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MAGNETIC AND EPR INVESTIGATIONS ON Co(II)-HEMOCYANIN: A MODEL FOR DEOXY-HEMOCYANIN

Magnetic exchange interaction of the binuclear copper active sites found in hemocyanin, tyrosinase and in some multi-copper oxidases is still an unresolved problem. Strong antiferromagnetic coupling between the two Cu(II)-ions of the oxy-form is concluded from the diamagnetic behaviour in magnetic and EPR investigations [1]. From series of dimeric model complexes it has been shown that a greater spin of the metal centers results in a decrease of the antiferromagnetic coupling [2]. This leads to a strong increase of the magnetic effect, which is an important advantage for the magnetic investigations in proteins because of the small concentration of metallic centers. Owing to this, metal substitution at the active site in these enzymes seems to be a useful tool to investigate their magnetic behaviour.

In this study we report on the magnetic and EPR properties of the Co(II)-derivative of *Limulus polyphemus* hemocyanin, where both Cu-ions of the active site were replaced by Co(II). Co(II)-hemocyanin was prepared by a modification of the method developed by S. SUZUKI and co-workers [3].

Because of the small concentration of magnetic centers we have developed two methods for sample preparation which will be presented. Using sedimentation and lyophilisation techniques we have obtained enzyme concentrations up to 35% by weight.

Initial information about the Co(II)-coordination arises from absorption and near-infrared spectra

suggesting tetrahedral coordination in Co(II)-hemocyanin which would be comparable to tetrahedral low molecular weight complexes. From the $\nu_3(^4A_2 \rightarrow ^4T_1(P))$ and $\nu_2(^4A_2 \rightarrow ^4T_1(F))$ transitions we have determined a strong tetrahedral ligand field comparable to Co(II)-tyrosinase [4]. The splitting of the ν_3 transition into four components reflects distortions from tetrahedral symmetry. Since Cu(I)-complexes often show tetrahedral coordination [5], the Co(II)-derivative of *Limulus polyphemus* hemocyanin may reflect the coordination geometry of the deoxy-form.

Magnetic measurements have been carried out between 4.2 and 315 K with a Faraday-system described previously [6]. The magnetic behaviour of oxyhemocyanin and its Co(II)-derivative will be presented.

The exchange coupling is discussed in relation to the limits of the used instrumental equipment. The general application of temperature dependent measurements of the magnetic susceptibility to proteins containing active sites with exchange coupled metal centers will be pointed out.

Temperature dependent X-band EPR spectra have been recorded between 4.2 and 80 K. At 4.2 K a broad signal has been observed which can be estimated on the basis of axial symmetry of the EPR spectra [7]. The signal typical for high-spin Co(II) complexes [8] is broadened significantly with increasing temperature and disappears at 76 K in the most concentrated sample measured. This is caused by the rapid spin-lattice relaxation typical for Co(II). The EPR spectra will be discussed in relation to the exchange coupling and to structural features of the Co(II)-derivative of hemocyanin.

From the results of the Co(II)-hemocyanin it can be concluded that Co(II) replaces Cu(I) in the active site in a distorted tetrahedral environment. Therefore this derivative can be used as a model for the deoxy-form which can be studied by common techniques in contrast to a Cu(I)-site.

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Fe(II)- AND Fe(III)-SUBSTITUTED HORSE LIVER ALCOHOL DEHYDROGENASE

The catalytic metal binding site in $H_4Zn(n)_2$ -HLADH (HLADH=horse liver alcohol dehydrogenase, EC 1.1.1.1), a derivative lacking the catalytic zinc ions, provides a pseudotetrahedral geometry for various metal ions [1]. Recently, Fe(II) ions have been inserted into this site. In the absence of added oxidizing or reducing agents, Fe(II) is quickly oxidized to a violet Fe(III)-enzyme; in a subsequent slow reaction the Fe(III)-enzyme is bleached ($t_{1/2} \sim 15$ min). In the presence of oxidizing or reducing agents

Fe-HLADHs can be prepared, in which iron is exclusively in the di- or trivalent state. The electronic structure of the iron ion is influenced by ligand binding and binding of coenzyme to the enzyme. In this communication we characterize Fe-HLADH by EPR-, Mössbauer-, UV/Vis-, CD- and resonance Raman [2] spectroscopy.

Fe(II)-HLADH

The reduced Fe-HLADH does not absorb in the visible region and is EPR-silent at low temperatures. Mössbauer spectra reveal two Fe(II) species in the high spin state ($S=2$). The Mössbauer parameters ($\delta(1)=0.86$; $\delta(2)=1.31$; $\Delta E_Q(1)=3.80$; $\Delta E_Q(2)=3.35$, {mm·s⁻¹}) indicate a coordination number of five for both species.

Fe(III)-HLADH

The oxidized Fe-HLADH shows a broad electronic absorption band with a maximum at 560 nm ($\epsilon=1500$ M⁻¹ cm⁻¹), consistent with a S-Fe(III) ligand-to-metal charge transfer. A shoulder is detected at 336 nm ($\epsilon=4000$ M⁻¹ cm⁻¹). In the CD-spectrum the visible absorption band is resolved into at least three transitions. Resonance Raman spectra are produced by excitation at 514 nm ($\nu_1=309$ (w), $\nu_2=357$ (s), $\nu_3=387$ (w) and $\nu_4=428$ (s) cm⁻¹). They are very similar to those of Ni- and Cu-HLADH [3]. The EPR spectrum of Fe(III)-HLADH at 10 K is characterized by absorptions at $g=9.5$ and $g=4.25$ and is typical for a rhombic symmetry of Fe(III) in the high spin state ($S=5/2$). At least two species with slightly different parameters are responsible for these signals. This heterogeneity is revealed by Mössbauer spectroscopy only in the paramagnetic hyperfine structure ($\Delta E_M(1)=390$ kG; $\Delta E_M(2)=470$ kG). The Mössbauer parameters are in the range expected for tetra- and pentacoordinate iron.

CONCLUSIONS

Fe(II)- and Fe(III)-HLADH do not show appreciable enzymatic activity in ethanol oxidation. Iron binds in the active site of HLADH to two sulfur atoms of cysteines, one histidine and one water molecule. The identity of the fifth ligand in the pentacoordinate structure is under scrutiny. Thus, Fe-HLADH illustrates a certain coordinati-