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Inactivation Mechanism of Tetrameric β-Galactosidase by γ-Rays Involves both Fragmentation and Temperature-Dependent Denaturation of Protomers†

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ABSTRACT: The radiation inactivation method is widely used to estimate the molecular size of membrane-bound enzymes, receptors, and transport systems in situ. The method is based on the principle that exposure of frozen solutions or lyophilized protein preparations to increasing doses of ionizing radiations results in a first-order decay of biological activity proportional to radiation inactivation size of the protein. This parameter is believed to reflect the "functional unit" of the protein defined as the minimal assembly of structure (protomers) required for expression of a given biological activity. We tested the functional unit as a concept to interpret radiation inactivation data of proteins with *Escherichia coli* β-galactosidase, where the protomers are active only when associated in a tetramer. γ-Irradiation of β-galactosidase at both −78 and 38 °C followed by quantitation of the residual unfragmented protomer band by SDS–polyacrylamide gel electrophoresis yielded the protomer size, indicating that only one protomer is fragmented by each radiation hit. By following the enzyme activity as a function of dose it was found that only the protomer that has been directly hit and fragmented at −78 °C was effectively inactivated. In contrast, at 38 °C, it was the whole tetramer that was inactivated. β-Galactosidase cannot have two different functional units depending on temperature. The inactivation of the whole β-galactosidase tetramer at 38 °C is in fact related to protomer fragmentation but also to the production of stable denatured protomers (detected by gel-filtration HPLC and differential UV spectroscopy) due to energy transfer from fragmented protomers toward unhit protomers. We conclude that β-galactosidase inactivation is the result of a two-step mechanism involving (1) fragmentation of the protomer directly hit by an ionizing radiation and (2) temperature-dependent radiation-induced denaturation of associated unfragmented protomers. Therefore, the radiation inactivation size reflects the size of the fragmented protomer and, when irradiation is carried out at higher temperatures, the transfer of energy from fragmented protomers toward other protomers inside the oligomer.

The radiation inactivation and fragmentation method is used extensively to estimate the molecular size of enzymes, receptors, and transport systems [reviewed by Kempner and Schlegel (1979); Beauregard et al., 1987a; Kempner & Fleisher, 1989]. The method is especially useful with membrane-associated proteins since it yields the molecular size of a protein as it exists in situ without prior purification or solubilization by detergent. The target theory is the basic framework used to analyze radiation inactivation data (Lea, 1955). It postulates that direct hit by an ionizing radiation on a protein will cause complete and irreversible inactivation whereas unhit molecules will retain full activity. The analysis of biological activity decay curves as a function of radiation dose gives the radiation inactivation size (RIS)[1] (Beauregard et al., 1987a) of the protein based on an empirical calibration curve established with proteins of known molecular weight (Kepner & Macey, 1968).

Most oligomeric proteins studied by radiation inactivation yield the protomer size, but RIS values corresponding to the whole oligomer have also been obtained (Kempner & Schlegel, 1979). In the latter case, it has been proposed that the integrity of each protomer in the oligomer is required for expression of biological activity and that the RIS reflects the minimal functional unit of the oligomer (Kempner & Schlegel, 1979; Steers et al., 1981). Alternatively, the energy absorbed by a hit on one subunit may be transferred to the other protomers in the oligomer, causing the loss of biological activity of the whole oligomer (Saccomani et al., 1981). The functional unit concept has been widely accepted to interpret radiation inactivation experiments, but its validity has not been thoroughly tested with well-characterized oligomeric proteins. To do so, we selected the homotetrameric enzyme β-galactosidase

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[1] Abbreviations: ONPG, *o*-nitrophenyl β-D-galactopyranoside; MUG, 4-methylumbelliferyl β-D-galactopyranoside; SDS, sodium dodecyl sulfate; GuHCl, guanidine hydrochloride; RIS, radiation inactivation size; TS, target size.

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from *Escherichia coli*. This enzyme has a functional unit that corresponds to the whole tetramer since protomer interaction is required for expression of enzymic activity in each protomer (Roth & Rotman, 1975; Conway de Macario et al., 1978).

The choice of β-galactosidase also allowed us to test for energy transfer between protomers and consequent fragmentation and/or denaturation (conformational change) of associated unhit protomers (Beauregard et al., 1987b). Because of its relatively large protomer size (116 kDa) (Wallenfels et al., 1963), β-galactosidase was particularly well suited to detect conformational changes in protomers by gel-filtration analysis or differential UV spectrometry. Our results do not validate the functional unit concept but suggest an alternative two-step inactivation mechanism for β-galactosidase involving (1) fragmentation of protomers directly hit by an ionizing radiation and (2) temperature-dependent denaturation (conformational change) in associated protomers.

Materials and Methods

Materials. *E. coli* β-galactosidase, *o*-nitrophenyl β-D-galactopyranoside (ONPG), and sodium dodecyl sulfate (SDS) were purchased from Sigma Chemical Co. (St. Louis, MO). Before use for irradiation experiments, the purity of the β-galactosidase preparation was tested by SDS–polyacrylamide gel electrophoresis according to Laemmli (1970). If necessary, the enzyme was further purified by HPLC under the conditions described below. The calibration standards for gel-filtration HPLC were purchased from Pharmacia Ltd. (Montréal, Canada). The 4-methylumbelliferyl β-D-galactopyranoside (MUG) was from Terochem Ltd. (Mississauga, Ontario); lactose and other chemicals were from Fisher (Montréal, Québec). The galactose determination kit used for the assay of β-galactosidase activity with lactose as substrate was purchased from Boehringer-Mannheim (Montréal, Québec).

Irradiation Conditions. β-Galactosidase solutions (0.4 or 0.8 mg/mL) in 10 mM tris(hydroxymethyl)aminomethane (pH 8) were either lyophilized or frozen rapidly in 1.5-mL Eppendorf microcentrifuge tubes. The lyophilized powders were flushed with dry nitrogen and capped before being irradiated in a Gammacell 220 ^{60}Co source (Atomic Energy of Canada, Ottawa) at a dose rate of about 1.5 Mrad/h. The temperature of the frozen samples was maintained at −78 °C with crushed dry ice. The lyophilized samples were irradiated either in dry ice or at the irradiation chamber temperature (38 °C). Enzyme activity was measured according to published procedures with ONPG, MUG, and lactose as substrates (Beauregard et al., 1987b).

Fragmentation of the β-galactosidase protomer as a function of increasing radiation dose was followed quantitatively by SDS–polyacrylamide gel electrophoresis in a 11% (w/v) gel (Laemmli, 1970) as previously described by Karlish and Kempner (1984). The proteins were stained with Coomassie Blue R-250 and destained, and the band corresponding to the intact protein was excised from the gel. The Coomassie stain was extracted overnight in 5% (w/v) SDS, and the optical density of the extract was determined at 605 nm. The relationship between OD at 605 nm and amount of protein was linear in the range between 2 and 40 μg of β-galactosidase. Identical results were obtained when the band intensity was quantitated by direct microdensitometry of the stained gels according to le Maire et al. (1990).

The logarithm of β-galactosidase activity or of protein stain intensity was plotted as a function of the radiation dose expressed in megarads, and the dose necessary to diminish the β-galactosidase activity or the stain intensity to 37% of its initial value at temperature t ($D_{37,t}$) was determined.

The radiation inactivation size (RIS), obtained by following the β-galactosidase activity, and the target size (TS), measured by following the stain intensity of the band corresponding to the native β-galactosidase protomer on a SDS gel, were computed from the equation (Beauregard et al., 1987a)

$$\log (\text{RIS or TS}) = 5.89 - \log D_{37,t} - 0.0028t \quad (1)$$

where t is the irradiation temperature in degrees Celsius. The radiation inactivation size (RIS) is defined as the mass of 1 mol of protein inactivated by a hit and target size (TS) as the mass of 1 mol of protein whose polypeptide chain has been fragmented by a hit (Beauregard et al., 1987a).

Model for Radiation Fragmentation of Tetrameric β-Galactosidase. The model predicts the behavior of the various oligomeric forms resulting from the irradiation of tetrameric β-galactosidase with increasing doses. It is based on the following assumptions: (1) according to the target theory (Lea, 1955), the various oligomeric forms of β-galactosidase are fragmented as an exponential function of dose, (2) no reequilibration of protomers occurs between the various oligomeric forms, and (3) each protomer is fragmented independently. We will see below that all of these assumptions were found to hold true under our experimental conditions. The decay of native tetramers (UF_4) and the resulting oligomers generated by successive protomer fragmentation were assumed to follow a unidirectional relationship yielding oligomers with three (UF_3), two (UF_2), and one (UF_1) unfragmented protomers.

$$UF_4 \text{ (tetramer)} \rightarrow UF_3 \rightarrow UF_2 \rightarrow UF_1 \rightarrow \text{peptide fragments} \quad (2)$$

The decay of the native tetrameric form in the preparation is described by the simple exponential equation

$$(UF_4)_D = (UF_4)_0 \exp(-4\mu D) \quad (3)$$

where $(UF_4)_D$ is the number of tetramers at dose D, $(UF_4)_0$ is the number at the dose 0, μ is the protomer decay constant, and D is the radiation dose expressed in megarads.

The fragmented oligomers decay as described by the following series of exponential equations derived from relation 2.

$$(UF_3)_D = 4(UF_4)_0 [\exp(-3\mu D) - \exp(-4\mu D)] \quad (4)$$

$$(UF_2)_D = 6(UF_4)_0 [\exp(-2\mu D) - 2\exp(-3\mu D) + \exp(-4\mu D)] \quad (5)$$

$$(UF_1)_D = 4(UF_4)_0 [\exp(-\mu D) - 3\exp(-2\mu D) + 3\exp(-3\mu D) - \exp(-4\mu D)] \quad (6)$$

This series of relations was used to predict the proportions of native tetramers and other oligomeric species generated by irradiation of β-galactosidase at both 38 and −78 °C, assuming μ values of 0.1695 Mrad^{-1} at 38 °C and 0.0867 Mrad^{-1} at −78 °C (Beauregard et al., 1987b).

The detailed derivation of relations 3–6 is given in an appendix to this paper.

High-Performance Liquid Chromatography. High-performance liquid chromatography of standard proteins and irradiated β-galactosidase was performed with a Waters Associates instrument Model 6000 A with 50 mM sodium phosphate buffer, pH 7.0, 0.1 M NaCl as eluant. The chromatographic column (4 × 300 mm) was a Waters Associates SW 300 gel-filtration type eluted at a flow rate of 0.8 mL/min. The following standard proteins was known Stokes' radii (R_s) were used to calibrate the column: thyroglobulin (8.6 nm), ferritin (6.3 nm), catalase (5.2 nm), aldolase (4.6 nm), albumin (3.5 nm), trypsin inhibitor (2.2 nm), and ribonuclease A (1.8 nm). The void and total volumes of the column were measured

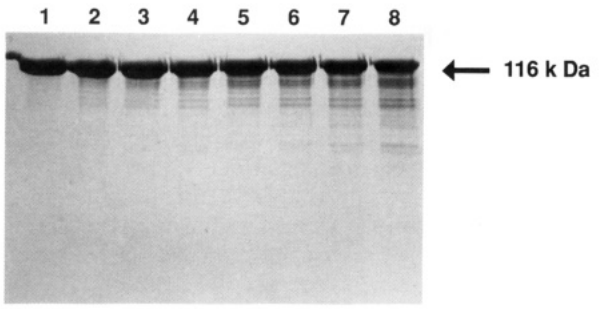

FIGURE 1: SDS–polyacrylamide electrophoretic analysis of radiolysis fragments of β-galactosidase on an 11% (w/v) gel. Samples containing 25 μg of β-galactosidase were applied in each lane after irradiation at −78 °C with the following doses: unirradiated control, lane 1; 0.87 Mrad, lane 2; 1.74 Mrad, lane 3; 2.61 Mrad, lane 4; 3.47 Mrad, lane 5; 4.57 Mrad, lane 6; 6.08 Mrad, lane 7; 10.42, lane 8.

with cytochrome oxidase and $NaNO_3$, respectively. The Stokes' radii of the standard proteins were plotted against their experimentally determined partition coefficients, and the data were fitted with a second-order polynomial.

Differential Ultraviolet Spectroscopy. Differential ultraviolet spectroscopy of β-galactosidase was carried out in a Perkin-Elmer Model λ 3 double-beam spectrophotometer between 230 and 300 nm. The sample cuvette contained β-galactosidase irradiated with a dose of 0.75 Mrad at 38 °C, and the reference cuvette contained β-galactosidase irradiated with 1.5 Mrad at −78 °C. After irradiation, the lyophilized powder was dissolved in tris(hydroxymethyl)aminomethane buffer, pH 8, and analyzed by spectrometry.

RESULTS

Radiation Fragmentation of β-Galactosidase. SDS–polyacrylamide gel electrophoretic analysis of β-galactosidase under increasing doses of γ-rays at −78 °C showed progressive decay of the 116-kDa protomer band into defined radiolysis fragments of molecular masses between 33 and 105 kDa (Figure 1). Identical fragments were obtained when the enzyme was irradiated either at 38 or −78 °C, indicating that the fragmentation pattern is independent of irradiation temperature (only data at −78 °C are shown in Figure 1). Such fragmentation of proteins into defined peptides has been previously reported with several other purified proteins exposed to ionizing radiations (Solomonson et al., 1987; le Maire et al., 1990). A semilogarithmic plot of the band intensity corresponding to intact β-galactosidase protomer versus radiation dose at −78 °C yielded a D_{37} of 13.8 Mrad, corresponding to a TS of 93 kDa according to eq 1 (Table I). A similar TS value was obtained when irradiation was carried out at 38 °C. At both temperatures, the TS values corresponded approximately to the β-galactosidase protomer (116 kDa), indicating that a single protomer is fragmented by each radiation hit.

Radiation Inactivation of β-Galactosidase. The fragmentation is accompanied by loss of β-galactosidase activity (Figure 2). Using three β-galactoside substrates, we found similar RIS values for β-galactosidase irradiated at −78 °C either in frozen solution or in lyophilized form, corresponding approximately to the protomer (Table I). The mean RIS value with the ONPG substrate was lower than those determined with the other β-galactosidase substrates. The difference was statistically significant (Student's t test, $P < 0.01$), suggesting that a smaller domain of the β-galactosidase protomer may be required for the binding and/or hydrolysis of ONPG as compared to the other substrates. Independent domains with RIS values of associated biological activity smaller than the protomer have been previously reported for other proteins

Table I: Radiation Inactivation Size (RIS) and Target Size (TS) of *E. coli* β-Galactosidase[a]

measured decay	physical state of sample	RIS (kDa)	TS (kDa)
	Irradiation at −78 °C		
Coomassie Blue	frozen		147, 93 (2)
activity, ONPG	frozen	80 ± 12 (5)	
activity, MUG	frozen	120, 156 (2)	
activity, lactose	frozen	101, 134 (2)	
average		101 ± 30 (9)	120 (2)
activity, ONPG	lyophilized	97 ± 30 (4)	
	Irradiation at 38 °C		
Coomassie Blue	lyophilized		102, 103 (2)
activity, ONPG	lyophilized	382 ± 62 (4)	
activity, MUG	lyophilized	369, 388 (2)	
activity, lactose	lyophilized	396 (1)	
average		383 ± 57 (7)	103 (2)

[a] ONPG, *o*-nitrophenyl β-D-galactopyranoside; MUG, 4-methylumbelliferyl β-D-galactopyranoside. The mean ± standard deviation values are shown with three and more determinations. The numbers of determinations are indicated in parentheses.

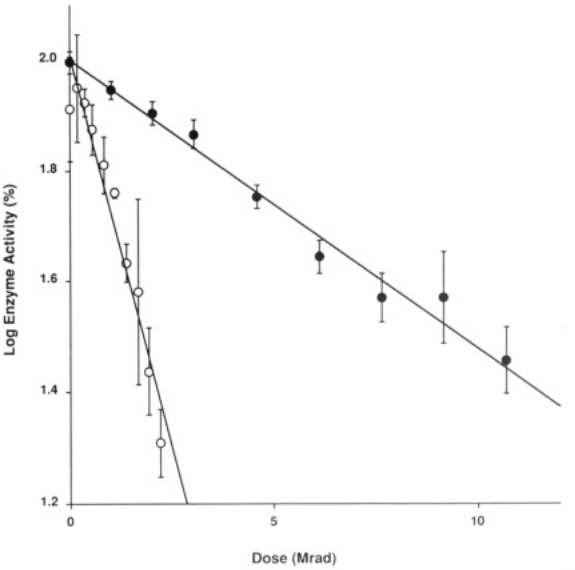

FIGURE 2: Radiation inactivation and fragmentation of β-galactosidase as a function of radiation dose. Inactivation of β-galactosidase with 4-methylumbelliferyl β-D-galactopyranoside as substrate (●) at −78 °C and (○) at 38 °C. The least-squares lines are shown, and the error bars represent means and standard errors of triplicate determinations.

(Solomonson et al., 1987; Kempner & Fleisher, 1989). However, because of the relatively high coefficient of variation of TS and RIS values observed with β-galactosidase and other proteins (le Maire et al., 1990) and the relatively small sample size, the observed difference may still be due to experimental variation. Thus, all values were pooled to calculate the mean and standard deviation (Table I).

In contrast, about 3.8-fold higher RIS values were obtained when irradiation was conducted at 38 °C. In this case, the coefficient of variation of RIS values was around 15%. These results suggest that a −78 °C a single protomer is inactivated by a hit as expected from the fragmentation of a single protomer whereas, at 38 °C, all the protomers associated in the tetramer have lost their enzymic activity. Since the functional unit of β-galactosidase is known to be the tetramer, the results at −78 °C cannot be explained on the basis of the functional unit concept because the same enzyme irradiated at two different temperatures cannot have different functional units. Therefore, other inactivation mechanisms of β-galactosidase were sought.

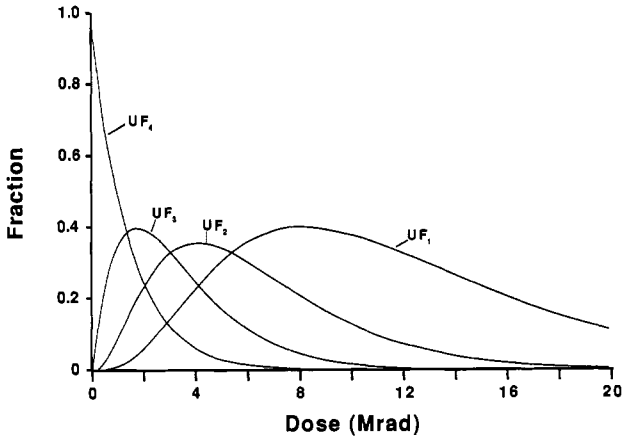

FIGURE 3: Computer model of protomer fragmentation of the β-galactosidase tetramer (UF_4) showing the variation of each oligomeric species as a fraction of the initial amount of tetramer at zero dose with three (UF_3), two (UF_2), and one (UF_1) unfragmented protomers as a function of radiation dose. The results of a simulation at 38 °C are shown assuming a μ value of 0.1695 $Mrad^{-1}$ for protomer decay.

The larger RIS value obtained when irradiation is conducted at 38 °C, by comparison to −78 °C, cannot be accounted for by the different physical state of the enzyme during irradiation (lyophilized versus frozen solution). That is, similar RIS values were obtained at −78 °C when the enzyme was irradiated either in lyophilized form or in frozen solution (Table I). Many other proteins and enzymes gave similar RIS values when irradiated in lyophilized form and frozen solution (Kempner & Schlegel, 1979; Kempner & Fleisher, 1989).

Model of Radiation Fragmentation of Tetrameric β-Galactosidase. To test the hypothesis of a conformational change in associated protomers, we needed a method to predict the distribution of β-galactosidase oligomeric species with three, two, and one unfragmented protomers as a function of radiation dose. Our goal was to determine a dose for which a maximum of oligomers with only one fragmented protomer will be obtained. With such a preparation, we would be able to compare the conformational state of the unfragmented protomers irradiated with equivalent doses at −78 and 38 °C by gel-filtration HPLC and differential UV spectroscopy. A mathematical model was constructed (see the appendix), and the equations programmed into a computer allowed us to trace a series of theoretical curves describing the evolution of each oligomeric species generated by irradiation (Figure 3). The optimal radiation doses to obtain a maximum of the oligomeric species with three unfragmented protomers (UF_3) were 1.5 Mrad at −78 °C and 0.75 Mrad at 38 °C. With such doses, the proportion of oligomers with three unfragmented protomers is expected to reach about 32% of the total number of tetramers in the preparation whereas the oligomeric species with two unfragmented protomers will represent only about 6% of the total tetramers (Figure 3). A second pair of doses (40 Mrad at −78 °C and 20 Mrad at 38 °C) was also selected so that β-galactosidase preparations contained tetramers with only one intact protomer (UF_1); all three others having been fragmented by radiation hits (Figure 3).

Irradiation Does Not Cause Dissociation of β-Galactosidase Tetramer. Considering that protomer dissociation inactivates β-galactosidase (Conway de Macario et al., 1978), it was first necessary to establish whether the loss of enzymic activity under radiation exposure may be caused by protomer dissociation. Gel-filtration HPLC analysis in nondenaturing conditions revealed similar Stokes' radii for the unirradiated control (R_s = 6.86 nm, peak I, Figure 4A) and the irradiated β-galactosidase (R_s = 6.91 nm) at both −78 °C (Figure 4D,J) and 38 °C (Figure 4G,M). This is true both at low radiation doses (0.75 and 1.5 Mrad, parts D and G of Figure 4), which, according to the model, yield 32% of the tetramers with one fragmented protomer and at high radiation doses (Figure 4J,M), where three of the four protomers of all oligomers were fragmented by radiation hits. From these experiments, we concluded that radiolysis fragments stay associated together in a conformation close to that of the native enzyme since, in nondenaturing conditions, β-galactosidase was eluted as a single peak on the HPLC column at a position corresponding

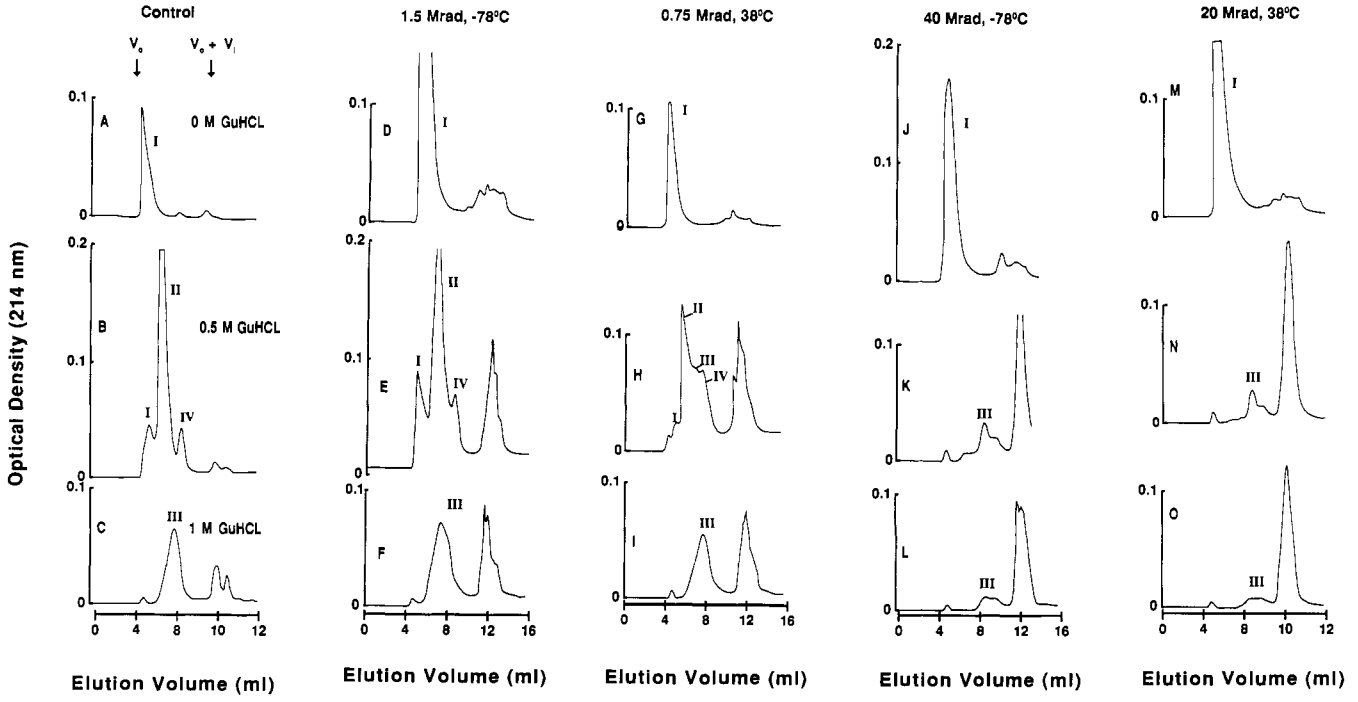

FIGURE 4: Gel-filtration HPLC analyis in the absence (A, D, G, J, and M) and in the presence of 0.5 M (B, E, H, K and N) and 1 M guanidine hydrochloride (C, F, I, L, and O) of β-galactosidase irradiated with 1.5 and 40 Mrad at −78 °C and 0.75 and 20 Mrad at 38 °C as indicated. V_0, void volume. $V_0 + V_i$, total volume of the gel-filtration column.

approximately to that of the native protein. It is only in the presence of GuHCl that the radiolysis fragments dissociated and were eluted between the native protein peak and the total volume ($V_0 + V_i$) of the gel-filtration HPLC column.

Effect of Guanidine Hydrochloride on Native and Irradiated β-Galactosidase. We used mild denaturing conditions to partially dissociate the β-galactosidase tetramer and detect changes in protomer conformation by gel-filtration HPLC analysis. In 0.5 M GuHCl, the native tetrameric β-galactosidase dissociated almost completely into species corresponding to dimeric and protomeric forms of β-galactosidase with R_s values of 3.89 and 3.09 nm, respectively (peaks II and IV in Figure 4B). In 1 M GuHCl, the dissociation is complete since only one major peak, III, is observed on the chromatogram (Figure 4CA). Peak III represents denatured protomers in an open conformation with an R_s of 3.30 nm, intermediate between R_s values of β-galactosidase protomers and dimers in 0.5 M GuHCl.

The peaks eluted from the HPLC gel-filtration column were characterized by their apparent R_s values. Wallefels et al. (1963) have reported that the R_s of native β-galactosidase was about 6.8 nm, in good agreement with our values obtained on the calibrated HPLC column with both the native and irradiated enzyme. Dissociation of tetrameric β-galactosidase occurs via the formation of dimers and then protomers (Conway de Macario et al., 1978). The R_s values for the dimer and monomer determined in the presence of GuHCl should be taken with caution and are given only for comparison purposes for a given eluant. That is, the column was calibrated with standard proteins eluted in buffer whereas the R_s values of the dimer and protomers were determined in GuHCl. The denaturant may affect both the enzyme and the gel-filtration medium, yielding erroneous R_s values.

Irradiation at −78 °C, with a dose sufficient to statistically hit and fragment about 32% of the tetramers (as predicted by the model), increased the quantity of the radiolytic peptides eluted at the total volume of the HPLC column when the irradiated β-galactosidase preparation is treated and the column is eluted with 0.5 M GuHCl (Figure 4E). However, irradiation by itself does not seem to affect the equilibrium between the tetrameric, dimeric, and protomeric species in the denaturant because the relative proportions of the peaks separated by HPLC (peak I, 19%; peak II, 70%; and peak IV, 11%) remained similar to those of the native enzyme in 0.5 M GuHCl (peak I, 15%; peak II, 73%, and peak IV, 12%) (Figure 4B). This finding suggests that the unfragmented protomers retain their capacity to form dimers in 0.5 M GuHCl. In 1 M GuHCl, the irradiated enzyme is found as a single peak at the same position on the HPLC chromatogram as the unirradiated enzyme although the peak showed more tailing, indicating the presence of lower R_s radiolysis fragments (R_s = 3.30 nm, peak III, Figure 4F).

When irradiation was conducted at 38 °C, β-galactosidase also gave a single peak on the HPLC column eluted in buffer (R_s = 6.81 nm, Figure 4G). In 0.5 M GuHCl, the enzyme irradiated at 38 °C (Figure 4H) dissociated into dimers (R_s = 3.89 nm, peak II) and protomers (R_s = 3.09 nm, peak IV) similarly to β-galactosidase irradiated at −78 °C, but, in addition, a new species was observed (R_s = 3.34 nm, peak III) that represents a denatured protomer. This species is incompletely resolved from the two other close peaks, but rechromatography indicated that the conformer is stable and differs from the dimer and protomer.

In 1 M GuHCl, the β-galactosidase preparations irradiated at 38 °C eluted as a single peak (R_s = 3.30 nm, Figure 4I). This result rules out the possibility that the intermediate peak III in the preparation irradiated at 38 °C may represent covalent reaction products between radiolysis fragments and intact protomers. This was also confirmed by SDS–polyacrylamide gel electrophoresis where the preparation irradiated at 38 °C showed no species with molecular masses above that of the native protomer (Figure 1).

The denatured protomers at 38 °C (peak III in Figure 4H) represents about 29% of the total surface under the protein peaks. Since the fragmentation model predicts that 11% of the protomers will be fragmented with 0.75 Mrad at 38 °C (Figure 3), the experimental value of 29% of transconformed protomers agrees well with the 32% predicted by the theoretical model (three protomers denatured for each protomer fragmented).

Higher radiation doses of 40 Mrad at −78 °C and 20 Mrad at 38 °C did not notably affect the elution profile of the enzyme eluted in nondenaturing conditions by comparison with the native enzyme (Figure 4J,M). However, in the presence of the denaturating agent GuHCl, the protomers dissociated and yielded large quantities of fragments that eluted at or close to the total volume of the gel-filtration column (Figure 4K,L,N,O). This again indicates that radiolysis fragments in nondenaturing conditions remain associated in the tetramer even though, at these relatively high radiation doses, most of the protomers in the tetramers were actually fragmented as revealed by SDS–polyacrylamide gel electrophoresis.

Differential Ultraviolet Spectrometry of Irradiated β-Galactosidase. Differential ultraviolet spectrometry detects protein unfolding by the increase of ultraviolet absorption of aromatic amino acid residues due to increased solvent interaction. The differential ultraviolet spectrum of β-galactosidase irradiated at −78 and 38 °C is consistent with a conformational change (denaturation) in β-galactosidase irradiated at 38 °C. The absorption peak was at 278 nm, and the difference in optical absorption between preparations irradiated at 38 and −78 °C was 0.041. Since equivalent doses were used at −78 and 38 °C and the fragmentation patterns observed by SDS–polyacrylamide gel electrophoresis were identical at both temperatures, then the higher ultraviolet absorption of β-galactosidase irradiated at 38 °C is unlikely to be due to different fragmentation or to chemical effects of irradiation but reflects a conformational change in unfragmented β-galactosidase protomers. Such results are consistent with the observation of denatured protomer species detected by gel-filtration HPLC analysis of β-galactosidase irradiated at 38 °C.

Discussion

β-Galactosidase is a tetramer of identical protomers of 116 kDa, each bearing an active site (Wallenfels et al., 1963), but enzyme activity is expressed in the protomers only when they are associated in a tetramer (Roth & Rotman, 1975; Conway de Macario et al., 1978). The simultaneous protomer decay due to fragmentation and loss of enzyme activity indicates that when irradiation is conducted at −78 °C, the protomers are fragmented independently by hits and both TS and RIS values correspond approximately to the protomer molecular mass (Figures 1 and 2 and Table I). We conclude that, at −78 °C, when one or several protomers are hit and fragmented, all other unhit protomers in the tetramer remain enzymatically active. Thus, the fragmentation of one or several of the protomers in the tetramer is not sufficient to cause inactivation of other associated unhit protomers even in β-galactosidase, where protomer association is required for expression of enzyme activity. The situation is completely different when the enzyme is irradiated at 38 °C because, in this case, all associated

protomers in the tetramer lose enzymic activity after a hit on one of the protomers. These results invalidate the functional unit concept to explain radiation inactivation of β-galactosidase since at both irradiation temperatures the radiation inactivation size of the enzyme should have corresponded to the whole tetramer (Roth & Rotman, 1975).

We propose a different mechanism of β-galactosidase inactivation that in addition to fragmentation of the protomer directly hit by the ionizing radiation also involves denaturation of associated protomers when irradiation is conducted at 38 °C. The loss of enzymic activity in the tetramers irradiated at 38 °C is not due to protomer dissociation but, as shown by gel-filtration HPLC (Figure 4) and differential ultraviolet spectroscopy, to denaturation of protomers associated to the protomer hit and fragmented by a γ-ray. A stable denatured β-galactosidase protomeric form of R_s 3.30 nm was identified by HPLC in mild dissociating conditions (0.5 M GuHCl) as a consequence of irradiation at 38 °C (Figure 4H). Thus, the irradiation at 38 °C in addition to destroying the enzymic activity in the whole tetramer results in unfolding of the inactivated but unfragmented associated protomers.

The assumptions made to construct the mathematical model of β-galactosidase fragmentation (see Materials and Methods section) were tested experimentally. First, the application of the target theory (Lea, 1955) was validated by the observed monoexponential decay of both enzymic activity and protomer fragmentation as a function of dose (Figure 2). Second, gel-filtration HPLC clearly showed that no reequilibration occurs between protomers under nondenaturing conditions. That is, the irradiated tetramers, even after relatively high doses (20 Mrad at 38 °C and 40 Mrad at −78 °C), were eluted into a single peak at the position of native β-galactosidase (Figure 4D,G,J,M). Third, the protomers were fragmented independently by radiation hits. The quantitation of the decay of native β-galactosidase protomer by SDS–polyacrylamide gel electrophoresis yielded the protomer size, thus indicating that a single protomer is fragmented by each hit at both −78 and 38 °C (Table I).

In conclusion, the experimental evidence provided in this paper suggests that inactivation of β-galactosidase is a two-step process. The first step involves fragmentation and inactivation of the protomers directly hit by a γ-ray. The second step is temperature-dependent and irreversible and involves a conformational change in the protomers associated to the fragmented protomer. This conformational change would be responsible for the loss of enzyme activity in all protomers of the tetramer after a hit. This second process occurs only at higher temperatures and probably results from energy transfer between noncovalently linked protomers. Venter (1985) proposed that changes in the secondary and tertiary structures of proteins without fragmentation are sufficient to cause inactivation by radiation exposure. However, this paper provides the first direct experimental evidence in favor of such an inactivation mechanism in β-galactosidase.

The mechanisms of energy transfer from one protomer to the other inside an oligomeric protein are still unclear. However, certain considerations can be made from our present knowledge of intra- and intermolecular energy transfer in theoretical chemistry (Vaidhyanathan & Jung, 1987). Under our experimental conditions where proteins are irradiated in frozen solution or in lyophilized form, the indirect action of free radicals on the protein is believed to be limited (Kempner & Schlegel, 1979). That is, under our experimental conditions, most free radicals formed by irradiation of water are immobilized in the sample and will be deactivated before reacting with a protein. The direct effect of an ionizing radiation is due to primary ionization in a sphere of about 20 Å around a protein molecule that will release an enormous amount of energy (66 eV), enough to break 15 C–H bonds. Ultimately, electron-excited states of about a few electron volts are obtained and the mode of deexcitation determines the biological damage to the protein. Energy transfer is visualized as taking place by one or more of the following basic mechanisms: (1) the energy may be emitted back as radiation (luminescence or fluorescence) and absorbed by another molecule with overlapping absorption bands, (2) transfer from one molecule or group to another by resonance, (3) photon lattice interaction known as excitons, or (4) solitary excitons, also named solitons (Davydov, 1973). Energies released by ionizing radiations are large enough to produce many solitons. Apart from the primary ionization event, which cannot be temperature dependent, all mechanisms of charge migration or energy transfer are apparently facilitated by torsional motion of the amino acid residues by increasing π-orbital overalp at higher temperatures (Davydov, 1973). Although the inter-protomer energy transfer may be effected by one or more of these mechanisms, we conclude from our study that in β-galactosidase and possibly other oligomers the energy transfer from one protomer to the other(s) after a hit determines the RIS value measured by the radiation inactivation method. Therefore, the RIS reflects the size of the structure inactivated by a hit, and thus inter-protomer energy transfer in the oligomer, and not the structure required for enzymic activity, which, for β-galactosidase, would be the whole tetramer (Roth & Rotman, 1975). More work is needed to identify the structural bases of inter-protomer energy migration and the effects on it by temperature or other experimental conditions.

Appendix: Detailed Derivation of Relations 3–6

(i) General Case, with Decay Constants μ_A, μ_B, μ_C and μ_E.

$$A \xrightarrow{\mu_A} B \xrightarrow{\mu_B} C \xrightarrow{\mu_C} E \xrightarrow{\mu_E} \text{fragments of E}$$

The transformations of A to B, of B to C, of C to E, and of E to fragments, with dose D follow Poisson distributions. Therefore, we can write

$$\frac{dN_A}{dD} = -\mu_A N_A$$

$$\frac{dN_B}{dD} = \mu_A N_A - \mu_B N_B \qquad (2')$$

$$\frac{dN_C}{dD} = \mu_B N_B - \mu_C N_C \qquad (3')$$

$$\frac{dN_E}{dD} = \mu_C N_C - \mu_E N_E \qquad (4')$$

The solution to differential eq 1′, with boundary conditions $N_A = N_{A_0}$ when $D = 0$, is

$$N_A = N_{A_0} \exp(-\mu_A D) \qquad (5')$$

The determination of the solution to eq 2′ is as follows. Substituting expression 5′ in eq 2′ and rearranging terms, we get the differential equation

$$dN_B - [\mu_A N_{A_0} \exp(-\mu_A D) - \mu_B N_B]\, dD = 0 \qquad (6')$$

Equation 6′ is a first-order linear differential equation of the form

$$M(N_B,D)\, dN_B + P(N_B,D)\, dD = 0 \qquad (7')$$

where $M(N_B,D) = 1$ and $P(N_B,D) = -[\mu_A N_{A_0} \exp(-\mu_A D) - \mu_B N_B]$. Equation 6′ may be thus integrable, according to Ayres (1952), by multiplying both sides of it by

$$\exp \int -\left[\frac{\frac{\partial M(N_B,D)}{\partial D} - \frac{\partial P(N_B,D)}{\partial N_B}}{M(N_B,D)}\right] dD = \exp \int \mu_B dD = \exp(\mu_B D) \quad (8')$$

And after grouping terms, this leads to

$$d[N_B \exp(\mu_B D)] = \mu_A N_{A_0} \exp[-(\mu_A - \mu_B)D]\, dD \quad (9')$$

whose solution is

$$N_B \exp(\mu_B D) = \frac{\mu_A N_{A_0}}{(\mu_B - \mu_A)} \exp[-(\mu_A - \mu_B)D] + C_B \quad (10')$$

The constant C_B may be evaluated by applying the boundary conditions $N_B = 0$ when $D = 0$ and then

$$N_B = \frac{\mu_A N_{A_0}}{\mu_B - \mu_A}[\exp(-\mu_A D) - \exp(-\mu_B D)] \quad (11')$$

The solution to eq 3′ is obtained by first substituting expression 11′ in eq 3′ and then proceeding as was done to resolve equation 2′. The boundary conditions are $N_C = 0$ when $D = 0$.

We get

$$N_C = \mu_A \mu_B N_{A_0}\left[\frac{\exp(-\mu_A D)}{(\mu_B - \mu_A)(\mu_C - \mu_A)} - \frac{\exp(-\mu_B D)}{(\mu_B - \mu_A)(\mu_C - \mu_B)} + \frac{\exp(-\mu_C D)}{(\mu_C - \mu_A)(\mu_C - \mu_B)}\right] \quad (12')$$

Similarly, the solution to equation 4′, with boundary conditions $N_E = 0$ when $D = 0$ is

$$N_E = \mu_A \mu_B \mu_C N_{A_0}\left[\frac{\exp(-\mu_A D)}{(\mu_B - \mu_A)(\mu_C - \mu_A)(\mu_E - \mu_A)} - \frac{\exp(-\mu_B D)}{(\mu_B - \mu_A)(\mu_C - \mu_B)(\mu_E - \mu_B)} + \frac{\exp(-\mu_C D)}{(\mu_C - \mu_A)(\mu_C - \mu_B)(\mu_E - \mu_C)} - \frac{\exp(-\mu_E D)}{(\mu_E - \mu_A)(\mu_E - \mu_B)(\mu_E - \mu_C)}\right] \quad (13')$$

(*ii*) *Particular Case.* If we have a system for which

$$\mu_A = 4\mu, \quad \mu_B = 3\mu, \quad \mu_C = 2\mu, \quad \text{and} \quad \mu_E = \mu \quad (14')$$

and if we use the notation

$$N_A \equiv (UF_4)_D,\ N_B \equiv (UF_3)_D,\ N_C \equiv (UF_2)_D,\ N_E \equiv (UF_1)_D,\ \text{and}\ N_{A_0} \equiv (UF_4)_0$$

then for that particular system, expressions 5′, 11′, 12′, and 13′ reduce to

$$(UF_4)_D = (UF_4)_0 \exp(-4\mu D) \quad (3)$$

$$(UF_3)_D = 4(UF_4)_0 [\exp(-3\mu D) - \exp(-4\mu D)] \quad (4)$$

$$(UF_2)_D = 6(UF_4)_0 [\exp(-2\mu D) - 2\exp(-3\mu D) + \exp(-4\mu D)] \quad (5)$$

$$(UF_1)_D = 4(UF_4)_0 [\exp(-\mu D) - 3\exp(-2\mu D) + 3\exp(-3\mu D) - \exp(-4\mu D)] \quad (6)$$

Registry No. GuHCl, 50-01-1; β-galactosidase, 9031-11-2.

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