AN EPR STUDY OF *NEUROSPORA* TYROSINASE

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1. Introduction

It has been shown recently, that mushroom tyrosinase (EC 1.14.18.1) contains a pair of copper ions at the active site [1]. In contrast, *Neurospora* tyrosinase has been reported to contain only one copper per functional unit [2,3]. However, based on a recent determination of the molecular weight [4] this enzyme contains close to two copper ions per polypeptide chain. In analogy to the mushroom tyrosinase the copper in *Neurospora* tyrosinase might also be present as a copper pair. To get more information on the state of copper in *Neurospora* tyrosinase an EPR study was undertaken.

A careful search at various temperatures revealed no EPR-detectable copper, in agreement with the results of others [2,3] and consistent with the existence of coupled dimeric copper.

The reaction of NO with the native and the reduced enzyme did not give rise to any EPR signals typical for the NO-complex of hemocyanin and mushroom tyrosinase [5]. On the other hand, the reaction of the native enzyme with β-mercaptoethanol leads to a green, enzymatically completely inactive complex [6]. This complex displays an EPR signal with rather unusual characteristics probably arising from paramagnetic copper. The green compound may represent a complex between a half reduced copper pair and β-mercaptoethanol.

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2. Materials and methods

2.1. *Protein*

Neurospora tyrosinase (thermolabile isoenzyme from *Neurospora crassa*, FGSC-strain NO 320) was prepared according to the method of Lerch (Lerch, K., unpublished). The freshly isolated enzyme consisted of a mixture of about 30% oxytyrosinase and 70% resting tyrosinase as has been reported by Gutteridge and Robb [3] using a different purification method. The enzyme was homogeneous according to the criteria of SDS-polyacrylamide electrophoresis [7] and isoelectric focusing [8]. The pure enzyme had an extinction coefficient $A^{1\%}_{1cm}$ of 22 at 280 nm [4]. The determination of the specific activity was carried out according to Fling et al. [2] and the obtained value, 1100 units per mg protein, is slightly higher than those reported by others [2,3,9].

2.2. *Analytical methods and anaerobic technique*

Total copper was determined with 2,2'-biquinoline [10]. The anaerobic technique used has been described elsewhere [11]. All chemicals were of analytic grade and were used without further purification. Solutions were made with deionized, distilled water.

2.3. *Electron paramagnetic resonance (EPR) measurements*

EPR measurements at 9 GHz were made at 77 and 10 K with a Varian E-3 spectrometer and a Varian E-9 spectrometer, respectively, and at 35 GHz and 90 K in a Varian V-4503 spectrometer. EPR-detectable copper was determined by double integration of the 9 GHz spectra and corrections for the different

g-factors were applied [12]. In all EPR experiments the concentration of the protein was 1.16 mM (2.1 mM in total copper) in 10 mM sodium phosphate with 0.5 M NaCl (pH 6.8).

3. Results and discussion

Based on the copper determination and the recently determined molecular weight obtained by different methods [4] *Neurospora* tyrosinase contains 1.8–1.9 Cu per polypeptide chain. EPR analysis of the native enzyme under anaerobic and aerobic conditions showed less than 3% EPR-detectable copper with the signal shape typical for a normal Cu^{2+} complex. The same results were obtained after treatment with hydrogen peroxide (1 mol of H_2O_2 per mol of protein) leading to the completely oxygenated form [4,6] under aerobic conditions or to the 'reduced' form (cf. ref. [1]) under anaerobic conditions. After reduction with hydroxylamine (1 mol of NH_2OH per mol of protein) EPR-detectable copper diminished to less than 0.5%. Under the above conditions no other EPR signal was detectable between 0 and 0.6 T at 9 GHz either at 77 or 10 K. These results show that the copper in the native and the reduced form is EPR-non-detectable, as has been found in hemocyanin and mushroom tyrosinase [5]. Since *Neurospora* tyrosinase contains close to two Cu/molecule of enzyme the results strongly suggest that copper is present in the form of a copper pair.

For mushroom tyrosinase evidence for a copper pair has been obtained from experiments with NO [5]. Therefore, the native and the reduced (1 mol of H_2O_2 mol of protein) *Neurospora* tyrosinases were treated anaerobically with NO (1 atm) according to the method used by Schoot Uiterkamp and Mason [5]. However, the typical EPR signals observed with hemocyanin and mushroom tyrosinase were not obtained. Since ceruloplasmin forms an EPR-detectable NO complex [13] only after treatment with an excess of reducing agent [Deinum, J. and Reinhammar, B., unpublished] experiments were performed with *Neurospora* tyrosinase using an excess of either H_2O_2, NH_2OH or ascorbic acid. Subsequent treatment with NO did not give rise to any EPR signals. However, these results still do not exclude the possibility that *Neurospora* tyrosinase contains a copper pair, since the tree and fungal laccases and ascorbate oxidase, which all have a copper pair [15,16], do not yield an EPR-detectable NO complex (Deinum, J. and Reinhammar, B., unpublished and ref. [14]). Furthermore, the CD spectra in the visible region of hemocyanin [17], mushroom tyrosinase [18] and *Neurospora* tyrosinase [6] are rather different, indicating differences in the copper site of these three proteins.

Upon anaerobic addition of β-mercaptoethanol to the native enzyme, a green, enzymatically completely inactive complex (fig.1) is formed, displaying a characteristic absorption with maxima at 355 nm (ϵ = 1700 M^{-1} cm^{-1}), 460 nm (ϵ = 800 M^{-1} cm^{-1}) and 700 nm (ϵ = 1900 M^{-1} cm^{-1}). This complex can be isolated by gel filtration and is stable for several days at 4°C [6]. This compound was now studied by EPR and was found to be paramagnetic. The EPR signal at 9 and 35 GHz is shown in figs.2 and 3. The signal remained unchanged in a sample that had been kept overnight at 0°C. The shape of the signal at 9 GHz was the same at 10 and 77 K, and no half-field line could be observed at even a 20 times higher amplification. At 10 K the signal is power saturated to about 50% with 20 mW microwave power. The signal is essentially axial as follows from the 35 GHz spectrum (fig.3). No hyperfine structure has been observed. Although the signal at 9 GHz somewhat resembles the $\Delta m = \pm 1$ line from a Cu^{2+}–Cu^{2+} pair [19], such an interpretation is not supported by the 35 GHz spectrum. The 9 and 35 GHz spectra are consistent with a paramagnetic species having a $S = 1/2$ with $g_{\parallel} = 2.19$ and $g_{\perp} = 2.05$. The linewidth has an aniso-

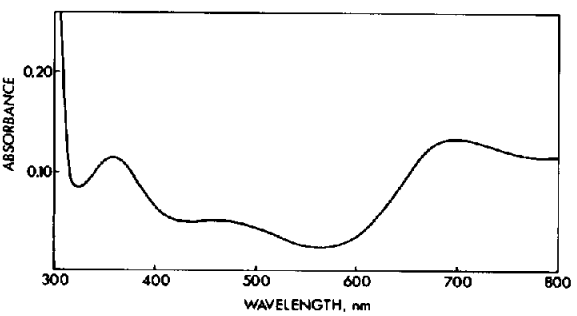

Fig.1. Absorption spectrum of β-mercaptoethanol treated *Neurospora* tyrosinase at 4°C. The concentration of the enzyme was 70 μM and a 10-fold molar excess of β-mercaptoethanol was used. The spectrum was recorded 18 h after addition of β-mercaptoethanol.

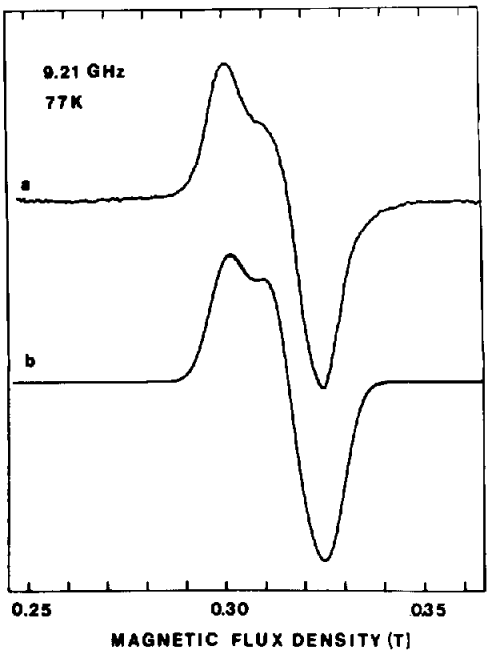

Fig.2. EPR spectrum of *Neurospora* tyrosinase (1.16 mM) treated anaerobically with a 5-fold excess of β-mercaptoethanol per mole of protein. (a) The spectrum was recorded at 77 K and 9.21 GHz. The microwave power was 2 mW, the modulation amplitude 0.2 mT. (b) Simulated spectrum of the signal in fig.2a. assuming axial g-tensor ($g_{\parallel} = 2.19$, $g_{\perp} = 2.05$, $A_{\parallel} = 1.5$ mT, $A_{\perp} = 3.5$ mT) and 8 mT line-width.

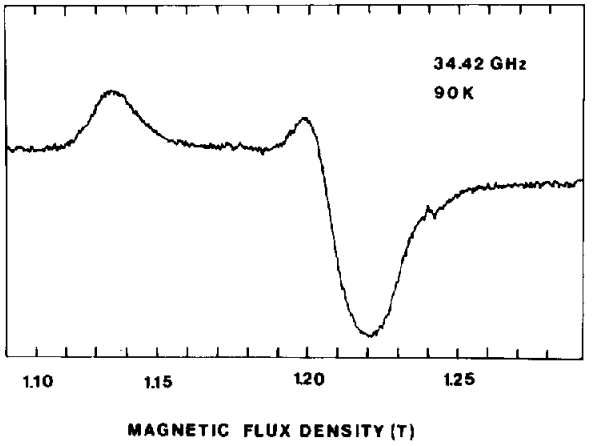

Fig.3. The spectrum was recorded at 90 K and 34.42 GHz. The microwave power was 40 mW, the modulation amplitude 0.2 mT. Other conditions as in fig.1.

tropy, which could arise from an anisotropic hyperfine coupling. Assuming that the unpaired spin couples to one Cu nucleus only, a reasonable good stimulation of the 9 GHz spectrum is obtained (fig.2b) if the hyperfine coupling constants are smaller or equal to: $A_{\parallel} =$ 1.5–2.0 mT, $A_{\perp} = 3.5$-4.0 mT. The integrated intensity of the signal amounts to 30% of the total copper content, i.e. less than one unpaired spin per pair of copper. The spectrum is rather unusual as compared to other copper proteins, but its parameters are such that is could arise from an isolated Cu^{2+}. For example, small hyperfine coupling constants have also been observed for the copper ion in stellacyanin [20]. However, the possibility that two copper ions are involved in the paramagnetic center must also be considered. If the protein is first completely reduced anaerobically with NH_2OH, subsequent addition of β-mercaptoethanol will not give rise to the green-colored complex and the new EPR signal is not formed. Since β-mercaptoethanol is known to be a reducing agent, the paramagnetic species is most likely a one-electron reduced form of this protein. However, this green species is not formed with other reducing agents and consequently it probably represents a complex between half-reduced protein and β-mercaptoethanol. As *Neurospora* tyrosinase does not have any disulfide bridges [2], only the copper can be reduced.

In summary, the absence of an EPR signal from the oxidized and reduced forms of the protein together with the new information that the protein contains two copper ions per molecule suggests the presence of a copper pair also in *Neurospora* tyrosinase. Since no paramagnetic NO-complex was observed the state of the two EPR-nondetectable copper ions is still not known with certainty. However, the rather unique observation of a paramagnetic species in *Neurospora* tyrosinase reacted with β-mercaptoethanol opens the possibility for further investigations of the copper site in the tyrosinases. Preliminary experiments with the mushroom enzyme also show that this tyrosinase forms an inactive, green, complex with β-mercaptoethanol [Lerch, K., unpublished]. EPR studies of this complex are currently being performed in this laboratory.

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References

[1] Jolley, R. J., Jr., Evans, L. H., Makino, N. and Mason, H. S. (1974) J. Biol. Chem. 249, 335–345.
[2] Fling, M., Horowitz, N. H. and Heinemann, S. F. (1963) J. Biol. Chem. 238, 2045–2053.
[3] Gutteridge, S. and Robb, D. (1975) Eur. J. Biochem. 54, 107–116.
[4] Lerch, K. (1976) FEBS Lett., submitted for publication.
[5] Schoot Uiterkamp, A. J. M. and Mason, H. S. (1973) Proc. Natl. Acad. Sci. USA 70, 993–996.
[6] Lerch, K. (1976) 11th Meeting of the Federation of European Biochemical Societies, Hamburg, Germany.
[7] Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412.
[8] Svenson, H. (1961) Acta Chem. Scand. 15, 325–341.
[9] Katan, T., Arnon, R. and Galun, R. (1975) Eur. J. Biochem. 59, 387–394.
[10] Brumby, P. E. and Massey, V. (1967) in: Methods of Enzymology (Estabrook, R. W. and Pullman, M. R. eds) Vol. X, pp. 463–474, Academic Press, New York.
[11] Carrico, R. J., Malmström, B. G. and Vänngård, T. (1971) Eur. J. Biochem. 20, 518–524.
[12] Aasa, R. and Vänngård, T. (1975) J. Magn. Res. 19, 308–315.
[13] Van Leeuwen, F. X. R., Wever, R. and Van Gelder, B. F. (1973) Biochim. Biophys. Acta 315, 200–203.
[14] Van Leeuwen, F. X. R., Wever, R., Van Gelder, B. F., Avigliano, L. and Mondovi, B. (1975) Biochim. Biophys. Acta 403, 285–291.
[15] Malmström, B. G., Andréasson, L. E. and Reinhammar, B. (1975) in: The Enzymes, (Boyer, P. D., ed) Vol. 12B, pp. 507–579, Academic Press, New York.
[16] Solomon, E. I., Dooley, D. M., Wang, R. H., Gray, H. B., Cerdonio, M., Mogno, F. and Romani, G. L. (1976) J. Am. Chem. Soc. 98, 1029–1031.
[17] Van Holde, K. E. (1967) Biochemistry 6, 93–99.
[18] Schoot Uiterkamp, A. J. M. (1973) Copper pairs, an EPR and optical study of hemocyanin and tyrosinase, Ph. D. Thesis, University of Groningen, Veenstra-Visser Offset, Groningen.
[19] Schoot Uiterkamp, A. J. M., Van der Deen, H., Berendsen, H. J. C. and Boas, J. F. (1974) Biochim. Biophys. Acta 372, 407–425.
[20] Vänngård, T. (1972) in: Biological Application of Electron Spin Resonance (Swartz, H. M., Bolton, J. R., Borg, D. C. eds) pp. 411–447, Wiley.