Radiation-damaged tyrosinase molecules are inactive

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ABSTRACT Target analysis of radiation inactivation of mushroom tyrosinase yields different target sizes for diphenoloxidase and monophenoloxidase activities, which correspond to the subunits H and HL_2 (or HL), respectively. After gel electrophoresis of irradiated samples, all diphenoloxidase activity is observed at the same position as seen in the original material. Radiolytic fragments contain no detectable activity, consistent with a fundamental assumption of target theory.

INTRODUCTION

The radiation inactivation technique can be used to determine the size of those molecules that are associated with a measurable function. The method requires that the loss of activity in irradiated samples must be due solely to radiation action directly on the molecules whose function is being measured. Under these conditions, it is assumed that radiation-damaged molecules are totally inactive (1, 2). Thus the only measurable activity surviving radiation exposure should be due to molecules that escaped radiation damage, and these are expected to retain all properties of the native enzyme. Measurements of two characteristics support this idea: irradiated enzymes show unaltered K_m values (3–6) and electrophoretic migration (7–9). However, the production of enzymatically active fragments by radiation is an untested possibility. The existence of such structures would increase the activity detected in irradiated samples and lead to molecular sizes smaller than those predicted by target theory.

Gel electrophoresis is the simplest technique for separating many radiolytic products from the starting material. Degradation products in irradiated enzyme samples have been detected by denaturing gel electrophoresis (7–9), but there have been no attempts specifically to seek active fragments after radiation exposure. In most cases this is not possible since enzyme activity is irreversibly lost after SDS treatment. Fortunately, mushroom tyrosinase can be used to examine this question since it has great stability and can regain activity after exposure to SDS (10). There are no previous target analyses of the radiation inactivation of tyrosinases, although a great deal is known both of the structure of the protein and the chemistry of its reactions (11, 12). In the present study we have successfully utilized this enzyme to provide a clear and unequivocal answer to the question of enzymatically active radiolytic fragments.

MATERIALS AND METHODS

Most of the studies were performed with a crude preparation of mushroom (*Agaricus bisporus*) tyrosinase obtained from Worthington Biochemical Corp. (Freehold, NJ) as a salt-free lyophilized powder and a few experiments utilized a preparation from Sigma Chemical Co. (St. Louis, MO). Human transferrin and BSA were obtained from Sigma Chemical Co. High and low molecular weight protein standards for gel electrophoresis were obtained from Bio-Rad Laboratories (Richmond, CA).

Proteins were dissolved in distilled water. For irradiation, 0.1–0.5-ml samples were placed in glass vials (No. 12012 LAB; Kimble Div., Owens-Illinois Inc., Toledo, OH), frozen at −80°C, and sealed with an oxygen-gas torch. Tyrosinase concentrations were 1.48 or 7.4 mg/ml. Some enzyme samples (0.1 ml) were diluted with 0.2 ml containing either distilled water, or transferrin (14.8 mg/ml), or BSA (7.4 mg/ml).

Frozen samples were irradiated with 13 MeV electrons at −135°C as described (4). Thawed samples were assayed for enzyme activity by two methods. The tyrosine + O_2 assay was as described in the Worthington Biochemical catalog (13). The reaction with 3-hydroxytyrosine (DOPA) was the DOPAchrome method as described (14). Surviving function after radiation exposure was fit with a least-squares analysis constrained to 1.0 at zero dose. Target analysis for the two enzyme assays was as described (4).

Samples containing different amounts of protein yielded widely different target sizes with both enzyme assays: the most dilute samples yielded very large target values (Fig. 1). At levels of 2 mg per vial and greater, the target values are independent of the quantity of protein. Virtually identical results have been reported for glutathione S-transferase (15) as well as glucose-6-phosphate dehydrogenase and acetylcholinesterase (unpublished studies from this laboratory). When different volumes of the same protein solution were irradiated (0.74 mg tyrosinase/100 μl and 2.22 mg tyrosinase/300 μl), the variation in target sizes was found to correlate with the absolute amount of protein per vial rather than the protein concentration. Addition of carrier protein to levels >2 mg protein per vial eliminates this effect and permits determination of the correct molecular size consistent with the known protein structure.

Protein samples were mixed with an equal volume of solution containing 2% SDS and 4 mM dithiothreitol (DTT) but were not heated; 240 μl (containing ~0.9 mg protein) were loaded in each well of a 10% polyacrylamide slab gel. Duplicate sets of samples from each radiation dose were placed on a single gel. Gel electrophoresis was performed

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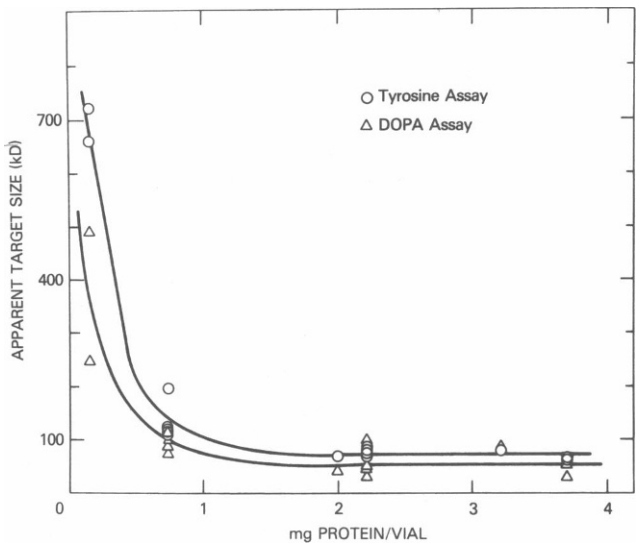

FIGURE 1 Target sizes of mushroom tyrosinase irradiated at −135°C in different protein solutions.

according to Maizel (16) at 100 V for 3 h until the phenol red dye front approached the bottom of the gel. Each gel was cut in half to separate the two sets. One set was stained for 1 h in a solution containing 0.1% Coomassie Blue R-250, 0.1% copper acetate, 10% acetic acid, and 25% isopropanol. Destaining took 72 h in a mixture of 7% acetic acid-5% methanol.

The other half of the electrophoretogram was washed for 1 h in Tris buffer (0.1 M, pH 6.8). These unstained gels were incubated in the dark for 1 h in 1 mM DOPA in 0.1 M phosphate buffer (pH 7) to reveal enzymatic activity and then stored in destaining solution.

Densitometry of gels was performed with a Zeineh scanning photometer (Biomed Instruments Inc., Fullerton, CA) with a laser light source. Similar results were obtained from scans of gel photographs.

RESULTS

Effects of freezing and thawing on DOPA and tyrosine reactions

Mushroom tyrosinase activity was measured before and after a freeze-thaw cycle at −80°C. Compared with fresh solutions, the frozen-and-thawed samples on the average retained 98 ± 20% of the DOPA reaction ($n = 19$) and 97 ± 15% of the tyrosine reaction ($n = 18$).

Survival of enzymatic activity after irradiation

Various frozen solutions of mushroom tyrosinase were exposed to different doses of high energy electron radiation. The surviving enzymatic activity in the thawed samples was measured by the tyrosine + O_2 reaction as well as the DOPA assay. The DOPA assay yielded more variable results than tyrosine. Fig. 2 shows the radiation

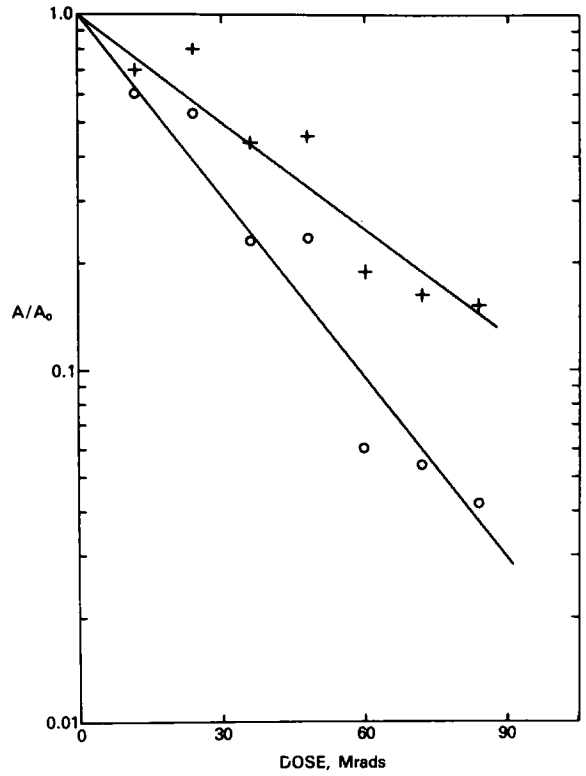

FIGURE 2 Radiation inactivation of frozen mushroom tyrosinase. Samples contained 2.0 mg tyrosinase in 500 μl distilled water. *Open circles*, from the tyrosine + O_2 assay; *crosses*, from DOPA assay.

inactivation from a typical experiment. With protein levels ≥2 mg per vial, the tyrosine + O_2 assay yields a target size of 74 ± 8 kD ($n = 8$). The target value of the DOPA function is 55 ± 23 kD; the large error (due to the variability in the DOPA assay) makes it appear that the two target sizes do not differ significantly. However, since the two assays were performed on aliquots taken from each irradiated sample, a paired t-test was employed and revealed that the two sets of data (and consequently the two target sizes) are significantly different ($P < 0.05$).

Gel electrophoresis of tyrosinase

Samples were treated with SDS and DTT without heating and subjected to electrophoresis as described. The Coomassie stain (Fig. 3, *right*) revealed multiple protein bands in this impure preparation, with predominant peaks near M_r 50,000 and 30,000. Smallest molecular weight compounds were retained near the bottom of the gel at the dye front. On the other half of the gel, enzymic activity was detected by the brown staining due to the product DOPAchrome (Fig. 3, *left*). Only one band of activity was observed, near M_r 70,000 (the Sigma preparation also contained a second, weaker band of activity near M_r

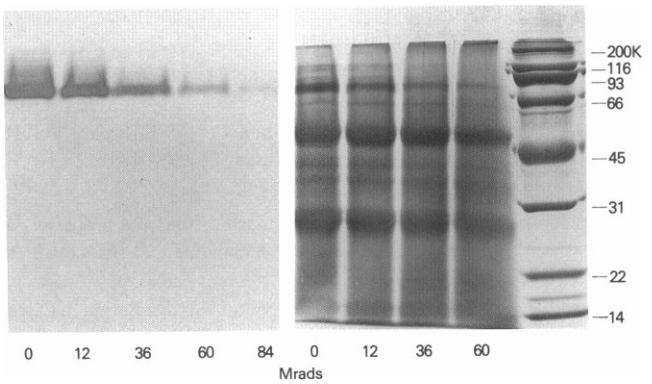

FIGURE 3 Enzyme activity (*left*) and protein pattern (*right*) in irradiated samples resolved by gel electrophoresis. Frozen tyrosinase solutions (2.22 mg in 300 μl) absorbed 0, 12, 36, 60, or 84 Mrads from high energy electrons. Diphenoloxidase activity (*left*) and Coomassie Blue staining (*right*) after electrophoresis is shown for samples with indicated radiation dose. Indicated M_r positions from Coomassie Blue–stained standards on the same gel. The front is seen at the bottom of the Coomassie Blue–stained gel.

40,000). No activity was observed near the major protein bands (which are due to contaminant proteins) nor was any activity recovered in samples heated with SDS and DTT before electrophoresis.

Irradiated samples were also subjected to the same gel electrophoresis (without heating). Enzymic activity was only observed at the position of the one band found in the controls, and the intensity decreased with radiation exposure (Fig. 3). The same results were obtained when the same preparation (Worthington Biochemical Corp.) was irradiated in a lyophilized state, or the Sigma enzyme in a frozen state. From densitometric scans of the DOPA-chrome reaction in Fig. 3, the DOPA activity remaining in this single band after 60 Mrads was 19% that of the control, the same value as found in the solution assay of the DOPA reaction in this sample. Thus all surviving activity is accounted by the single electrophoretic band. This result eliminates the possibility that active fragments are not detected on electrophoretograms because of the SDS + DTT treatment.

DISCUSSION

The tyrosinase reaction with DOPA is a measure of the diphenoloxidase activity, whereas the tyrosine substrate additionally involves the monophenoloxidase step (12). The monophenoloxidase activity yielded a target size of 74 kD, whereas the diphenoloxidase reaction to a value of 55 kD. The native form of mushroom tyrosinase is a tetramer described as an H_2L_2 structure, where the H subunits are 45,000–48,000 each and the smaller L subunits are 13,000–15,000 (12, 17) with no disulfide linkages between subunits (18). Active enzyme sedimentation of *Agaricus* tyrosinase gave the tetramer, M_r 110,000 (10, 17). Since tyrosinase subunits do not readily dissociate (17), this tetrameric value may only represent the smallest active unit which sediments under the experimental conditions. Indeed, Arnaud (10) also reported heat depolymerization of tyrosinase to an active form sedimenting at M_r 62,000, and Strothkamp et al. (18) reported the creation of an active M_r 69,000 structure they describe as HL_2. The target sizes observed in the present study are clearly smaller than the H_2L_2 tetramer. This implies that radiation-deposited energy is not transferred among all four subunits, and that enzymatic activity does not require the entire tetramer. The simplest model for interpretation of these target sizes is that monophenoloxidase activity requires a single H unit, while the additional step involved in the diphenoloxidase increases the required structure to HL or HL_2.

Coomassie and silver staining of irradiated proteins after gel electrophoresis has been widely reported (7–9, 19–21). Stain intensity of the original band decreases with radiation exposure (7–9, 22–25), and new areas of staining are observed lower on the gels (8, 9, 19–21). These results confirm the degradation of the original polypeptide structure by radiation with the production of smaller molecular weight fragments. A similar loss in Coomassie staining intensity was observed in the present study near the site of the original tyrosinase band, but this may be contaminated with non-tyrosinase protein. The presence of radiolytic fragments is obscured by the intense Coomassie staining of impurities in these samples. We have extended these observations by detection of enzymatic activity on gel electrophoretograms of samples which had been treated with SDS and DTT without heating. In both unirradiated and irradiated samples, tyrosinase activity is restricted to a single band with a mobility comparable to a protein of M_r 70,000, similar to the HL_2 structure previously described (18). The intensity of this enzymatic reaction in the gel band decreases with exposure to radiation in the same fashion as discussed above for protein staining. In contrast, however, no activity is detected in any protein fragment.

Two proteins which are known to retain enzymatically active domains after proteolysis also have been studied by radiation inactivation. Irradiation of the intact *Neurospora arom* complex yielded a target size for dehydroshikimic reductase activity corresponding to the entire dimeric structure rather than the fragment (26, 27). A similar result also has been obtained with the *E. coli* homoserine dehydrogenase II (methionine) activity which can be detected in a fragment (28), but again the target size is the complete structure (29). In these two cases, the correspondence of the target size with the holoenzyme

suggests that fragments do not contribute significantly to measured activity, but denaturation with SDS precludes a direct measurement.

The present results with tyrosinase confirm one of the fundamental assumptions of target theory: a protein molecule damaged directly by high energy radiation loses all holofunction. No fragments are produced that retain detectable activity. Together with the observations that irradiated enzymes show no changes in K_m (3–6), and that enzymes with active domains show target sizes corresponding to the entire molecule (26, 29), these results strongly suggest that the only surviving activity in irradiated proteins is due to those molecules that have escaped primary ionizations.

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