Radiation inactivation of galactose oxidase, a monomeric enzyme with a stable free radical

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Abstract: To determine the radiation sensitivity of galactose oxidase, a 68 kDa monomeric enzyme containing a mononuclear copper ion coordinated with an unusually stable cysteinyl-tyrosine (Cys-Tyr) protein free radical. Both active enzyme and reversibly rendered inactive enzyme were irradiated in the frozen state with high-energy electrons. Surviving polypeptides and surviving enzyme activity were analyzed by radiation target theory giving the radiation sensitive mass for each property. In both active and inactive forms, protein monomer integrity was lost with a single radiation interaction anywhere in the polypeptide, but enzymatic activity was more resistant, yielding target sizes considerably smaller than that of the monomer. These results suggest that the structure of galactose oxidase must make its catalytic activity unusually robust, permitting the enzymatic properties to survive in molecules following cleavage of the polymer chain. Radiation target size for loss of monomers yielded the mass of monomers indicating a polypeptide chain cleavage after a radiation interaction anywhere in the monomer. Loss of enzymatic activity yielded a much smaller mass indicating a robust structure in which catalytic activity could be expressed in cleaved polypeptides.

Keywords: galactose oxidase; radiation; target sizes; enzymatic activity; surviving monomers

Introduction

A group of proteins has been identified which share the unique property of maintaining a free radical in their native structure; the radical is required for enzymatic activity. An extensive literature has accumulated concerning the structure and catalytic function of many of these proteins.^{1–4} Detailed structural analyses have shown that in many of these molecules, the free radical resides on a specific amino acid residue near a metal ion that may be involved in the formation or stabilization of the radical. The

biological activity of the protein depends not only on the structural integrity of the protein but also on the presence of the free radical.

Radiation inactivation is a unique technique for study of the structure and function of macromolecules that has been very useful in determining the functional mass of proteins.⁵ Gamma rays and high-energy electrons ionize randomly throughout irradiated matter, principally with valence electrons. In each primary ionization, large amounts of energy (average 60 eV = 1500 kcal/mol) are transferred, resulting in structural damage to the molecules. The greater the number of electrons in a molecule, the greater the probability of a primary ionization. In the frozen state, the chance of such events is proportional to the mass of the macromolecule.

Radiation inactivation has previously been used to investigate a free-radical enzyme, ribonucleotide

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reductase, that is an $\alpha_2\beta_2$ heterotetramer; the smaller subunit contains the stable free radical. A radiation inactivation study of this enzyme⁶ described the radiation target sizes associated with both the function and the structure of the holoenzyme as well as those of the recombinant subunits. Among the results reported, one atypical feature was observed: the smaller subunit in isolation displayed a radiation sensitivity of enzymatic function which was vastly greater than the mass of the subunit, whereas the target size based on structure was comparable to the mass of the subunit. This very unusual result was interpreted in terms of an indirect effect of the ionizing radiation: radiation products generated elsewhere in the sample were proposed to have eliminated the free radical on this subunit and thereby prevented enzymatic catalysis. The holoenzyme did not show this extreme radiation sensitivity. It was suggested that in the isolated subunit, the solvent was accessible to the free radical, whereas in the holoenzyme, the free radical is buried deep inside the protein structure.

Both the unusual observation and the unique explanation suggested that other free-radical containing enzymes might display similar phenomena. Galactose oxidase (GO) was selected because of the detailed knowledge of its structure⁷⁻⁹ and because it is a monomeric protein. Each 68 kDa polypeptide contains 639 amino acids, a small amount of carbohydrate, and a copper ion located near an unusual cysteinyl-tyrosine thioether, which is the site of the free radical. The enzyme can also be prepared in an "inactive" state with neither copper nor free radical. This form can be "reactivated" by replacement of copper and oxidation to regenerate the free radical.

This project was undertaken to answer two questions: is the radiation sensitivity of this metalloenzyme much greater than that expected on the basis of its mass and does this sensitivity change if the free radical is removed?

Results

Multiple radiation experiments of GO were performed. Surviving monomers and surviving enzymatic activity were determined in three independent radiation experiments of native GO. Surviving monomers were determined in five radiation experiments of inactive enzyme; in four of these experiments, enzymatic activity was determined after reactivation by reconstituting with copper and treatment with ferricyanide to regenerate the metalloradical complex that is required for catalysis.

Denaturing gel electrophoresis showed GO monomers with mobility consistent with their molecular weight (Fig. 1). After radiation exposure, decreasing amounts of surviving GO monomers were observed. In addition, samples of both native and inactive GO exposed to large doses of radiation also showed a

Figure 1. Denaturing gel electrophoresis of native GO. Lane S, MW standards. Lanes 1-5: increasing amounts of unirradiated GO (0.2, 0.4, 0.6, 1.0, and 1.0 μ g) for calibration. Major band appears near M_r 68,000. Lanes 6-11: samples exposed to 3, 7.8, 15.6, 31.8, 65.1, and 92.9 Mrads, respectively.

smear of protein staining at lower molecular weights. This reflects a broad, nearly continuous distribution indicating random cleavage of GO. Because of the spread in molecular weights, staining of these cleavage products appears relatively faint compared with the discrete monodisperse protein band.

In the native enzyme containing both the free radical and copper ions, it was observed that the amount of surviving intact GO molecules decreased exponentially with radiation dose (Fig. 2). Inactive GO also displayed surviving monomers decreasing as an exponential function of radiation dose (Fig. 3). The radiation target size calculated in each of these experiments averaged 68.0 kDa (Table I). This value is very close to the known mass of this protein and indicates that a single primary ionization anywhere in the polypeptide chain leads to scission of the polymer backbone. This result is consistent with previous radiation studies of other proteins.⁵ Therefore, the presence of free radicals or of copper ions has no effect on the radiation sensitivity of the individual polypeptides.

Monomer measurements which yield radiation target sizes equal to that of a known polypeptide mass indicate that a radiation hit anywhere in the polypeptide leads to scission of the backbone, but does not necessarily mean that the scissions occur throughout the monomer. Preferential loci for chain scission would be revealed on denaturing gel electrophoresis of irradiated samples by the appearance of new, lower molecular weight bands rather than a broad smear of many different-sized fragments. Very faint discrete new bands could be observed in the stain smear in the GO experiments, but only after

Figure 2. Radiation inactivation of native GO. Surviving monomers (■) and surviving enzymatic activity (□) were measured from the same irradiated samples. Data combined from three independent experiments, shown as average $\pm$ S.D.

large radiation exposures equivalent to an average of more than two primary ionizations per molecule. The very small amounts of protein in these new

Figure 3. Radiation inactivation of inactive GO. Surviving monomers (●) and (after reactivation) surviving enzymatic activity (○) were measured from the same irradiated samples. Data combined from four to five independent experiments, shown as average $\pm$ S.D.

Table I. Radiation Target Sizes (in kDa) for GO; Average $\pm$ S.D. for $n = 3-5$

	Native	Inactive
Enzymatic activity	32.1 ± 14.9	29.0 ± 14.1 (after reactivation)
Monomer	68.0 ± 5.8	73.9 ± 9.7

bands and the limited radiation range in which they were visible precluded significant radiation analysis of these products in this study.

Activity measurements in irradiated GO samples (Figs. 2 and 3) showed considerable variation in the several experiments, but lead to a radiation target size in native GO samples of the order of 32 kDa—much smaller than the monomer target size or the known protein structure. Activity measurements in the irradiated inactive samples are especially difficult because of the many steps involved in reactivation. Nevertheless, with all the control and corrections included, it appears that similar activity results are observed; a radiation target size of the order of 29 kDa is calculated from the activity measurements. Thus, this extremely unusual result does not depend on the “activated state” of GO during irradiation. Similar results were obtained with both native and reactivated samples using the “direct” benzyl alcohol assay, indicating that the reactivation treatment was complete.

Irradiated samples of native GO were also analyzed by size-exclusion chromatography. A single peak containing all the surviving protein and enzymatic activity was observed in both the unirradiated and irradiated samples. No additional peaks of lower molecular weight were found in irradiated samples (Fig. 4). This finding is consistent with all reports that irradiated protein molecules do not dissociate even though there are scissions of the polypeptide backbone.^{10,11}

Discussion

In the present experiments, the direct actions of ionizing radiation on GO were the same whether the sample contained the native form of the enzyme or the inactive form in which copper and the free radical had been removed. The anticipated large radiation sensitivity of enzymatic activity seen in the isolated monomer of ribonucleotide reductase⁶ was not observed for GO.

Radiation destruction of GO polypeptides was found to be independent of the presence of free radicals or copper. The calculated radiation target size corresponded well with the mass of the polypeptide. This result is consistent with those found in all other proteins. A single radiation hit anywhere in a polypeptide causes one or more scissions of the polymer backbone; denaturing gel electrophoresis separates the surviving original polypeptide monomers

Figure 4. Size exclusion column analysis of enzymatic activity of native GO samples after various radiation exposures in the frozen state. Samples receiving 0 (○), 20 (●), 53 (◆), or 83 Mrads (■) are shown.

from the fragments arising from radiation-damaged monomers.

In the same irradiated samples, the surviving enzymatic activity of both native and inactive forms was the same, but was more resistant to radiation damage than the surviving polypeptides. Molecules that had suffered a scission of the polymer backbone were still capable of enzymatic catalysis. Such phenomena have been reported only rarely and explanation of such molecular events was not clear. Activity measurements yielding target sizes smaller than a single polypeptide have been reported for nitrate reductase,^{12,13} but it was only observed with measurements of “partial reactions,” not the native reaction. Related observations were reported for a few other enzymes,^{14,15} but none reported target sizes for loss of monomers.

The possibility of enzymatically active fragments is not normally expected,¹⁶ but must be considered. Preferential loci for chain scission would be revealed in irradiated samples by the appearance of new, lower molecular weight bands on SDS PAGE (rather than a broad smear of many different-sized fragments). Almost all proteins irradiated in air do not show any specific new fragments. No specific fragmentation of GO was seen after exposures in air to radiation which averaged one primary ionization per polypeptide, although small amounts were seen after radiation doses corresponding to two or more interactions per molecule. This result suggests that under these radiation conditions, specific fragmentation in GO is an uncommon event occurring in only a few molecules.

The target size for GO activity (irradiated in a nitrogen atmosphere and assayed by the method of Kosman *et al.*¹⁷) has been repeatedly reported as 68 kDa^{18–22} although it is not clear whether these were independent experiments or referred to one common set of experiments. In none of these was there any analysis of the loss of monomers. In an interesting pulse radiolysis study of GO²³ irradiation was in the liquid state where target analysis does not apply.

Size exclusion column analyses of irradiated GO was similar to results previously observed in many other proteins.^{10,11} Even though there are scissions of the polypeptide backbone, the radiation-fragmented protein molecules do not dissociate except under denaturing conditions. Only a single peak eluted from these size-exclusion columns; it contained essentially all the surviving protein and enzymatic activity. No additional peaks of lower molecular weight were found in irradiated samples, excluding the possibility of dissociated radiation fragments that are enzymatically active.

While the monomer peak seen on SDS PAGE is due to only the intact subunits, the size exclusion peak was composed of both native and scissioned protein molecules. Since irradiated proteins do not lose their native hydrodynamic properties,¹¹ the radiation fragments must remain attached in essentially the original form. In all previous cases, biological activity was rapidly lost.

The randomness of radiation damage throughout a polypeptide has been established in many different proteins; the absence of specific fragments (especially those of ~30 kDa, the target size for surviving activity in GO samples) indicates that random damage also holds for irradiated GO. If a single ionization anywhere in a polypeptide leads to cleavage of the backbone, and in the same material biological activity is only lost when a small structure is hit, it suggests that some of the polypeptide structure is not needed for function. Molecular explanation of the small target size for activity therefore turns attention to the polypeptide itself, suggesting that GO might have some unusual structure. The X-ray crystal structure shows that there are three separate domains in the GO architecture.^{7,9} An N-terminal targeting domain is structurally quite independent and may possibly be expendable; the C-terminal may also undergo chain cleavage without much impact on active site properties. It is the middle domain (residues 155–552, 40.55 kDa) whose structural integrity is most important for catalytic function. This region forms a β -propeller containing seven repeats of a kelch motif⁹ each of which has a mass of ~5.5 kDa. Thus, the observed target size may correspond to several repeats in the middle domain. Since specific fragments of ~30 to 40 kDa were not released on denaturation of irradiated GO, it is conceivable that the middle region of GO can

suffer random chain breaks and still remain enzymatically active. Such a situation might be akin to RNase peptides in which ribonuclease activity was restored by subtilisin-derived fragments.²⁴

The original questions stimulating this project have been answered: the radiation sensitivity of GO does not change if the free radical is removed and is not greater than that expected on the basis of its mass. Indeed, the radiation sensitivity suggests that the enzymatically active structure is smaller. GO is an unusual enzyme. The direct action of ionizing radiation on both native and inactive GO molecules reveal enzymatically active structures surviving in molecules with cleaved polymer backbones, suggesting a unique molecular structure and an opening for study of the mechanism of radiation energy transfer.

Materials and Methods

GO from *Dactylium dendroides* was purchased as a crude preparation from Worthington Biochemical Company. It was further purified using Sepharose 6B or Sepharose CL-6B.²⁵ These preparations showed only one band on denaturing gel electrophoresis.

Some of the purified enzyme was inactivated by removal of copper and free radicals; this inactive enzyme was reactivated by restoring both, as previously described.²⁶

Denaturing gel electrophoresis (SDS PAGE) was performed as described²⁷; protein was determined by Coomassie stain.

Enzymatic activity was determined by the o-tolidine method.²⁸ Briefly, an aliquot of enzyme was pipetted into an assay mixture containing galactose (50 mg/mL), horseradish peroxidase (5 µg/mL), and o-tolidine (2.8 mg/mL) in 50 mM potassium phosphate buffer, pH 6. The absorbance of the assay mixture was monitored at 425 nm and enzymatic activity of calculated: one unit of activity is defined as the amount of enzyme that yields an increase of 1 O.D. 425 nm per min in the coupled assay.

Samples that had been frozen at -80°C and thawed retained enzymatic activity. Because of the many steps involved in reactivation, samples were corrected for variations in both volume and protein (as determined by the enhanced bicinchoninate (BCA) method²⁹). Inactive samples displayed a small amount of enzyme activity that increased 300-fold on reactivation. Additional enzyme activity measurements were performed with the "direct" assay using 3-methoxy benzyl alcohol.³⁰

Liquid chromatography analysis used size exclusion columns as described.¹¹ Aliquots of irradiated GO were loaded on agarose-sephadex columns. Samples were collected in the effluent and analyzed. Protein was quantitatively detected by ultraviolet absorption; enzymatic activity in eluted fractions was determined by the o-tolidine method.

Samples of 0.25 mL containing 2 mg protein/mL were placed in glass ampoules, frozen at -80°C , and sealed with an oxygen-gas torch. Frozen samples were held at -80°C except when irradiated at -135°C . Irradiations were performed with a beam of high-energy (13 MeV) electrons at the Armed Forces Radiobiology Research Institute (Bethesda, MD) as described.³¹

Analysis of irradiated sample measurements were performed as described.³¹

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