ON THE NATURE AND MODE OF ACTION OF THE COPPERPROTEIN, TYROSINASE

I. EXCHANGE EXPERIMENTS WITH RADIOACTIVE COPPER AND THE RESTING ENZYME

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SUMMARY

1. Radioactive ^{64}Cu and ion-exchange techniques have been applied to study the incorporation of radioactive copper into resting tyrosinase.

2. Under the experimental conditions, the radiocopper incorporation into the enzyme was found to be small and decreased with increasing purification of the enzyme.

3. The data are used to present the view that the functional copper of the enzyme is bonded to the protein in a non-dissociable bond.

4. The results are explained in terms of two types of copper sites (active and non-exchangeable; inactive and exchangeable) in the impure and/or partially inactivated enzyme.

INTRODUCTION

The enzyme tyrosinase, also called polyphenol oxidase[1,2], has long been known to contain copper as a factor essential for its activity[3–5]. However, very little is known as to the nature of the bond between the copper and the protein. The mode of function of the tyrosinase copper during its catalysis of the aerobic oxidation of mono- and *o*-dihydric phenols is not understood.

A number of years ago, JOSELOW AND DAWSON[6,7] used ^{64}Cu and ion-exchange techniques in an investigation of the nature of the copper–protein bond in the enzyme ascorbic acid oxidase. They found that the copper–protein bond in the resting enzyme is non-dissociable, as evidenced by the fact the non-functioning enzyme did not exchange its copper when exposed to radioactive cupric ions. The approach used in those studies has provided methods for obtaining valuable information about the properties of other copper proteins.

Tyrosinase is particularly interesting because it is a copper protein that possesses two essentially different catalytic activities, *i.e.*, monophenolase and *o*-dihydric-

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phenolase activity[1]. It plays an important role in the pigmentation processes of many plants and animals and is believed to be involved in the respiration of some plant species[8,9]. The present investigation deals with exchange experiments between radioactive cupric ions and resting (non-functioning) tyrosinase.

EXPERIMENTAL

Tyrosinase

The common mushroom, *Psalliota compestris*, served as the enzyme source. The method of preparation used was that described earlier by workers in these laboratories[10]. The catecholase activity of the enzyme was measured by the chronometric method[11]. The cresolase activity was measured manometrically[12] and the protein content of the enzyme preparations was estimated by determination of the dry weights of dialyzed aliquots[10]. The characteristics of the enzymes used in this investigation are summarized in Table I.

TABLE I

CHARACTERISTICS OF THE TYROSINASE PREPARATIONS USED IN THE EXCHANGE EXPERIMENTS

Enzyme preparation No.	mg/ml dry wt.	μg Cu/ml	% Cu	Catecholase activity per			Cresolase activity per			Catecholase cresolase activity ratio
				ml	mg	μg Cu	ml	mg	μg Cu	
C308	4.9	7.0	0.14	11700	2390	1670	315	64	45	37.2
C305D2	4.4	7.4	0.17	10350	2450	1400	285	64	37	36.4
C312-2	2.2	4.5	0.20	5730	2600	1280	118	53	26	48.6
C305D	4.4	7.4	0.17	12100	2730	1640	280	64	38	43.2
C313-1	4.8	7.1	0.15	13100	2740	1850	165	34	23	79.3
C311-2	5.9	8.8	0.15	19100	3240	2170	365	62	42	52.3
C314-M	7.2	11.7	0.16	25100	3500	2140	510	71	44	49.2
C306D	5.0	4.4	0.09	3640	728	830	348	70	79	10.5
C307D	6.3	7.3	0.12	10100	1600	1390	935	148	128	10.8

In general, two types of tyrosinase preparations can be distinguished[13]. Those of lower molecular weight having a ratio of catecholase to cresolase activity of about 20 or higher are called "high catecholase" enzymes. Those having a higher molecular weight and a catecholase to cresolase activity ratio of about 10 or lower are called "high cresolase" enzymes. Intermediate preparations can also be obtained. In Table I the seven "high catecholase" and the two "high cresolase" preparations are arranged in the order of increasing catecholase activity per milligram of dry weight (Column 6) which is a good measure of the purity of the preparation.

Measurement of copper

The method of WARBURG AND KREBS[14] was used to estimate the copper content of the several enzymes employed, of the cupric salt solutions used and of the ion-exchange column effluents. The method was satisfactory for the enzyme and standard copper solution analysis, detecting as little as 0.01 μg of copper with a precision of ± 5 %. However, the analysis of the column effleunts was less satisfactory (precision estimated to be ± 8 %). Several attempts to apply colorimetric methods[15,16] which were found to give good results with standard copper solutions, were unsuccessful in the estimation of the trace amounts of copper in the effluents.

Ion exchange resin

Analytical grade Amberlite IR-100*, a cation exchanger, was used in columns of the type described by JOSELOW AND DAWSON[6]. These columns contained about 3 g of resin (air dried) which had a capacity for copper far in excess of the actual needs of the experiment. The resin was conditioned and used in the Na^+ form as previously described[6]. The removal of cations by these columns depends on the rate of flow. It was established that a rate of flow through the resin column of about one drop per 40 sec (corresponding to 10 ml of effluent collected in 70 to 100 min) guaranteed the complete removal of much larger amounts of cupric ions than used in the exchange experiments. The enzyme was not deactivated or retained by the resin. When enzyme solutions were passed through the Amberlite IR-100 column, all of the tyrosinase activity and copper was recovered in the effluent. It was also found that the Amberlite resin removed added cupric ions quantitatively from acetate buffer solutions (pH 5.7) containing enzyme. The pH 5.7 was selected because it is above the isoelectric point of tyrosinase and in the range of optimum enzyme activity.

Radioactive copper solutions

The Brookhaven National Laboratories of Long Island, N.Y., supplied the ^{64}Cu (half-life 12.8 h) in wire form. The wire was dissolved in hot dilute nitric acid and dilutions of the resulting stock solution to the desired copper concentration were made with 0.1 *M* acetate buffer of pH 5.7. The short half-life of this isotope made necessary the completion of each experiment within a relatively short time. The radioactivity of the ^{64}Cu did not impair the catalytic activities of tyrosinase**.

The counting procedure

The manipulations were similar to the previously described operation[7]. Aliquots of the radioactive solutions were pipetted into the center well of aluminum planchets designed to fit a flow-type (Q-gas) counter. The samples were evaporated under an infrared lamp, then allowed to cool in a desiccator over phosphorus pentoxide before counting. Duplicate specimens were prepared and counted for all samples. All counts were corrected for decay and background. In none of the experiments was the counting rate high enough to necessitate coincidence corrections.

Actual exchange experiments

In each experiment a given amount of tyrosinase was mixed with an amount of radioactive cupric ions equivalent to the amount of enzyme copper. These solutions, buffered to pH 5.7 with 0.1 *M* acetate buffer, were let stand at room temperature (24–26°) for a certain length of time with frequent shaking and then they were passed through the cation exchange columns. The column effluents were subsequently assayed for radioactivity, enzyme activities, and copper content. The radioactive comparison standard solutions were identical with the mixtures investigated but were not passed through the Amberlite columns. As a measure of the exchange taking place, the counting rate of the effluents was expressed as a percentage of the counting rate of the comparison standard. This percentage is reported as the percent

* Rohm and Haas Co., Philadelphia, Pa. (U.S.A.).

** FRIEDEN[17] has previously reported that added cupric ions do not affect the activity of tyrosinase.

radioactivity incorporated into the enzyme. It should be kept in mind that, in the case where an equivalent amount of radioactive copper and enzyme are involved and no adsorption of cupric ions to the protein occurs, the percentage radioactivity incorporated into the enzyme can never exceed 50 %, *i.e.*, one half the radioactivity of the comparison standard. In this case of maximum exchange there has been a complete random distribution of the total added radioactivity between the enzyme copper and the ionic copper in the solution.

Other details

The solutions of reagents and buffer salts were prepared with distilled water that had been redistilled using an all Pyrex glass still with a hot wire around the reflux condenser to break the film. Such "copper-free" water contained less than 0.01 μg of copper /ml. The buffer salts were twice crystallized from copper-free water. Solutions of ascorbic acid (Chas. Pfizer Company) were freshly prepared before each experiment in which they were involved. Catechol and *p*-cresol (Eastman-Kodak Company) were resublimed and redistilled, respectively, prior to use.

RESULTS

The exchange experiments between resting tyrosinase and radioactive cupric ions at 40–50 min exposure time are summarized in Table II. The degree of purity of the enzymes, expressed in specific activities, is listed in column 2 of this table. The copper content of the cation exchange columns corresponded, within experimental error, to the copper content of the enzyme (columns 3 and 4). Small amounts of radioactive copper were incorporated into the enzyme (column 7). The data in Table II have been arranged in the order of decreasing exchange so as to indicate the inverse relationship that was found to exist between the specific activity ("purity") and the extent of radiocopper incorporation. This inverse relationship appears to be essentially linear

TABLE II

PERCENT RADIOACTIVITY INCORPORATED INTO THE RESTING ENZYME AFTER 40–50 MIN OF CONTACT WITH RADIOCOPPER

The amount of ^{64}Cu involved was the same as the copper content of the enzyme. Amberlite IR-100 column used.

Enzyme preparation No.	*Catecholase activity per mg dry wt.*	*Cu in enzyme before* μg	*Cu in effluent after* μg	*% in effluent of the original*		*% radioactivity* incorporated into the enzyme*
				Catecholase activity	*Cresolase activity*	
C306D	728	2.2	2.0	—	—	9.7 ± 0.8
C307D	1600	3.7	3.7	67	81	7.1 ± 1.3
C305D2	2450	3.7	3.4	69	57	5.0 ± 0.6
C305D	2730	3.7	3.3	73	73	3.6 ± 0.3
C308	2390	3.5	4.0	103	—	3.4 ± 0.4
C312-2	2600	4.5	4.1	106	61	1.9 ± 1.3
C313-1	2740	3.6	3.9	97	66	1.9 ± 0.7
C314M	3500	4.7	4.7	81	—	1.9 ± 0.3
C311-2	3240	4.4	3.9	67	48	1.1 ± 0.1

* The error analysis of the radioactive assay was carried out according to G. FRIEDLANDER AND J. W. KENNEDY, in *Introduction to Radiochemistry*, J. Wiley, New York, 1949.

when the % radioactivity incorporated into the enzyme is plotted against the catecholase activity/mg dry wt. Extrapolation of the linear relationship in the direction of higher enzyme activity indicates that an enzyme preparation having a specific activity of about 4000 catecholase units/mg dry wt. would not undergo exchange with radioactive cupric ions. This value of specific activity was that found by MALLETTE AND DAWSON[13] for homogeneous ("pure") tyrosinase. Unfortunately, an enzyme of comparable purity was not available for this investigation.

In another series of experiments, the dependence of the amount of radioactive copper incorporated into the enzyme on the length of contact time between the radiocupric ions and tyrosinase was established. The results of these experiments are presented in Table III. When these data are plotted, % incorporation *vs.* time, they indicate that a limiting value of exchange is approached after a period of time which appears to differ for each enzyme preparation. The limiting value again shows the inverse relationship between radioactive copper incorporation and the purity of the preparation, if the relatively high values for the highly purified preparation C 314 M are ascribed to a temperature effect.

TABLE III

PERCENT RADIOACTIVITY INCORPORATED INTO THE RESTING ENZYME ON VARYING THE TIME OF CONTACT WITH RADIOCOPPER

The amount of ^{64}Cu involved was the same as the copper content of the enzyme.

Enzyme preparation No.	*Catecholase activity per mg dry wt.*	*Time of contact with ^{64}Cu (min)*	*Cu in enzyme before µg*	*Cu in effluent after µg*	*% in effluent of the original*		*% radioactivity* incorporated into the enzyme*
					Catecholase activity	*Cresolase activity*	
C306D	728	40	2.2	—	—	—	9.7 ± 0.8
C306D	728	270	2.2	2.0	—	68	14.3 ± 0.9
C308	2390	45	3.5	4.0	103	—	3.4 ± 0.4
C308	2390	165	3.5	3.6	—	—	5.0 ± 0.5
C308	2390	285	3.5	3.7	83	—	6.7 ± 0.5
C308	2390	480	3.5	4.1	—	—	6.6 ± 0.5
C308	2390	1350	3.5	3.3	81	—	6.8 ± 0.5
C305D	2730	40	3.7	3.3	73	73	3.6 ± 0.3
C305D	2730	260	3.7	3.4	57	62	5.7 ± 0.6
C305D	2730	840	3.7	3.2	—	—	10.0 ± 0.1
C311-2	3240	45	4.4	3.9	67	48	1.1 ± 0.1
C311-2	3240	100	4.4	—	76	59	1.9 ± 0.2
C311-2	3240	180	4.4	3.9	—	—	2.4 ± 0.7
C311-2	3240	300	4.4	3.9	67	56	2.3 ± 0.7
C311-2	3240	480	4.4	4.2	—	—	2.8 ± 0.8
C311-2	3240	1170	4.4	4.1	74	59	5.0 ± 0.4
C314 M**	3500	45	4.7	4.7	81	—	1.9 ± 0.3
C314 M	3500	180	4.7	4.8	—	—	3.1 ± 0.4
C314 M	3500	360	4.7	4.9	58	78	4.6 ± 0.5
C314 M	3500	540	4.7	4.5	—	—	5.5 ± 0.4
C314 M	3500	1200	4.7	—	69	68	7.3 ± 0.4

* See footnote under Table II.

** All experiments with Prep. No. C314 M were carried out at 32° rather than 24–26° as with all other preparations.

DISCUSSION

Purified tyrosinase preparations do not lose their copper on dialysis against copper-free water under physiological conditions[2]. The cation exchange resin Amberlite IR-100 did not remove copper from the enzyme (Table II). These facts indicate that the copper is firmly bound to the tyrosinase protein. The low incorporation of radioactivity into highly purified tyrosinase (and the inferred failure of homogeneous tyrosinase to exchange copper) are further indication of the firm bond of copper to the tyrosinase protein. Indeed, these findings are evidence that the copper involved in the enzyme function is non-dissociable from the protein.

Previously, it has been shown that samples of resting ascorbic acid oxidase of varying degrees of purification did not exchange their copper with radioactive cupric ions[7]. Thus, tyrosinase behaved differently, since some incorporation of radioactivity was found. An inverse relation between the extent of exchange and the "purity" of tyrosinase was apparent. The enzyme lost activity during most experiments (Table II, columns 4 and 5), presumably due to the diluting of the enzyme stock solution and due to bringing it from refrigerator (storage) to room temperature. However, there does not appear to be any simple relationship between the enzyme inactivation and the amount of radiocopper incorporated into the preparation. Thus, as can be seen in Table II, the losses in catecholase and cresolase activities of enzymes C305 D2 and C 311-2 were quite similar but the amounts of radioactive copper incorporated were significantly different.

The observed incorporation of small amounts of radioactivity into tyrosinase might be explained on the basis of earlier observations and interpretations of KUBOWITZ[3]. This pioneer worker in the field of copper containing enzymes found that 100% reactivation of apo-enzyme (*i.e.*, enzyme from which the copper atoms had been removed with cyanide) required much more copper than was originally bound to the protein. He could not remove this excess copper by dialysis against water and concluded that copper could occupy inactive positions ("falsche Stellen") on the enzyme. Consequently it seems likely that there may be two types of copper-bonding sites in tyrosinase; active and inactive sites. If one assumes that the copper bound to the active sites is the more strongly bonded, it is to be expected that the purification of the enzyme will preserve these active sites preferentially. Thus, with increasing purification (higher specific activities), the number of more weakly bonded copper atoms decreases. It may be that these inactive copper sites are the only ones which undergo exchange with radioactive cupric ions. In other words the data obtained in this investigation suggests that resting tyrosinase undergoes exchange with radioactive cupric ions only when the enzyme contains copper bonded at inactive sites, *i.e.*, only when the enzyme is impure and/or partially inactivated.

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