RESEARCH ARTICLE

Radiation Inactivation of Glutamate Dehydrogenase Hexamer: Lack of Energy Transfer Between Subunits

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Abstract. *The effects of ionizing radiation on glutamate dehydrogenase and on fluorescein isothiocyanate–tagged glutamate dehydrogenase were analyzed by target theory. Enzymatic activity, fluorescence, and the survival of the 56,000-dalton monomer subunit were determined on frozen samples irradiated at −135°C and on lyophilized samples irradiated at either −135°C or +30°C. The effects of temperature were the same for all three parameters. Enzymatic activity was lost after small doses of high-energy electrons, whereas fluorescence and monomer subunits survived much larger doses of radiation. Target analysis revealed that the functional unit size for enzymatic activity was the hexamer, confirming both the earlier radiation study and conventional biochemical analyses. Target sizes obtained from fluorescence and subunit structure measurements were close to that of the monomer. These results indicate that the primary ionization caused by electron bombardment results in damage to a single polypeptide strand and that there is no massive transfer of radiation energy to other units in the hexamer. The large target size observed for enzymatic activity appears to be a structural requirement for the simultaneous presence of six intact subunits rather than the result of the spread of energy from the initial site to adjacent chains with consequent damage to other subunits.*

Ionizing radiation deposits large amounts of energy at each interaction with matter, and dissipation of this energy within the target material results in structural damage to the affected molecules. The mechanism of energy dissipation is an important problem in several scientific areas; for example, biologically active molecules such as enzymes and receptors lose the ability to function, but details of the structural disruption are not completely understood. Synthetic polymers display altered physical and chemical characteristics after exposure of bulk plastics to ionizing radiation, and details of the mechanisms involved in the alterations have been a subject of great interest in this field for many years. The transfer of energy from the site of the primary ionization to other areas of the affected polymer involves several different physical processes; many of these are comparable to those observed in ultraviolet and laser irradiation, which have also been widely studied.

Enzymes can offer an especially advantageous system for the study of some aspects of these problems. Determining the effects of radiation on the biochemical activity of these molecules offers one experimental approach; another is analyzing the residual structure after irradiation. Since the individual molecules of a single enzyme species have identical and precisely defined structures, an enzyme population can be examined for the presence of common radiation products or effects. We used this approach to answer a question fundamental to the understanding of radiation effects on all macromolecules.

The structure of glutamate dehydrogenase from bovine liver is well known. Six identical monomers of 56,000 daltons are arranged as two sets of three chains to form the enzymatically active hexamer (*1*). The chains are joined by noncovalent bonds (*2*). The amino acid sequence of the enzyme is known (*3*), as is the binding site of the substrate (*4*). At high concentrations, the purified enzyme forms supramolecular associations of more than 2×10^6 daltons—octamers of hexamers (*5*).

Earlier study of the radiation inactivation of the lyophilized enzyme (*6*) confirmed that the hexamer is the functional unit for enzymatic activity. This result indicates that a primary ionization occurring anywhere in the complex of six subunits results in the complete loss of function of the hexamer. It has previously been pointed out (*7*) that there are alternative explanations for this observation. The enzymatic activity might require the intact structure of all subunits. Alternatively, only one subunit might be active, but massive amounts of energy could be transferred from the damaged monomer to the other five subunits, resulting in severe structural damage to all. The latter explanation was experimentally unsupported. The two models lead to different predictions for the state of the five remaining subunits in each enzyme molecule. These two models are tested in the present study.

Materials and Methods

The enzyme L-glutamate dehydrogenase (E.C.1.4.1.3) type III from bovine liver was obtained from the Sigma Chemical Company as a lyophilized powder containing approximately 30 percent by weight of a citrate buffer salt. Fluorescein isothiocyanate (FITC) isomer I bound to Celite was also obtained from Sigma.

Lyophilized glutamate dehydrogenase (GlDH) was dissolved in 0.05*M* carbonate buffer, *p*H 9.4, to a concentration of 6 nmole/ml. Coupling of the enzyme to FITC was accomplished by the method of Rinderknecht (*8*). FITC-Celite powder was added directly to the enzyme solution to a concentration of 60 nmole of FITC per milliliter—ten times the concentration of the enzyme. After incubation on ice for 5 minutes, the Celite was removed by centrifugation. Subsequent steps were performed in the cold room. The solution (2.5 ml) was applied to a column (Pharmacia PD-10, Sephadex G-25) previously equilibrated with 0.02*M* potassium phosphate buffer, *p*H 7.8. The GlDH-FITC compound was eluted with this buffer and collected in a sample of approximately 4 ml. Other samples of enzyme were treated identically except that FITC-Celite was not added and the 5-minute incubation was omitted.

Samples of GlDH or GlDH-FITC (250 μl) were put in thin-walled 2-ml glass vials, and the solutions were rapidly frozen by immersion in a slurry of dry ice

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and ethanol. Some samples were lyophilized to dryness. Vials were sealed with an oxygen-gas flame while the samples were still at very low temperature.

Radiation exposures were performed at the Armed Forces Radiobiology Research Institute (Bethesda, Maryland) with a linear accelerator producing 13-MeV electrons. The beam was spread with a water scatterer to provide a uniform field of radiation at the samples. Dose measurements were made with thermoluminescent dosimeters, dye films, and ionization chambers. During irradiation, samples were maintained at a constant temperature of +30°C by a stream of air or at −135°C by a stream of cold nitrogen gas.

After irradiation, vials were opened and lyophilized samples were suspended in distilled water. Portions were removed for independent analysis of different parameters.

Enzyme activity was assayed by a modification of the instructions provided by Boehringer Mannheim. Each cuvette contained 0.59 ml of 20 m*M* phosphate buffer, *p*H 7.8; 0.10 ml of 2 m*M* nicotinamide adenine dinucleotide (reduced form); 0.10 ml of 10 m*M* adenosine diphosphate; 0.10 ml of 1*M* ammonium acetate; 0.10 ml of 200 m*M* α-ketoglutarate; and 0.01 ml of enzyme solution. The change in absorbance was monitored at 340 nm at room temperature. Blanks without enzyme solution were used to correct for nonenzymatic changes in absorbance.

Fluorescence of FITC was determined in a spectrophotofluorometer (Aminco-Bowman) set at 490-nm excitation and 520-nm emission wavelengths. Procedures for gel electrophoresis were those of Maizel (*9*). Samples were prepared by mixing the protein solution with an equal volume of 0.1*M* tris-HCl buffer, *p*H 6.8, containing 2 percent sodium dodecyl sulfate (SDS), 20 percent glycerol, 2 percent β-mercaptoethanol, and 0.002 percent phenol red. Samples were heated for 1.5 minutes on a steam bath. Tube or slab 15 percent gels at room temperature were used for SDS–polyacrylamide gel electrophoresis (SDS-PAGE). Up to 90 μg of protein was loaded onto each tube. Gels were stained with 0.25 percent Coomassie brilliant blue R-250 and destained by diffusion or an electrophoretic destainer (Canalco); a mixture of 5 percent methanol and 7 percent acetic acid was used for both methods. Photographic color transparencies (4 by 5) were taken of the gels. Both gels and transparencies were scanned with a laser densitometer (Zeineh). The integrated absorbance of the monomer peak was obtained for each gel sample. On tube gels, the intensity of Coomassie blue staining was proportional to concentration up to 110 μg of protein.

Table 1. Radiation sensitivity of several parameters of glutamate dehydrogenase. Target size is expressed as the mean ± standard deviation. The number of experiments is shown in parentheses.

Sample	Parameter	Condition of enzyme	D_{37} (Mrad)		Target size (kD)
			+30°C	−135°C	
GlDH	Enzyme activity	Lyophilized	2.4 (6)	6.8 (4)	257 ± 23
		Frozen		6.6 (4)	
GlDH-FITC	Enzyme activity	Lyophilized	2.4 (5)	8.9 (4)	
		Frozen		6.8 (5)	
GlDH-FITC	Fluorescence	Lyophilized	10.1 (5)	31.1 (4)	59 ± 3
		Frozen		31.6 (6)	
GlDH-FITC	Monomer band on SDS-PAGE	Lyophilized	6.8 (1)	23.3 (4)	72 ± 9
		Frozen		27.1 (7)	

Measurements of enzymatic activity, fluorescence, and the Coomassie stain intensity of the monomer peak on SDS-PAGE were determined on control and irradiated samples. Each measurement (A) was normalized to the value obtained in the unirradiated control sample (A_0). A least squares fit, constrained to pass through 1.0 at zero radiation, was calculated from $\ln (A/A_0) = -k'D$, where D is the radiation dose. The radiation dose that reduces activity to 37 percent of that of the unirradiated sample is D_{37}. Thus $\ln (0.37\,A_0/A_0) = -1 = k'D_{37}$. The slope of the inactivation curve, k', is dependent on temperature (*10, 11*) in a predictable manner (*12*).

$$k' = k/S_t = (D_{37})^{-1}$$

where S_t is a temperature correction factor, which is equal to 1.0 for irradiations at +30°C and equal to 2.8 for irradiations at −135°C. The radiation target size was determined from

$$\text{Molecular weight} = 6.4 \times 10^{11}\, k$$

obtained from the phenomenological relation of Kepner and Macey (*13*).

Results

The enzymatic function of glutamate dehydrogenase decays as a single exponential function of radiation dose to at least 1 percent surviving activity. This simple dose-response relation was found in lyophilized samples irradiated at +30°C and −135°C as well as in frozen preparations irradiated at −135°C (Table 1). The only difference in radiation sensitivity among these samples is that due to temperature during exposure. The calculated target size, after the temperature effect is taken into account, is slightly (but significantly) less than that of the known molecular weight of 330,000 of the hexameric complex. This result is in very close agreement with the original radiation study of the loss of enzymatic activity (*6*).

A fluorescent tag can be covalently bound to protein, permitting measurement of a new "function" associated with the enzyme structure. Both FITC and fluorescamine were used. Attempts to couple glutamate dehydrogenase to pure FITC gave variable results, with most of the loss of enzymatic activity attributable to the long exposure to high *p*H and to the loss of material during subsequent column elutions. The use of FITC-Celite significantly reduced this variability. Experimental parameters were optimized to obtain high recovery of enzyme activity (70 percent), minimize loss of material, and remove unbound fluorescent compounds after termination of the coupling reaction. Concentrations of reactants were then manipulated so that, on the average, one fluorescent moiety was attached to each monomer.

As with the native enzyme, the loss of enzymatic activity after exposure to ionizing radiation was monitored in lyophilized samples irradiated at +30°C and at −135°C and in frozen samples at the lower temperature. Results with the fluorescently labeled glutamate dehydrogenase were the same as those obtained with the unlabeled enzyme (Fig. 1 and Table 1). In addition to these measurements, the loss of fluorescence after exposure to high-energy electrons was determined. Enzyme activity disappeared after low doses of radiation, but fluorescence persisted even after large radiation exposures (Fig. 1). Similar results (not shown) were obtained with fluorescamine. The sensitivity of fluores-

cence to radiation varied with temperature during exposure. Target analysis for the fluorescence "function" led to a molecular weight of 59,000, which is comparable to the molecular weight of the monomer.

Radiation damage to the structure of the monomeric subunits was examined by electrophoresis on denaturing gels. After SDS-PAGE, the residual protein structures were detected by Coomassie blue staining and also by fluorescence of the FITC tag. The unirradiated samples revealed a largely uniform population of 56,000-dalton monomers. After exposure of the samples to increasing doses of ionizing radiation, there was a progressive appearance of Coomassie blue–reactive material of lower molecular weight. This clearly indicates the degradative action of radiation on the protein structure. The absence of bands of molecular size greater than 56,000 daltons implies a minimal amount of radiation cross-linking, even among protein chains that must have been closely apposed in the hexameric conjugate and supramolecular associations.

Photometric scan of the gels can be used to estimate the radiation destruction of the protein structure (*14*). In the case of fluorescently tagged glutamate dehydrogenase, the disappearance of the 56,000-dalton monomer band after irradiation was much less than the decrease in enzymatic activity (Fig. 2). However, the same temperature effects were observed. The decrease in stain intensity in the monomer band led to a radiation target size considerably smaller than that of the enzymatically active hexamer. These gel measurements are subject to considerable difficulties; for example, the accuracy, reproducibility, and linearity of the Coomassie stain; the identification of the monomer band; and distinguishing the unaffected units from those that have been damaged but are close in molecular weight (or at least close in electrophoretic mobility). Nevertheless, the apparent target size of 72,000 daltons is close to that of the monomer.

The survival of fluorescence in these irradiated polymers was dependent on temperature during irradiation; enzymatic activity and also the "structural" measurements of the monomer display the same temperature relation (Table 1). The mechanism for this effect must therefore depend neither on conformation nor (from experiments with monomeric enzymes) on the presence of other subunits.

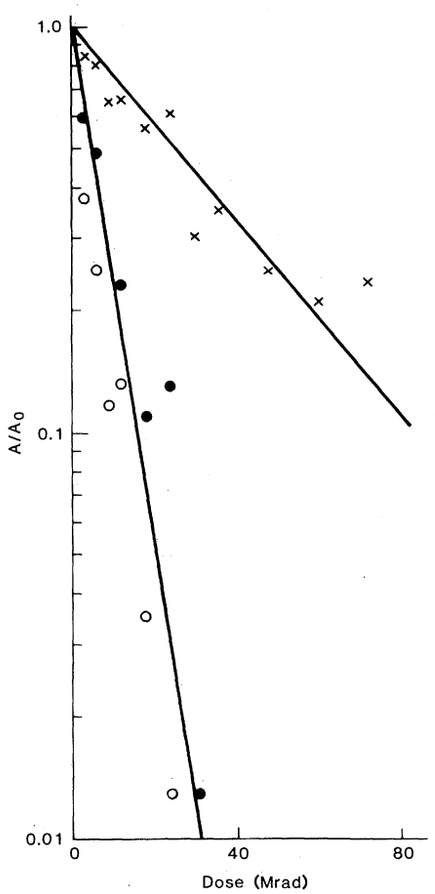

Fig. 1. Effects of high-energy electron irradiation at −135°C on lyophilized samples of glutamate dehydrogenase and fluorescein isothiocyanate–coupled glutamate dehydrogenase. Surviving enzymatic activity of GlDH (○) and GlDH-FITC (●) as well as the fluorescence of GlDH-FITC (X) as a function of radiation exposure.

Discussion

The smallest structure of bovine liver glutamate dehydrogenase that can catalyze the reaction between α-ketoglutarate and ammonia is a hexamer of identical subunits (*15*). A similar size was obtained by radiation inactivation (*6*).

The validity of target analysis depends on the random nature of radiation damage. High-energy electrons and gamma rays pass unabated through free space. Only when radiation impinges on matter is there a release of energy to the target material. For the energy range used for these inactivation studies, this release is usually via the orbital electrons. Because the average distance between ionizations is very large (about 2500 Å for high-energy electrons in water) compared to the size of macromolecules, the damage occurs randomly throughout the mass of the irradiated material. The original measurement of radiation exposure, the roentgen, was initially defined in terms of ionizations per unit volume; the development of target analysis by Crowther (*16*), Lea (*17*), and Pollard (*18*) perpetuated this concept. A newer unit of radiation absorption, the rad (defined as the absorption of 100 ergs of energy per gram of material) was adopted, in part to remove the volume concept. This distinction was not appreciated by Kempner and Schlegel (*7*) or by other authors (*19, 20*). The target size is determined by the mass and is independent of the shape or volume of the target. Theories that make use of the latter parameters or that involve partial molal volumes are therefore incorrect.

In the direct action of radiation on macromolecules, all damage derives from the initial interaction of ionizing radiation with the target molecule. High-energy electrons or gamma rays cause primary ionizations (principally with orbital electrons) in which there is a deposition of 1500 kcal/mole on the average. The dissipation of this energy in the target molecule results in damage to the structure, with consequent loss of biochemical function. With enzymes or other biologically active structures that are composed of several molecular subunits, it is not clear whether other subunits are

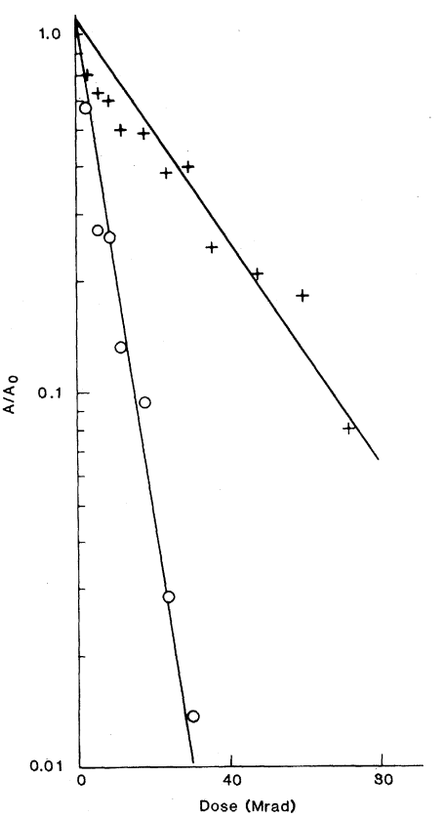

Fig. 2. Effects of high-energy electron irradiation at −135°C on frozen solutions of fluorescein isothiocyanate–coupled glutamate dehydrogenase. Surviving enzymatic activity (○) and intensity of Coomassie blue stain of the monomer subunit on tube gel electrophoretograms (+) as a function of radiation exposure.

destroyed (due to energy transfer from the chain undergoing the primary ionization), or whether the destruction of one chain results solely in the dissolution of the complex without breakage of covalent bonds in adjacent chains (*7*). When the enzyme is composed of several subunits and the radiation inactivation leads to a target size comparable to that of one subunit, it is clear that major energy transfer between chains has not occurred. This has been indicated for tryptophan synthase, cytochrome oxidase, and more than a dozen other enzymes (*7*, *11*). However, an equally long list of enzymes indicates radiation targets equal to the size of the entire oligomeric structure (*7*). Are these a special group of proteins whose structure permits energy transfer between chains? Glutamate dehydrogenase is one of the group of large molecules whose entire structure is required for a "functional unit." It is therefore appropriate to test the structure of this enzyme after exposure to ionizing radiation and to seek evidence for radiation damage to the several polymers of which it is composed.

The frozen samples irradiated in this study contained the enzyme at a concentration and in a buffer reported to maintain glutamate dehydrogenase as a heptamer of hexamers, 1.8×10^6 daltons (*5*). Enzymatic activity was lost after radiation damage to any part of a single hexamer, confirming both the earlier radiation study and the results of the conventional biochemical analyses. This observation was found to be valid for both the native enzyme and the fluorescent adduct. The radiation sensitivity varied with temperature in the same quantitative manner as found for other enzymes. The observed target size indicates that very little energy transfer could have occurred among the seven self-associated hexamers.

Loss of structure of the individual monomers can be caused by breakage of covalent bonds in either the polymer backbone or in the side groups. The former will result in significant changes in molecular weight that can be detected by electrophoresis under denaturing conditions. Although the quantitative analysis of gel scans has many inherent technical difficulties, an estimate of surviving protein can be obtained by photometric measurements of protein-bound dye. After absorption of ionizing radiation energy in one polymer chain of a complex, significant damage should be found in the other chains if there had been massive energy transfer to adjacent members. Subsequent denaturation and electrophoresis would reveal a progressive loss of intact monomers with increasing radiation dose. Target analysis would show a sensitive molecular weight of two, three, or more units depending on the extent of energy transfer. Alternatively, if radiation damage is confined to a single polymer, the five other subunits of glutamate dehydrogenase would be structurally undamaged, at least as far as their molecular weight was concerned. Thus the denaturing gels should show a loss of monomer units leading to a target size equal to that of one subunit. The experimental results of this test were unequivocal; the observed target size was close to that of the monomer. There is thus no evidence for large transfers of energy from one subunit to another.

An alternative measure of damage to individual polymers was obtained from the fluorescence experiments. By covalently binding one fluorescent moiety to each subunit, a compound was created which retained enzymatic activity, but also had a new assayable property. The fluorescence of each chain is independent of the presence of other subunits and independent of the conformation of the polymer to which it is attached. Radiation destruction of enzymatic activity occurred much more rapidly than the loss of fluorescence. If the primary ionization resulted in large energy transfers to adjacent chains and significant bond breakage, then each radiation "hit" would cause the loss of several fluorescent centers. However, target analysis led to a molecular size of only 59,000 daltons, the size of the monomer unit. Therefore a second test of energy transfer between chains gave a negative result.

The principal conclusion of these studies of irradiated glutamate dehydrogenase is that there is no massive transfer of energy (from ionizing radiation) from one monomer subunit to another. The large target size observed for glutamate dehydrogenase activity as well as for the activity of many other enzymes (*7*) may indicate that in these proteins the expression of enzymatic function requires the simultaneous presence of several intact subunits.

On the basis of our current results, we hypothesize that direct radiation damage is confined to the individual polymer chains in which the primary ionization occurs. The transfer of radiation energy among multisubunit structures predicted by others (*19*) is not supported by the present observations.

References and Notes

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