NEUROSPORA TYROSINASE: MOLECULAR WEIGHT, COPPER CONTENT AND SPECTRAL PROPERTIES

K. LERCH

Biochemisches Institut, Universität Zürich, Zürichbergstrasse 4, CH-8028 Zürich, Switzerland

Received 28 June 1976

1. Introduction

Tyrosinase is a Cu-containing mixed-function oxidase catalyzing the hydroxylation of monophenols and the dehydrogenation of *o*-diphenols. The mushroom enzyme has been shown to contain a copper pair at the active site based on EPR measurements [1], magnetic susceptibility studies [2], and on the stoichiometric reaction of one H_2O_2 per two Cu-ions [3]. On the other hand, *Neurospora* tyrosinase has been reported to contain one copper per molecular weight of 33 000 [4,5] which has been shown to be the functional unit [5]. Since the presence of either one or two copper ions per functional unit of tyrosinase is of great importance for the mechanism of this enzyme *Neurospora* tyrosinase was reinvestigated.

The results of this investigation in context of sequence studies demonstrated that the copper content and the molecular weight are greater than previously thought [4,5]. The spectral properties of the H_2O_2-treated enzyme were found to be very similar to oxytyrosinase from mushroom [3] and helix-pomatia hemocyanin [6]. The reaction of H_2O_2 with *Neurospora* tyrosinase indicated a stoichiometry of one H_2O_2 per two copper ions reacted as reported for mushroom tyrosinase [3]. Based on the results presented in this report *Neurospora* tyrosinase is assumed to contain a copper pair rather than one copper per functional unit.

2. Materials and methods

2.1. *Preparation of enzyme*

Tyrosinase from *Neurospora crassa* wild-type strain (FGSC No. 320) was purified by salt fractionation and a combination of ion-exchange and hydroxyapatite chromatography (Lerch, K., to be published). The enzyme was homogeneous according to the criteria of SDS-polyacrylamide electrophoresis [7] and isoelectric focussing [8]. Freshly isolated enzyme contained about 30% oxytyrosinase as has been reported by Gutterridge and Robb [5] using a different purification method. The specific activity of the purified enzyme measured by the procedure of Fling et al. [4] was slightly higher than the ones reported by Gutteridge and Robb [5], Katan et al. [9], and Fling et al. [4]. The enzyme was stored in 10 mM sodium phosphate buffer, pH 7.2, + 0.5 M NaCl at 4°C.

2.2. *Molecular weight*

The minimal molecular weights of the pure enzyme and its cyanogen bromide fragments (Lerch, K., to be published) were determined by SDS-polyacrylamide electrophoresis [7] and by gel filtration in 6 M guanidine hydrochloride [14] using the reduced and carboxymethylated derivatives of bovine serum albumin (68 000), catalase (60 000), aspartate aminotransferase (46 000), aldolase (40 000), lactate dehydrogenase (36 000), chymotrypsinogen A (25 700), myoglobin (17 200), ribonuclease (13 700), cytochrome *c* (11 700), and insulin chain B (3500) as marker proteins.

The molecular weights of the three smaller cyanogen bromide fragments were also obtained from amino acid sequence data (Lerch, K., to be published).

2.3. *Analytical methods*

Amino acid analysis was carried out according to

Moore and Stein [10] using norleucine as an internal standard [11]. Samples were analyzed on a Durrum D-500 automatic amino acid analyzer after hydrolysis in 6 N HCl at 110°C for periods of 24, 48, and 72 h. Protein concentrations were determined gravimetrically according to [12], refractometrically with a Beckman analytical ultracentrifuge [13], and from amino acid composition [14]. The copper content was determined with 2,2-biquinoline [15] using an Instrumentation Laboratory Inc. reference standard. Titrations of the enzyme with hydrogen peroxide (Merck Superoxol, 30% H_2O_2) were carried out at 4°C using a Cary model 15 spectrophotometer. The concentration of hydrogen peroxide was determined titrimetrically with a standardized $KMnO_4$ solution (Titrisol, Merck). All chemicals were of analytical grade and were used without further purification. Solutions were made with deionized, distilled water.

3. Results and discussion

Neurospora tyrosinase has been reported to contain one copper atom per molecular weight of 33 000 by different investigators [4,5]. During sequence analysis of this enzyme it became apparent that spectrophotometric measurement of protein concentration, using the extinction coefficient $A^{1\%}_{1\,cm} = 15$ at 280 nm employed by these authors [4,5] resulted in large overestimates. Therefore, the extinction coefficient $A^{1\%}_{1\,cm}$ at 280 nm was determined by three independent methods. The results are shown in table 1. The data are in good agreement with an extinction coefficient $A^{1\%}_{280\,1\,cm}$ of 22 ± 1. Furthermore, from partial sequences of fragments the molecular weight of the protein was found to be significantly higher than the one obtained by the same authors [4,5]. Table 2 presents a summary of the molecular weights of the native enzyme

Table 1
Extinction coefficient $A^{1\%}_{2801\,cm}$ of *Neurospora* tyrosinase

Method	Extinction coefficient $A^{1\%}_{2801\,cm}$
Gravimetric determination after TCA-precipitation[a]	21.8 ± 0.8
Refractometric measurements[b]	22.2 ± 0.6
Amino acid composition[c]	23.0 ± 1.0

[a]Hoch and Vallee [12]
[b]Babul and Stellwagen [13]
[c]Cohen et al. [14]

Table 2
Molecular weights of *Neurospora* tyrosinase and its cyanogen bromide fragments

	Gel filtration in 6 M guanidine hydrochloride[a]	Amino acid sequence[b]	SDS gel electrophoresis[c]
CNBr-I	21 500		24 000
CNBr-II	9000	9100	9000
CNBr-III	7500	7400	7000
CNBr-IV	4000	4200	4000
Sum of fragments	42 000	–	44 000
Untreated enzyme	42 000	–	52 000[d]

[a]Determined according to Mann and Fish [16]
[b]Lerch, K., to be published
[c]Determined according to Weber and Osborn [7]
[d]The higher mobility in SDS is probably attributable to its high proline content [17]

Table 3
Copper content of 3 different preparations of *Neurospora* tyrosinase

Preparation	Copper content (nmoles copper per mg protein)	g atoms Cu/42 000 g protein
30/4	46.4	1.95
29/3	43.6	1.85
10/5	44.4	1.88

and its cyanogen bromide fragments using a number of different methods. The data are in general agreement with a molecular weight of about 42 000. Table 3 shows the copper content of three different preparations as well as the number of Cu ions per molecule. Based on the new molecular weight *Neurospora* tyrosinase contains close to two g atoms of copper per functional unit of mol. wt. 42 000. *Neurospora* tyrosinase thus resembles mushroom tyrosinase for which the presence of a copper pair has been amply documented [1–3].

The similarity to mushroom tyrosinase is also supported by spectroscopic data. In the presence of air both *Neurospora* tyrosinase [5] and mushroom tyrosinase [3] have been shown to react stoichiometrically with hydrogen peroxide to yield an oxygenated complex, characterized by a strong absorption band at 345 nm and a weaker one at 600 nm which disappear upon removal of oxygen. The spectra of the native and the hydrogen peroxide treated *Neurospora* tyrosinase are given in fig.1. The extinction coefficients at 345 and 600 nm referring to the Cu content are $9 \cdot 10^3$ M^{-1} cm^{-1} and $5 \cdot 10^2$ M^{-1} cm^{-1}, respectively. These values are in good agreement with the ones reported for mushroom tyrosinase [3] and hemocyanin [6] whereas the molar extinction coefficient at 345 nm given by Gutteridge and Robb for *Neurospora* tyrosinase [5] is smaller. The reaction of H_2O_2 with concentrated *Neurospora* tyrosinase (80–120 μM enzyme) using small amounts of hydrogen peroxide (10 μM H_2O_2) is documented in fig.2. The increment in absorbance at 345 nm was constant over several additions indicating a stoichiometric reaction of H_2O_2 with the enzyme. The extinction coefficient with respect to H_2O_2 calculated from the slope is $16.9 \cdot 10^3$ M^{-1} cm^{-1} (table 4) (average from 3 titrations). This value compares favorably with the one obtained from mushroom tyrosinase [3] but is quite different from the one reported by Gutteridge and Robb [5]. The stoichiometry of the H_2O_2 reacted per Cu as given by the

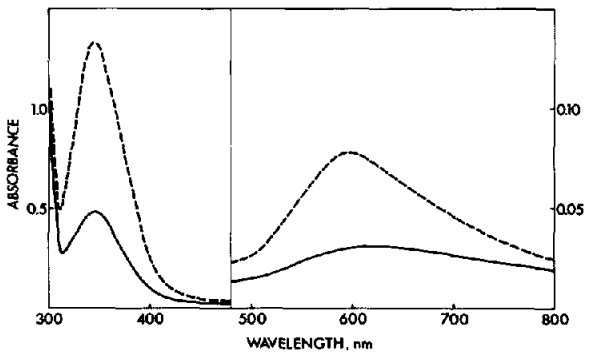

Fig.1. Absorption spectrum of native (——) and H_2O_2 treated (- - - -) *Neurospora* tyrosinase at 4°C. The concentration of the enzyme was 75 μM and a 5-fold molar excess of H_2O_2 was used. The spectrum was recorded 10 min after addition of H_2O_2.

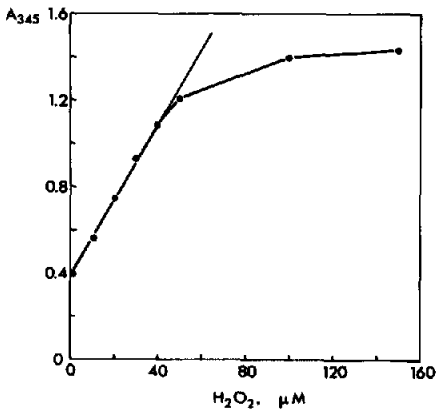

Fig.2. Determinations of the molar extinction coefficient of the oxygenated complex at 345 nm with respect to H_2O_2 reacted. Aliquots of 10 μM H_2O_2 were added to a concentrated solution of tyrosinase (82 μM) at 4°C. The sloper (extinction coefficient ϵ_{345, H_2O_2}) is $17.2 \cdot 10^3$ M^{-1} cm^{-1}.

Table 4
Extinction coefficients of H_2O_2 treated mushroom and *Neurospora* tyrosinase at 345 nm and stoichiometry of the reaction

	ϵ_{345}, H_2O_2 (M^{-1} cm^{-1})	ϵ_{345}, Cu (M^{-1} cm^{-1})	H_2O_2/Cu
Mushroom[a]	$17.2 \cdot 10^3$	9.1	0.53
Neurospora	$16.9 \cdot 10^3$	9.0	0.53

[a]Jolley et al. [3].

ratio of the extinction coefficients $\epsilon_{345, Cu}$ and ϵ_{345, H_2O_2} is listed in the same table. Again, this value compares favorably with mushroom tyrosinase [3] but differs considerably from the one reported by Gutteridge and Robb [5] for *Neurospora* tyrosinase. The discrepancy of the values given by the latter authors and the ones reported in this paper is in part explained by the use of a different molecular weight and a different extinction coefficient at 280 nm.

Acknowledgements

I would like to thank Miss Claudia Longoni for excellent technical assistance, Miss Erika Boller for preparing the enzyme, and Dr J. Kägi for valuable discussions. This study has been supported by the Swiss National Science Foundation, grant 3.409-0.74.

References

[1] Schoot Uiterkamp, A. J. M. and Mason, H. S. (1973) Proc. Natl. Acad. Sci. USA 70, 993.
[2] Makino, N., McMahill, P., Mason, H. S. and Moss, T. H. (1974) J. Biol. Chem. 249, 6062.
[3] Jolley, R. J., Jr., Evans, L. H., Makino, N. and Mason, H. S. (1974) J. Biol. Chem. 249, 335.
[4] Fling, M., Horowitz, N. H. and Heinemann, S. F. (1963) J. Biol. Chem. 238, 2045.
[5] Gutteridge, S. and Robb, D. (1975) Eur. J. Biochem. 54, 107.
[6] Lontie, R. and Witters, R., in Peisach, J., Aisen, P. and Blumberg, W. E. (1966) The Biochemistry of Copper, Academic Press, New York and London, 455.
[7] Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406.
[8] Svensson, H. (1961) Acta Chem. Scand. 15, 325.
[9] Katan, T., Arnon, R. and Galun, E. (1975) Eur. J. Biochem. 59, 387.
[10] Moore, S. and Stein, W. H. (1963) Methods Enzymol. 6, 819.
[11] Walsh, K. A. and Brown, J. R. (1962) Biochem. Biophys. Acta 58, 596.
[12] Hoch, L. and Vallee, B. L. (1953) Anal. Chem. 25, 317.
[13] Babul, J. and Stellwagen, E. (1969) Anal. Biochem. 28, 216.
[14] Cohen, P., Duewer, T. and Fischer, E. H. (1971) Biochemistry 10, 2683.
[15] Poillon, W. N. and Dawson, C. F. (1963) Biochim. Biophys. Acta 77, 27.
[16] Mann, K. G. and Fish, W. W. (1972) Methods Enzymol. 26, 28.
[17] Furthmayr, H. and Timpl, R. (1971) Anal. Biochem. 41, 510.