QUATERNARY STRUCTURE OF MUSHROOM TYROSINASE

K. G. Strothkamp, R. L. Jolley and H. S. Mason

Department of Biochemistry, School of Medicine, University of Oregon Health Sciences Center, Portland, Oregon 97201

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SUMMARY

The quaternary structure of Agaricus bispora tyrosinase has been investigated by sodium dodecylsulfate-acrylamide gel electrophoresis. The enzyme was found to contain two types of polypeptide chains, referred to as Heavy, molecular weight 43,000 ± 1,000, and Light, molecular weight 13,400 ± 600. In aqueous solution the predominant form of tyrosinase m.w. 120,000, has the quaternary structure L_2H_2.

INTRODUCTION

Concentration-dependent association-dissociation equilibria of fungal tyrosinases have been reported by several investigators (1 - 3). Some preparations of enzyme from the same source (mushroom or Neurospora) appear to dissociate more or less extensively than others (1, 4-11). In the case of mushroom tyrosinase, the minimum molecular weights observed by sedimentation methods have been 26,000 - 32,000 (1, 4) and the highest, about 120,000 - 128,000 (5, 6, 7). It has been widely assumed that mushroom tyrosinase consists of four subunits m. w. about 30,000, each containing one copper atom (1, 5), because the molecular weight of the associated form of the enzyme is about 120,000, and it contains four copper atoms. Active monomeric tyrosinases from species other than fungi appear to contain a single copper atom (12, 13, 14). On the other hand, recent work has clearly shown that copper atoms in tyrosinase occur in pairs (15 - 19). Since the number of copper atoms at the active site has mechanistic and functional importance, we have now reinvestigated its quaternary structure using SDS* polyacrylamide gel electrophoresis.

*SDS: sodium dodecylsulfate

METHODS AND MATERIALS

The α-, β-, and δ-isozymes of tyrosinase were prepared from mushroom (Agaricus bispora) (17, 20). The preparations had the specific activities and copper contents previously described. Analytical SDS gel electrophoresis was performed with the Ortec system (Ortec Inc., Oak Ridge, Tenn.) in 8% acrylamide gels containing 0.1% sodium dodecyl sulfate. Samples were prepared by diluting them 1:1 with 3% SDS, 40% sucrose and Tris-acetate, (0.01 M, pH 8.0); β-mercaptoethanol was added to give a 1% solution, and EDTA (pH 8.0) to give 10^{-3} M. The mixture was placed in boiling water for 5-10 min, or held at 37°C for several hours and let cool to room temperature. Final protein concentrations were about 1.5 mg per ml. Proteins used as molecular weight markers were prepared in an identical way. A marker peptide containing residues 1 - 65 (m. w. = 7,650) was prepared by cyanogen bromide cleavage of Sigma Type III cytochrome *c* (21, 22). Gels were stained with Coomassie blue R-250 for protein, and with a mixture of 4-tert-butyl catechol and L-proline (for catecholase activity) or p-cresol and L-proline (for cresolase activity) (23).

Pure subunits of tyrosinase were prepared by column SDS-acrylamide gel electrophoresis, using the same gel and buffer compositions as in the analytical work. A section of gel was rapidly stained for protein with Coomassie blue and the positions of the bands determined. The bands were cut out and eluted from the unstained gel as previously described (24). The homogeneity of isolated subunits was monitored by analytical SDS electrophoresis.

Samples for amino acid analysis were subjected to hydrolysis for 24, 48, and 72 hours at 110°C using constant boiling HCl containing 0.01% phenol. Cysteine and cystine were determined as cysteic acid after performic acid oxidation (25). All analyses were performed on a Beckman Model 120C amino acid analyzer. Attempts to reconstitute active tyrosinase subunits were made using procedures of Weber and Kuter (26). The molar ratio of the two subunits was determined by integrating densitometer scans of the analytical gels of whole enzymes. Concentration ranges were used which had been shown to be stained linearly with subunit concentration. A Transidyne integrating densitometer was used for this work.

RESULTS

Figure 1 shows typical SDS electrophoretic patterns of tyrosinase. Under strongly denaturing conditions (high SDS/protein ratio, heating at 100°C) two main bands were seen, along with several faint bands. The faint bands varied in intensity and position from one sample to the next. Some were intensified when mercaptoethanol was omitted from the denaturation mixture, and probably represented fragments produced by disulfide coupling, or, in some cases, proteolysis during preparation. Nine molecular weight markers were used to obtain a calibration curve (Figure 2). In all cases unknowns and standards were run simultaneously to overcome the possibility of small day-to-day variations in gel composition. No significant differ-

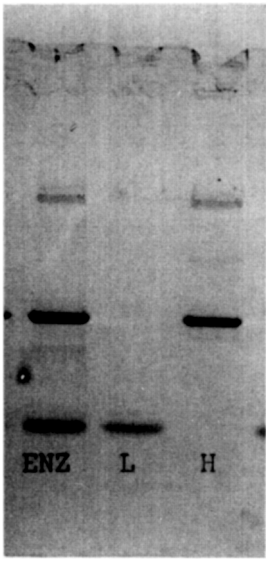

Figure 1. Analytical SDS-polyacrylamide gel of β-tyrosinase and its isolated subunits. The proteins were treated with 1% SDS at 100°C for 3 minutes. Enz, holoenzyme, 10μg; L, light subunit, 2μg; H, heavy subunit, 6μg.

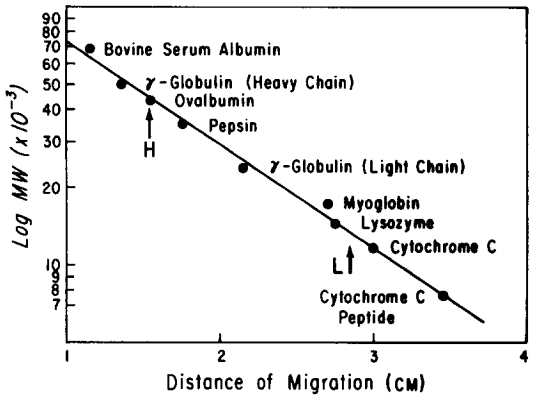

Figure 2. A typical molecular weight determination for the L and H subunits of β-tyrosinase. Nine molecular weight markers were used, and the best straight line was determined by the least squares approximation. Arrows mark the positions of the H and L subunits on the calibration curve.

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ences were observed in the subunits among the α-, β-, and δ-isozymes of tyrosinase based on 24 determinations of molecular weight. The molecular weights were, for the heavy subunit, H, 43,000 ± 1,000, and for the light subunit (L), 13,400 ± 600. These subunits did not have catecholase or cresolase activity.

TABLE I

Amino acid composition of H and L subunits and β-tyrosinase

Number of residues (to nearest integer)

Amino Acid	H	L	H_2L_2	β-tyrosinase*
LYS	18	7	50	44
HIS	13	2	30	28
ARG	18	6	48	48
ASP	45	16	122	144
THR	25	9	68	76
SER	23	10	66	60
GLU	41	11	104	124
PRO	32	6	76	76
GLY	19	17	72	88
ALA	26	7	66	68
1/2 CYS	2	1	6	8
VAL	21	8	58	64
MET	12	2	28	20
ILU	19	7	52	56
LEU	29	8	74	80
TYR	19	4	46	44
PHE	23	6	58	56
TOTAL	385	127	1,024	1,084
MW	43,700	13,600	114,600	120,400

*Taken from Jolley, R. L., et al., J. Biol. Chem., 244, 3251 (1969).

When the β- and δ-isozymes were incubated with 3% SDS at 37°C, or at low SDS/protein ratios, a band with an apparent molecular weight of 69,000 was observed; this band possessed both catecholase and cresolase activities (cf. 5). The α-isozyme did not form this band. The apo-enzyme prepared by dialysis against cyanide (18), gave the same electrophoretic pattern in SDS as native enzyme gave. Addition of NaCN, β-mercaptoethanol, EDTA, or β-mercaptoethanol plus EDTA to incubation mixtures of either native or apoenzyme produced no change in the electrophoretic pattern. Attempts to renature isolated subunits by removal of SDS to give apoprotein, followed by addition of Cu^{2+} at pH 8 (borate buffer), Cu^{2+} + cysteine at pH 8.6, $CuCl_4^{-3}$ at pH 5.6 (citrate-phosphate), or $Cu[CH_3CN]_4$ at pH 5.6, were unsuccessful.

Eletrophoresis of SDS-free subunits without denaturant indicated that more than 90% of the protein aggregated to species with very high molecular weights under these conditions.

The amino acid compositions of purified H and L subunits of β-isozyme are given in Table I, compared with that of native enzyme. The molar ratios of H to L, determined by densitometry, based upon analytical electrophoresis of nine separate enzyme preparations, and molecular weights of 43,000 and 13,400 respectively, was 1.0 ± 0.1 (S.D.).

DISCUSSION

We have found that native mushroom tyrosinase consists of two types of polypeptide chains, H (molecular weight 43,000) and L (molecular weight 13,400). These results are not due to proteolysis of native enzyme because these two species account for more than 90% of the protein seen on electrophoretic gels in all mushroom tyrosinase preparations examined including both freshly isolated enzymes and enzyme stored under sterile conditions. The electrophoretic pattern was not altered by omitting β-mercaptoethanol treatment, or by treatment with cyanide. Accordingly, disulfide bonds do not hold the subunits in the native enzyme together.

In the light of these results, it is evident that there is no "monomer" with m.w. 30,000 and containing one copper atom, as previously assumed. The proteins with reported molecular weights of 26,000 (1), 34,500 (4), and 32,000 (11) must be dimeric L_2 aggregates. The active enzyme, apparent m.w. 69,000, may then be L_2H (m.w. 69,800). The distribution of copper among the L and H subunits, and the function, regulatory and/or catalytic, of the association-dissociation phenomenon, remain to be determined.

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