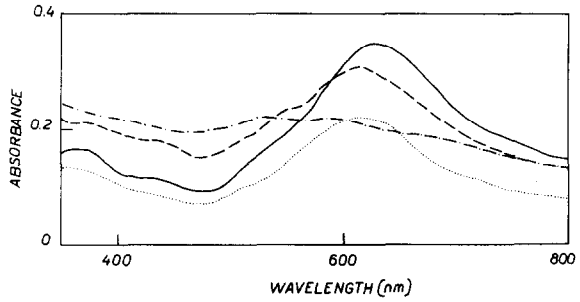

Fig.2. EPR spectra (a–d) of cupric horse liver alcohol dehydrogenase complexes with CN^-, imidazole, N_3^- and 2-mercaptoethanol, respectively. The same concentrations as in fig.1 were used; 2-mercaptoethanol was added in stoichiometric amounts.

which creates at least 3 component bands. The coordination of cyanide anions causes also the most distinguished change in the EPR spectrum of the cupric enzyme (fig.2, table 1). Besides the complexes with cyanide, also the complexes with imidazole, pyrazole, and 2-mercaptoethanol show large increase of the $A_{\parallel}$ value (table 1). The complex of Cu-LADH with azide is the only one, for which the $A_{\parallel}$-value remains below the 100 G limit (table 1). In contrast to the room temperature optical spectra, the EPR spectra are almost completely accounted for by the liganded species. This is reflected by the reversible loss of the blue colour of the solutions and appearance of a rose colour upon freezing. Addition of NAD^+ in anaerobiosis gives rise to a spectrum (fig.3) resembling those

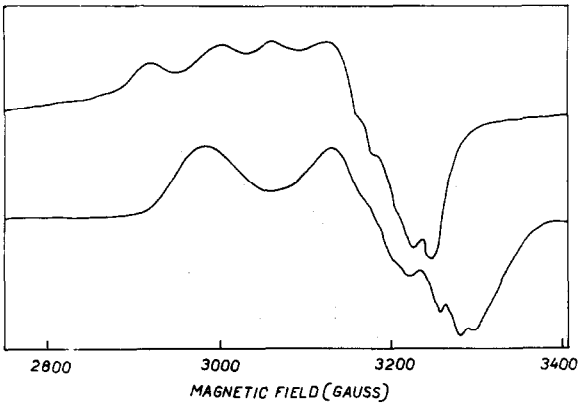

Fig.3. EPR spectra of cupric horse liver alcohol dehydrogenase complexes with NAD^+ (upper trace) and NADH (lower trace): enzyme was 190 μM/monomer; NAD^+, 6.14 mM; NADH, 1.1 mM.

of anion derivatives although with a significantly smaller $A_{\parallel}$-value. On the other hand, the complex of the cupric enzyme with NADH shows an EPR spectrum more similar to that of mononuclear blue proteins [4].

4. Discussion

The data in section 3 suggest the direct coordination of the small ligand molecules to the cupric ion in Cu-LADH. The coordination of these ligands is reflected in the change of both $A_{\parallel}$- and ϵ-values, and to some extent also of $g_{\parallel}$-values. The magnitude of the changes with respect to all 3 parameters is increasing in the series $N_3^- <$ 2-mercaptoethanol < pyrazole < imidazole < CN^-. The considerable increase of $A_{\parallel}$-values suggests an increasing tendency towards a tetragonal arrangement of the donor set in this series of complexes [4]. However, the $A_{\parallel}$-values of the anion complexes are in the range that excludes a square-planar geometry [4] and is compatible with distorted 5-coordinate geometries, as suggested for the 'non-blue' copper binding sites of superoxide dismutase, Cu(II)-carbonic anhydrase and laccase [5]. Contrary, the binding of the coenzyme NADH, which does not coordinate to the metal ion [6], leads to a much lower $A_{\parallel}$-value, comparable to that observed in small 'blue' proteins, in particular stellacyanin. It suggests a rather distorted tetrahedral coordination around the metal ions as it was established for plastocyanin and azurin [7,8]. The binding of NAD^+, which also does not coordinate to the metal ion, leads to a larger $A_{\parallel}$-value, which is however still compatible with tetrahedral coordination. The low $g_{\parallel}$-values for all complexes studied may be related to sulfur coordination [9]. It is remarkable that 2-mercaptoethanol coordinates to the cupric ion of Cu-LADH without reducing it. It is believed that the trithiolate coordination to copper in thioneins stabilizes cuprous ions [10]. The stabilization of the copper oxidation state in the complex of Cu-LADH with 2-mercaptoethanol may originate from the geometrical situation in the catalytic center, e.g., the more square planar arrangement of the donor set around the metal ion. The relatively close $A_{\parallel}$-values obtained for imidazole, pyrazole and 2-mercaptoethanol complexes with Cu-LADH may suggest that symmetry rather than high covalency of the copper–sulfur bond is responsible for the unusually small $A_{\parallel}$-values of the blue copper centers.

In conclusion, Cu-LADH is confirmed to be a good 'biological' model of copper centers existing in natural proteins; its EPR spectra in the presence of anion ligands and coenzymes display a wide range of $A_{\parallel}$-values, as those observed in stellacyanin (NADH), type 1 copper of blue oxidases (NAD^+ and azide), and the most distorted type 2 copper centers (other ligands).

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