Volume 309, number 1, 92-96 FEBS 11489 August 1992

Copper(II)-substituted horse liver alcohol dehydrogenase: structure of the minor species

Grazyna Formicka[a], Michael Zeppezauer[a], Frank Fey[b] and Jürgen Hüttermann[b]

[a]*Department 12.4 Biochemistry, University of Saarland, D-6600 Saarbruecken, Germany* and [b]*Dept. 3.6 Biophysics, University of Saarland, D-6650 Homburg/Saar, Germany*

Received 15 May 1992; revised version received 15 July 1992

Oxygen treatment of horse liver alcohol dehydrogenase EE isozyme substituted with Cu(II) at the catalytic site leads to bleaching with concomitant reduction to Cu(I) of ~90% of total Cu(II). The Cu(II) of the remaining 'minor species' cannot be reduced nor does it interact with exogenous ligands, e.g. 2-mercaptoethanol, imidazole, pyrazole, or azide ions. The EPR spectrum is axial with a super-hyperfine splitting of 15.6 G indicating binding of one nitrogen atom to Cu(II). These data as well as the energies and intensities of the absorption and CD spectra suggest the Cu(II) ion of the minor species to be located in the catalytic site of HLADH in a position and geometry different from that of the major species.

Alcohol dehydrogenase; Copper(II)-protein; EPR; CD; Absorption spectra

1. INTRODUCTION

The electronic structure of the copper(II) ion substituted at the catalytic site of the horse liver alcohol dehydrogenase (HLADH) EE isozyme has been studied extensively with different spectroscopic techniques [1-6]. A characteristic feature of the specimens studied so far is their similarity to type 1 copper centers in blue copper proteins [1]. The coordination sphere of the metal ion in HLADH can be perturbed by conformational changes in the protein due to coenzyme binding or by exogenous ligand binding to the copper(II) ion. These perturbations can be monitored spectroscopically, which renders this system a universal model for blue copper proteins.

The copper substitution in HLADH produces two different species [4]: a major species with a rhombic EPR spectrum and a minor species with an axial EPR spectrum which accounts approximately for 10% of the total copper. The major species is mainly responsible for the spectroscopic features of the blue copper center. This species is metastable and can be reduced (bleached) in a process accelerated by oxygen or hydrogen peroxide; for a possible mechanism see [4]. In contrast, the minor species cannot be bleached in this way. Also, the Cu(II) ion in the minor species was reported to be insensitive towards the interaction with exogenous ligands, suggesting a buried coordination site. However, no evidence for or against its binding in the active site was given so far. In the major species all available data support the replacement of the catalytic zinc ion by Cu(II) in its coordination sphere with two cysteines, one histidine, and one water molecule as ligands. As to the minor species we now present data suggesting binding of the Cu(II) ion to the active site; however, in a position different from that of the Cu(II) ion in the major species.

Correspondence address: G. Formicka, Dept. 12.4 Biochemistry, University of Saarland, D-6600 Saarbruecken, Germany. Fax:(49) (681) 302 2097.

2. EXPERIMENTAL

Horse liver alcohol dehydrogenase, EE isozyme (EC 1.1.1.1) was prepared from horse liver according to the method described earlier [7]. All chemicals used were of the purest grade commercially available. The preparation of the cupric horse liver alcohol dehydrogenase ($Cu(c)_2Zn(n)_2$ HLADH) was described earlier [1,6]. All experiments were carried out in 25 mM TES/Na^+ buffer, pH 7.0. Absorption spectra were recorded on a Perkin Elmer Lambda 9 spectrophotometer at 4–8°C. The circular dichroism spectra were taken on a Jasco J-20 recording spectropolarimeter. Absorption coefficients (ε and $\Delta\varepsilon$) values were calculated on the assumption that 10% of the copper is bound in the minor species. EPR spectra were recorded with a Bruker FT-EPR 380 spectrometer at 77 K.

3. RESULTS

The absorption, circular dichroism (CD) and electron paramagnetic resonance (EPR) spectra of the major species have been published earlier [4,6] and were remeasured in order to compare them with previous data and with the novel spectra of the minor species. All spectra of the major species were obtained directly after dissolving $Cu(c)_2$ $Zn(n)_2$ HLADH crystals under anaerobic conditions. The copper(II)-substituted HLADH prepared in this investigation appeared to be rather stable; 21° standing on open air was not sufficient to

 Published by Elsevier Science Publishers B.V.

fully bleach the samples, compared to about 6° reported earlier [4]. Therefore direct treatment with oxygen was necessary to obtain the minor species. Oxygen was blown slowly over an open sample placed on ice and 3 h after this treatment full reduction of the major species was achieved. During oxygen treatment the samples developed some turbidity invisible to the eye, but indicated by an apparent 15% increase of the absorption at 280 nm presumably due to denaturation of the major, Cu(I) species. Therefore absorption spectra were recorded directly after preparation and repeatedly during the oxygen treatment.

The absorption spectra obtained before and after oxygen treatment are shown in Fig. 1. The major species has its main absorption band at 624 nm and a less well resolved shoulder at about 790 nm. The second and fourth derivative of this spectrum reveal one more band at 530 nm, which is hidden in the absorption spectrum. The two other bands at 443 and 377 nm are about five times less intense than the band at 624 nm (Table I).

The absorption spectrum of the minor species, left after oxygen treatment, has four bands of similar intensity at 626, 530, 405 and 340 nm (Table I). Absorption coefficients for these bands are calculated on the assumption that the minor species contains 10% of the total copper. This is based on the integration of the EPR spectra which reveals the minor species to contain 8–10% of all copper ions. The bands at 620 and 530 nm in the minor species are found at similar wavelengths as those bands of the major species. They cannot be the remains of the major form left after the bleaching process, as can be seen from a comparison of their mutual band intensities (Table I). In the minor form all bands have comparable intensities. Bands at lower wavelengths are additionally affected by the slight increase of the baseline around 300 nm, as a result of some denaturation, indicated by a small increase of the protein band at 280 nm. The tail of this band is lifting the bands at 300–400 nm, which leaves the estimation of intensities at these wavelengths rather unprecise. However, if one would assume that the transitions at 620 and 530 nm are only due to the remains of the major form, not fully reduced, then the intensity at 624 nm after bleaching should account for 20% of the major form left. In such a case the intensity of the 530 nm band should be equal to about 0.018, instead of observed 0.056. Additionally the EPR spectrum recorded for the sample treated 3 h with oxygen shows no signs of the remains of the major form. Therefore we assume that the minor form is absorbing at 626, 530, 405 and 340 nm, as shown in Table I. The absorption intensities given in the Table I are only estimates due to the above-mentioned difficulties with protein denaturation and lack of an independent method for measuring precisely the concentration of the minor species.

The copper(II) ion in the minor species does not bind external ligands, i.e. pyrazole, imidazole, azide, 2-mercaptoethanol, and it cannot be reduced by ascorbate. The EPR spectrum of the minor form is shown in Fig. 2A. It is of the axial type with a hyperfine splitting of 10.4 mT. The magnitude of the hyperfine splitting as well as the $g_x/|A_x|$ ratio are similar to those of the binary complex of the major species with pyrazole (see Table II) which was suggested to be pentacoordinate [8]. The EPR spectrum of the minor species also shows three super-hyperfine lines with a spacing of 15.6 G which could be due to one nitrogen atom coordinating to the copper(II) ion. The spectrum of the major species (see Fig. 2B) was obtained via subtracting the spectrum of the minor species from that of the sample before oxygen treatment. A new simulation of this spectrum provided data which differ slightly from those reported earlier. The EPR spectrum of the major species has a rhombic form with a high $g_x/|A_x|$ ratio, similar to other type 1 Cu(II) in blue copper proteins. For comparison, Table II includes the data for the binary complexes of the major species with NADH and with pyrazole and its ternary complex with NAD^+ and pyrazole.

Circular dichroism spectra of the main and the minor species are presented in Fig. 3. One should notice that both species have opposite signs of their Cotton effects with exception of the band at the lowest wavelength.

An additional experiment was performed in order to address the question of the possible location of the copper(II) ion in the minor species. Cu(II) ions were added slowly to the native $Zn(c)_2Zn(n)_2$ HLADH under anaerobic conditions. As can be seen in Fig. 4 the copper binding to peripheral sites creates a species absorbing at 476 and about 540 nm, values distinctly different from those of the minor species.

From the ratios Cu:protein for each absorbancy increment it can be seen that the Cu(II) binding to peripheral sites is weak. At Cu:protein ratios larger than 5:1 the protein started to precipitate, therefore a quantitative evaluation of the number and intrinsic affinities of the peripheral sites was not possible.

Table I

Absorption band positions and molecular absorptivities for the major and minor species

	λ[nm]	ε[$M^{-1}cm^{-1}$]
'main species'	~790 sh	~1,400
	~624	5,700
	~530*	
	~443	980
	~377	1,200
	~280	19,200
'minor species'	~626	9,300
	~530	8,100
	~405	10,000
	~340	10,500

* Visible only on the 2nd and 4th derivative of the spectrum
** Calculated for 10% copper concentration

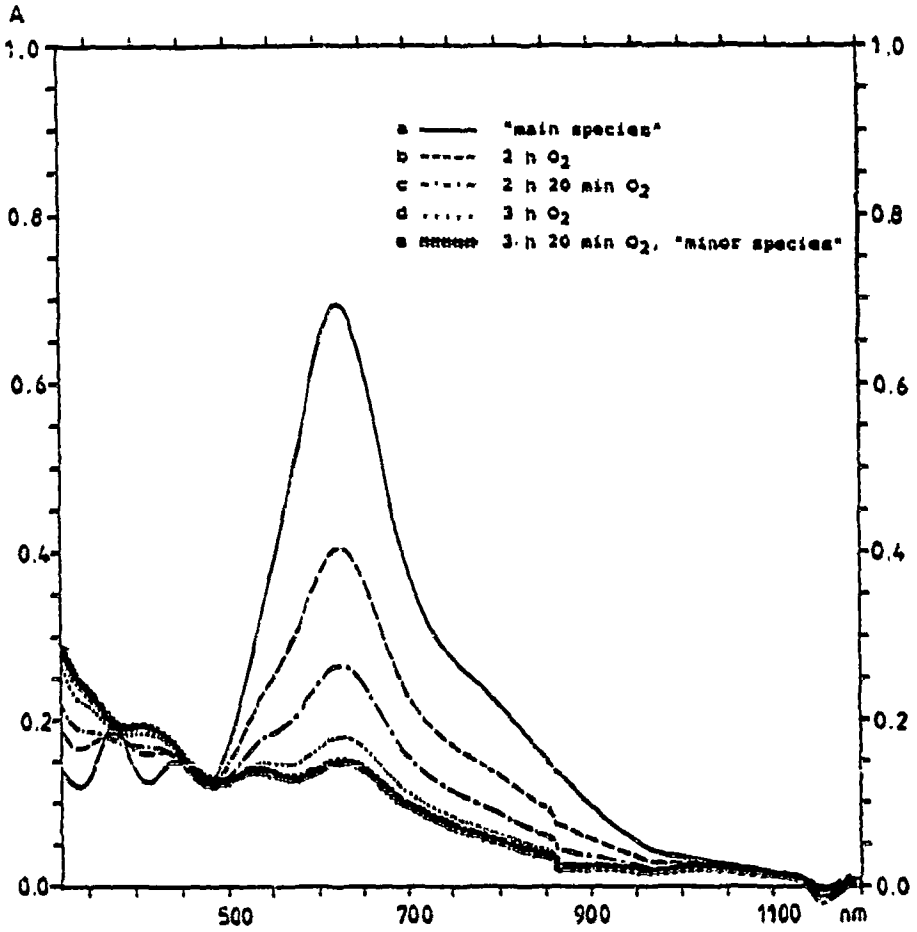

Fig. 1. Electronic absorption spectra of Cu(c)$_2$ Zn(n)$_2$ HLADH. of the main species (a) and after oxygen treatment (b–e), (the minor species (e)) in 25 mM TES/Na^+, pH 7.0 at 283 K. Enzyme concentration 48.6 μM.

4. DISCUSSION

Our data prove that the minor species represents an interesting novel copper binding site. The absorption and CD band positions, as well as their high intensities indicate thiolate–copper coordination. The spectral

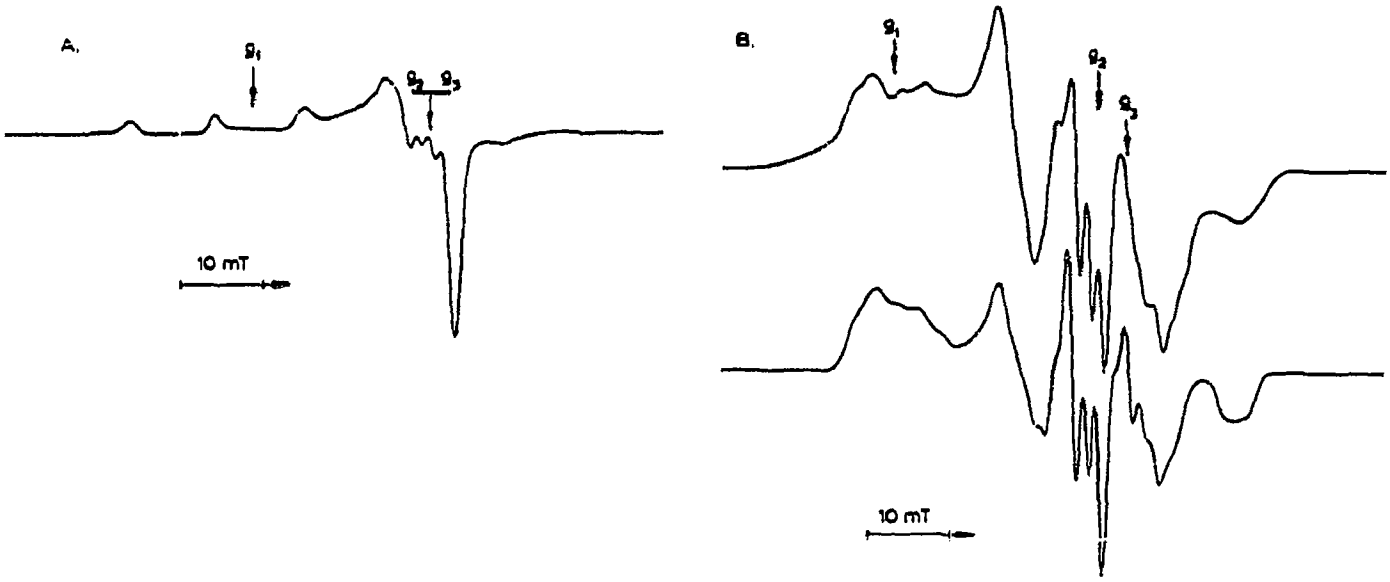

Fig. 2. EPR spectra of cupric horse liver alcohol dehydrogenase at 77 K. Concentration of enzyme 2 mM. (A) Experimental spectrum of the minor species. (B) Experimental (upper trace) and simulated (lower trace) spectra of the main species.

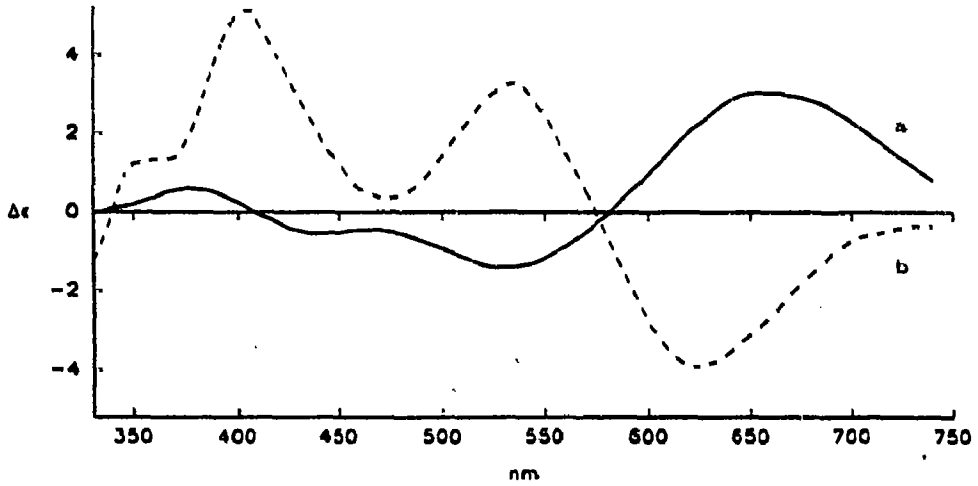

Fig. 3. Circular dichroism spectra of the major (a) and the minor (b) species. Enzyme concentrations: (a) 373 μM (b) 25 μM.

data of Cu(II) bound to peripheral sites of the native enzyme seem to exclude the location of Cu(II) to those sites in the minor species.

It is difficult to deduce the symmetry of the copper coordination sphere in the minor species from the available spectroscopic data. Similar problems were faced in the studies of other blue copper proteins, e.g. plastocyanin and azurin. By recent X-ray investigations [9–12] their copper(II) sites were found to be distorted tetrahedral and pentacoordinated, respectively, despite of their similar spectral properties [13].

Concerning the chemical behaviour of the minor species, its copper(II) site cannot be reduced by ascorbate, obviously due to a higher oxidation–reduction potential compared to the major species. The other feature characteristic of the copper(II) site of the minor species is its inaccessibility to external ligands. These facts taken together suggest that in the minor species the Cu(II) ion is located in the catalytic site of HLADH, however with different geometry and different chemical reactivity compared to the major species.

Two alternative structure models may be envisaged: (i) the Cu(II) site has a tetracoordinate structure, the water ligand being exchanged by an endogenous protein side chain – glutamate-68 seems to be the closest candidate [14]; (ii) the Cu(II) ion is pentacoordinate in a distorted trigonal bipyramidal structure similar to azurin. Either Glu-68 might provide two oxygen atoms as ligands, or another ligand would have to be found which is not exchangeable by external ligands. Even water molecules in the interior of the catalytic site seem to be possible candidates [15].

In both cases a necessary conformational change would hide the copper ion more deeply inside the protein. The circular dichroism spectra with similar band positions but opposite signs for the major and minor species support this idea of a protein conformation difference for both species. Exchange of the water ligand for an oxygen atom of Glu-68 would change the chirality of the coordination sphere if the other ligands remain unchanged, and render the copper ion inaccessible for external ligands.

The reason of the protein conformation change in the minor species of Cu(II)-substituted HLADH may be understood after comparing the X-ray structure of apo-HLADH, i.e. $H_4Zn(n)_2$ HLADH with the structure of the metal-substituted species. In the apo-zinc enzyme structure the carboxyl group of Glu-68 is moved closer towards the empty metal site [14]. It is conceivable that in the minor species the copper is bound to the oxygen

Table II

EPR parameters of different forms of Cu(II) substituted HLADH

	g_z (A_z)*	g_x (A_x)	g_y (A_y)	$g_z/\lvert A_z\rvert$**
Minor species	2.16 (10.4)	2.02	2.02	222
Main species	2.23 (2.5)	2.04 (8.7)	2.06 (3.2)	953
E · NADH [6]	2.19 (2.6)			842
E · NAD⁺ · py [6]	2.20 (4.0)			550
E · py*** [1]	2.20 (11.5)			191

*A values are given in mT (1 mT = 10 G).
**$g_z/\lvert A_z\rvert$ ratios are given in cm in order to facilitate comparison with older literature.
***py, pyrazole; E, main species.

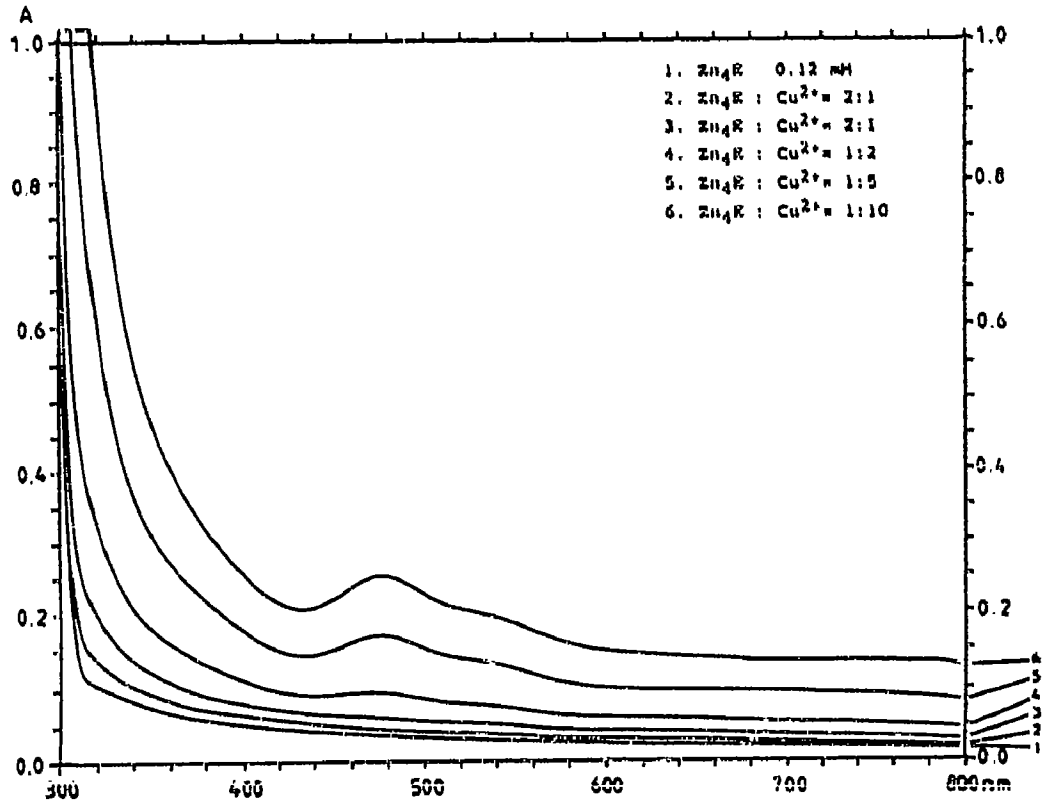

Fig. 4. Effect of Cu(II) ion addition to the native HLADH on the absorption spectra at 276 K. Enzyme concentration 120 μM.

atom of Glu-68 in such a fashion that the protein remains in the 'apo'-conformation. Attempts under way in our laboratory to resolve both species on a preparative scale and to crystallize them should help answering the open questions concerning the molecular structure of the minor form of Cu(II) HLADH.

Acknowledgements: We thank N. Weber for preparation of native HLADH, C. Klein for help in the preparation of the manuscript, and Prof. R. Hartmann and Dr. P. Dorfmüller for the possibility to use the J-20 spectropolarimeter. The work was supported by the BMFT, the Zentrale Forschungskommission, Universität des Saarlandes, and Deutsche Forschungsgemeinschaft, Schwerpunktprogramm Bioanorganische Chemie.

REFERENCES

[1] Maret, W., Dietrich, H., Ruf, H.H. and Zeppezauer, M. (1980) J. Inorg. Biochem. 12, 241–252.
[2] Maret, W., Zeppezauer, M., Desideri, A., Morpurgo, L. and Rotilio, G. (1981) FEBS Lett. 136, 72–74.
[3] Maret, W., Zeppezauer, M., Sanders-Loehr, J. and Loehr, T.M. (1983) Biochemistry 22, 3202–3206.
[4] Maret, W., Zeppezauer, M., Desideri, A., Morpurgo, L. and Rotilio, G. (1983) Biochim. Biophys. Acta 743, 200–206.
[5] Maret, W., Shiemke, A.K., Wheeler, W.D., Loehr, T.M. and Sanders-Loehr, J. (1986) J. Am. Chem. Soc. 108, 6351–6359.
[6] Maret, W. and Kozlowski, H. (1987) Biochim. Biophys. Acta 912, 329–337.
[7] Adolph, H.W. (1989) Dissertation, University of Saarland, Saarbruecken.
[8] Andersson, I., Maret, W., Zeppezauer, M., Brown III, R.D. and Koenig, S.H. (1981) Biochemistry 20, 3424–3432.
[9] Guss, J.M. and Freeman, H.C. (1983) J. Mol. Biol. 169, 521–563.
[10] Norris, G.E., Anderson, B.F. and Baker, E.N. (1986) J. Am. Chem. Soc. 108, 2784–2785.
[11] Nar, H., Messerschmidt, A., Huber, R., van de Kamp, M. and Canters, G.W. (1991) J. Mol. Biol. 218, 427–447.
[12] Baker, E.N. (1988) J. Mol. Biol. 203, 1071–1095.
[13] Gray, H.B. (1980) in: Methods for Determining Metal Ion Environments in Proteins (Darnall, D.W. and Wilkins, R.G. eds.) Adv. Inorg. Biochem., vol 2, pp. 1–25, Elsevier, New York, Amsterdam, Oxford.
[14] Schneider, G., Eklund, H. Cedergren-Zeppezauer, E. and Zeppezauer, M. (1983) Proc. Natl. Acad. Sci. USA 80, 5289–5293.
[15] Cedergren-Zeppezauer, E.S., Samama, J.-P. and Eklund, H. (1982) Biochemistry 21, 4895–4908.