Target Size Analysis by Radiation Inactivation: The Use of Free Radical Scavengers

GENE C. NESS,*[,1] LAURA C. PENDLETON,* AND MICHAEL J. MCCREERY†

*Department of Biochemistry and Molecular Biology, College of Medicine, University of South Florida, Tampa, Florida 33612; and †Medical Research Institute of Chemical Defense, U.S. Army Edgewater Area, Aberdeen Proving Ground, Maryland 21010

Several model systems were employed to assess indirect effects that occur in the process of using radiation inactivation analysis to determine protein target sizes. In the absence of free radical scavengers, such as mannitol and benzoic acid, protein functional unit sizes can be drastically overestimated. In the case of glutamate dehydrogenase, inclusion of free radical scavengers reduced the apparent target size from that of a hexamer to that of a trimer based on enzyme activity determinations. For glucose-6-phosphate dehydrogenase, the apparent target size was reduced from a dimer to a monomer. The target sizes for both glutamate dehydrogenase and glucose-6-phosphate dehydrogenase in the presence of free radical scavengers corresponded to subunit sizes when determinations of protein by sodium dodecyl sulfate–polyacrylamide gel electrophoresis or immunoblotting were done rather than enzyme activity. The free radical scavengers appear to compete with proteins for damage by secondary radiation products, since irradiation of these compounds can result in production of inhibitory species. Addition of benzoic acid/mannitol to samples undergoing irradiation was more effective in eliminating secondary damage than were 11 other potential free radical scavenging systems. Addition of a free radical scavenging system enables more accurate functional unit size determinations to be made using radiation inactivation analysis. Exp Biol Med 230:455–463, 2005

Key words: radiation inactivation analysis; free radical scavengers; glucose-6-phosphate dehydrogenase; glutamate dehydrogenase; target size determinations; functional units of proteins

Over the past three decades, the technique of radiation inactivation analysis using high-energy electrons (13 MeV) has often been used to determine the functional sizes of a wide variety of proteins including membrane proteins, receptors, and glycoproteins (1–6). Since this technique does not require a pure protein, it has been extensively used to estimate functional sizes of difficult-to-solubilize/purify membrane-associated enzymes and receptors (3). Thus, target sizes for low density lipoprotein (LDL) receptor (7), 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase (8, 9), asialoglycoprotein receptor (10, 11), H^+/K^+ ATPase (12), Na^+, K^+ ATPase (13), and insulin receptor (14) have been reported. The technique has also been applied to determination of target sizes *in situ*. Irradiation of liver pieces or isolated mitochondria was used to determine the sizes of HMG-CoA reductase (15) and ferrochelatase (16), respectively. Liver microsomes have been irradiated to determine the size of glucose-6-phosphatase (17).

Many of the functional sizes obtained by radiation inactivation correspond to dimers. These include glucose-6-phosphate dehydrogenase (used as an internal standard) (5), hepatic lipase (18), HIV-1 reverse transcriptase (19), and the disulfide-linked HMG-CoA reductase (8, 15). A trimer form was reported for glutathione S-transferase (20). β-Hydroxybutyrate dehydrogenase (21) and the dopamine transporter (22) behaved as tetramers, while the target size of glutamate dehydrogenase corresponded to a hexamer (23). Monomer target sizes were observed for vertebrate hyaluronan synthase (24) and HMG-CoA reductase from rats given statin drugs (8). In the case of ribonucleotide reductase, the target size agreed with twice the sum of the molecular weights of its large and small subunits, consistent with the proposed $\alpha_2\beta_2$ structure (25). Targets sizes of less than subunit masses were observed for DNA polymerase I, NADH:nitrate reductase, and sulfide oxidase irradiated in the presence of free radical scavengers (26). These smaller sizes may represent discrete protein domains. In the case of nitrate reductase, which contains heme, flavin adenine dinucleotide (FAD), and molybdopterin domains

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[1] To whom correspondence should be addressed at Department of Biochemistry and Molecular Biology, College of Medicine, University of South Florida, 12901 Bruce B. Downs Boulevard, Tampa, FL 33612. E-mail: gness@hsc.usf.edu

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that can catalyze partial activities such as NADH:ferricyanide reductase, measurable with suitable substrates, it is apparent that damage to one domain may not affect another domain joined in the polypeptide by a hinge, for example (27). When analyzed by sodium dodecyl sulfate (SDS) gels, radiation of nitrate reductase produces discrete lower molecular weight products rather than complete destruction (27). When the internal standard, glucose-6-phosphate dehydrogenase, was irradiated with a free radical scavenging system, the target size closely agreed with the subunit size (26). Thus, a very wide range of functional sizes has been obtained for proteins using the technique of radiation analysis.

The determination of functional size of a protein by radiation inactivation analysis is based on the concept that the probability of a molecule being destroyed by the high-energy electrons increases directly with the mass of the molecule (28). Thus, a larger protein is destroyed with a lower dose of radiation than is a smaller protein. Target theory predicts an exponential decrease in biochemical activity (enzyme/binding activity or immunoreactive or Coomassie blue stainable protein) with increasing dose of radiation. Based upon studies of known proteins, an empirical formula that related functional molecular mass to the radiation dose required to decrease the amount of surviving protein to 37% was established (29). This relationship is expressed as $Mr = 6.4 \times 10^5 \times T_f/D_{37}$, where $T_f \cong 2.8$ for irradiation at −135°C (30) and D_{37} is the radiation dose in megarads that reduces activity or amount of protein to 37% of the unirradiated sample. The D_{37} value is determined from a semi-log plot of activity remaining versus radiation dose.

The technique assumes that if a molecule is "hit" that the damage is so extensive that all biological activity is lost and that there are only direct effects of the radiation. This view is supported by a lack of change in the Km of irradiated enzymes; there is only a decrease in Vmax (4). An average of 1500 kcal/mol of energy is deposited in the target molecule as a result of being hit with an electron of 13 MeV (4). It has been estimated that about half of this energy results in breakage of covalent bonds. The rest generates radiolytic products of water. Although the free radicals that form as a result of the radiation can cause secondary damage, leading to an overestimation of target size, it was thought that at −135°C these radiolytic products of water were not free to diffuse, so that direct action was the only mechanism by which damage occurs (4). However, previous experiments with free radical scavengers demonstrated that this was not the case (26). The free radical scavengers "protect" the protein being irradiated by serving as alterative targets for these radical species, thus diluting the secondary damage. The scavengers generally have double bonds, hydroxyl or thiol groups.

It is has been reported that if the total protein concentration in the samples undergoing irradiation is at least 2 mg/ml no secondary damage will occur (19, 31) and that addition of free radical scavengers to limit secondary damage is not needed under these conditions (19). In the previous study on the effects of protein concentration (30), it was found that even at 40 mg/ml protein, the target size for glucose-6-phosphate dehydrogenase remained slightly above 100 kDa. In contrast, a target size of 57 kDa, in good agreement with the subunit size of 51 kDa, was observed for glucose-6-phosphate dehydrogenase by inclusion of a benzoic acid/mannitol free radical scavenging system (26), even with lower than 40 mg/ml total protein concentrations. In the absence of free radical scavengers, a target size of 120 kDa was observed for this enzyme (26). These observations indicate that considerable secondary damage occurs even at −135°C and with high protein concentrations, and that effective free radical scavenging may eliminate these secondary effects.

In the current study, we investigated the effects of free radical scavengers on the target size of glutamate dehydrogenase as well as glucose-6-phosphate dehydrogenase, whether radiolytic products of water persist in the frozen samples, and the effects of several potential free radical scavenging systems in addition to mannitol/benzoic acid.

Materials and Methods

Materials. Glutamate dehydrogenase from bovine liver and Torula yeast glucose-6-phosphate dehydrogenase were purchased from Sigma Chemical Co. (St. Louis, MO). Anti–glucose-6-phosphate dehydrogenase whole antiserum produced in rabbits was obtained from Sigma Chemical. The enhanced chemiluminescence kit and goat anti-rabbit horseradish peroxidase (HRP)-labeled secondary antibody were purchased from Amersham (Chicago, IL). Benzoic acid, mannitol, dithiothreitol, hydrogen peroxide, catalase from bovine liver, monothioglycerol, superoxide dismutase from *Escherichia coli,* ascorbic acid, diamide, bovine serum albumin, glutathione peroxidase from bovine erythrocytes, and glutathione were also purchased from Sigma Chemical.

Sample Preparation. Aliquots (0.25 ml) of purified glutamate dehydrogenase in citrate buffer (Sigma Chemical) at a concentration of 2 mg/ml were placed in 2-ml ampules ± 50 m*M* benzoic acid and 50 m*M* mannitol. Several sets of ampules containing 25 U/ml of glucose-6-phosphate dehydrogenase, 0.4 mg/ml of bovine serum albumin, 0.25 *M* sucrose, and the indicated concentrations of potential free radical scavengers were prepared. Sets of ampules containing 0.25 *M* sucrose ± 50 m*M* benzoic acid and 50 m*M* mannitol without added glucose-6-phosphate dehydrogenase were also prepared. All samples were quickly frozen in a dry ice/ethanol bath and sealed under vacuum. They were then shipped on dry ice to the Armed Forces Radiobiology Research Institute (Bethesda, Maryland) for irradiation.

Irradiation Procedure. Irradiation was carried out with a 13-MeV electron beam from a Varian V-7725 linear accelerator before a 1-cm water scatterer. The accelerator

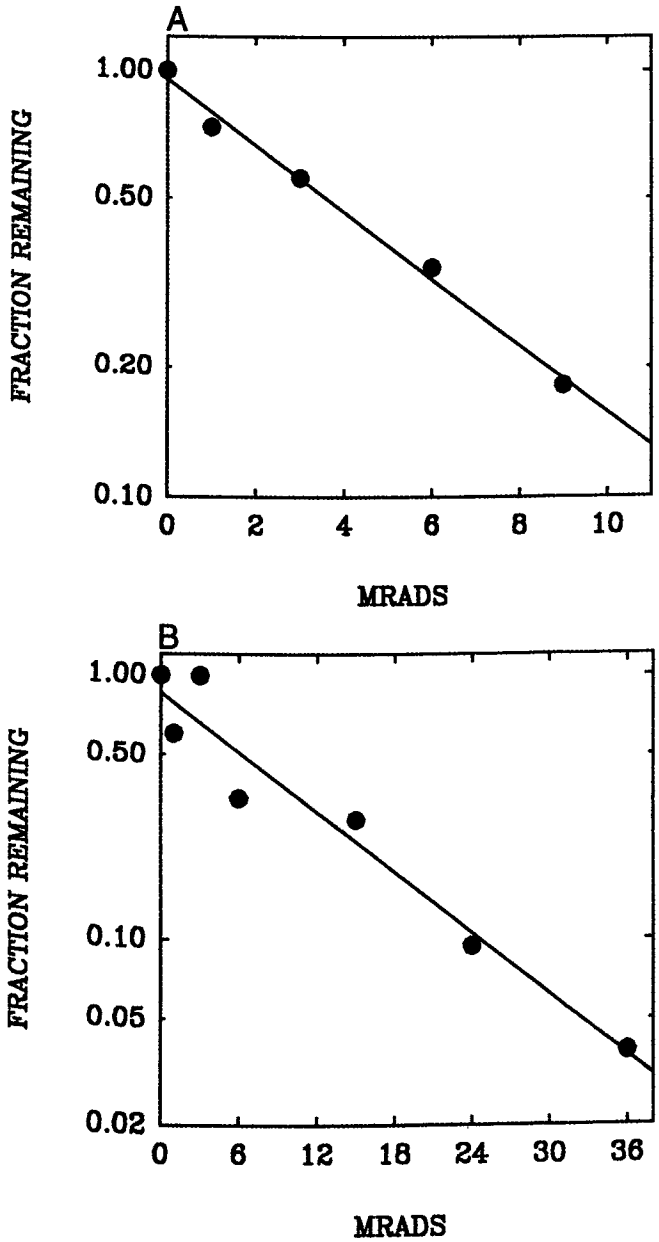

Figure 1. Radiation inactivation plots for bovine glutamate dehydrogenase activity. Samples were irradiated in the absence (A) or presence (B) of 50 m*M* benzoic acid and mannitol. The initial activity of bovine glutamate dehydrogenase was 60 units/ml, 30 units/mg. The target size in A was 325 kDa, with a correlation coefficient of −0.996. The target size in B was 161 kDa, with a correlation coefficient of −0.970. The target sizes were found to be statistically different ($F_{2,4} = 83.02$, $P = 0.0119$).

parameters were as follows: 4 μsec/pulse, 200 rads/pulse, and 30 pulses/sec. The reported irradiation doses are cumulative. Dosimetry was performed before and after each experiment with thermoluminescence detectors. During irradiation, the samples were held in a horizontal orientation in an 8 × 8-in. array at −135° ± 2°C. After irradiation the samples were shipped on dry ice to the University of South Florida.

Enzyme Analysis. Glucose-6-phosphate dehydrogenase activity in the freshly thawed samples was determined as previously described (26). Glutamate dehydrogenase activity was measured in the reverse direction at 340 nm using NADH, ammonium acetate, and α-ketoglutarate in potassium phosphate buffer at pH 7.8, as described (32). Neither enzyme activity was affected by addition of 50 m*M* benzoic acid/mannitol to the enzyme sample before assay. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS PAGE) on 7.5% gels was run as previously described (33). The gels were stained with Coomassie blue to detect glutamate dehydrogenase. Immunoblotting for glucose-6-phosphate dehydrogenase was done as previously described, except that 10% SDS PAGE gels were used. (33).

Calculations. To determine target sizes, the logarithm of the activity or protein remaining (A_D/A_0) was plotted against the radiation dose in megarads. The best fit lines for these plots were determined by linear regression analysis. Correlation coefficients were also obtained and are given in the figure legends. From the plots, D_{37} values were obtained and the functional or target molecular weights calculated using the equation $Mr = 6.4 \times 10^5 \times T_f/D_{37}$, where T_f is 2.8 for irradiation done at −135°C and D_{37} is expressed in megarads.

Statistical Analysis. To determine whether the target molecular weights obtained ± free radical scavengers were significantly different from one another, multiple linear regression analysis was performed using Microcal software (Origin 6.1; Northampton, MA), where $P < 0.05$ was considered significant.

Results

Since the reported functional molecular weight obtained from radiation inactivation analysis for glutamate dehydrogenase activity is about six times (23, 34) the subunit molecular weight of 55,393 determined from the amino acid sequence (35), this enzyme was selected for further investigation of the effects of free radical scavengers on apparent target sizes obtained by this method. When bovine liver glutamate dehydrogenase was irradiated in the absence of the benzoic acid/mannitol free radical scavenging system, enzyme activity measurements revealed a target size of 325 kDa (Fig. 1A). This agrees with previous reports that a hexamer is the functional unit of glutamate dehydrogenase (22, 34, 36). However, when glutamate dehydrogenase was irradiated in the presence of 50 m*M* benzoic acid and 50 m*M* mannitol, the target size for enzyme activity was 161 kDa (Fig. 1B). Association-dissociation of glutamate dehydrogenase hexamer and trimer has been described (37). The ability of NADH to bind to the trimer and for this form to undergo characteristic structural changes induced by ADP and GTP indicated previously that the smallest functional unit of glutamate dehydrogenase might be the trimer rather than the hexamer (38). The present radiation inactivation study provides additional independent data to support this idea.

The amount of glutamate dehydrogenase protein remaining after irradiation was determined by SDS PAGE

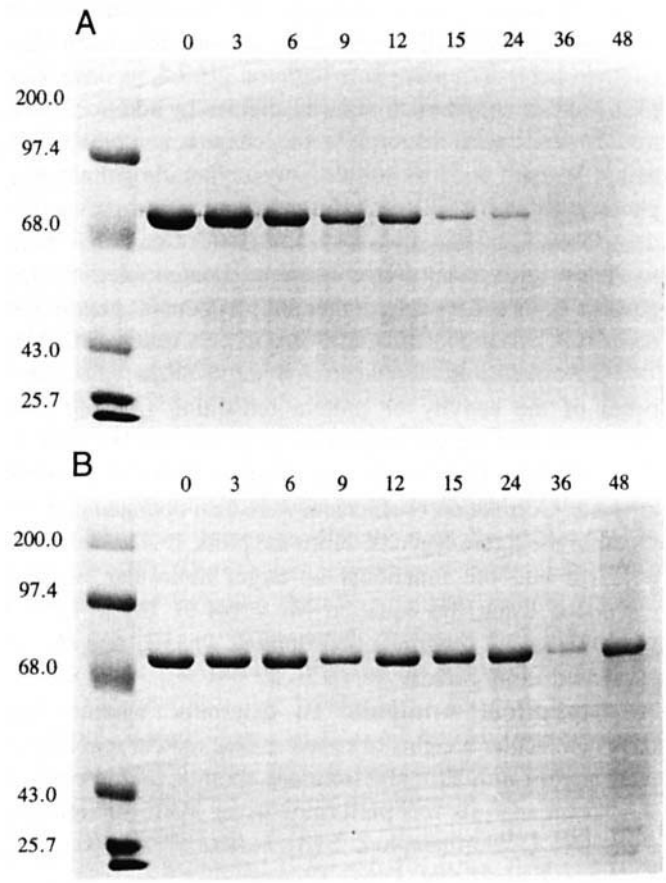

Figure 2. SDS PAGE of irradiated bovine glutamate dehydrogenase. (A) Samples were irradiated in the absence of free radical scavengers. (B) Samples were irradiated in the presence of 50 m*M* benzoic acid and mannitol. Glutamate dehydrogenase protein was detected by Coomassie blue staining. The irradiation dose in megarads is given at the top of each lane. Molecular weight markers are shown on the left sides of each gel.

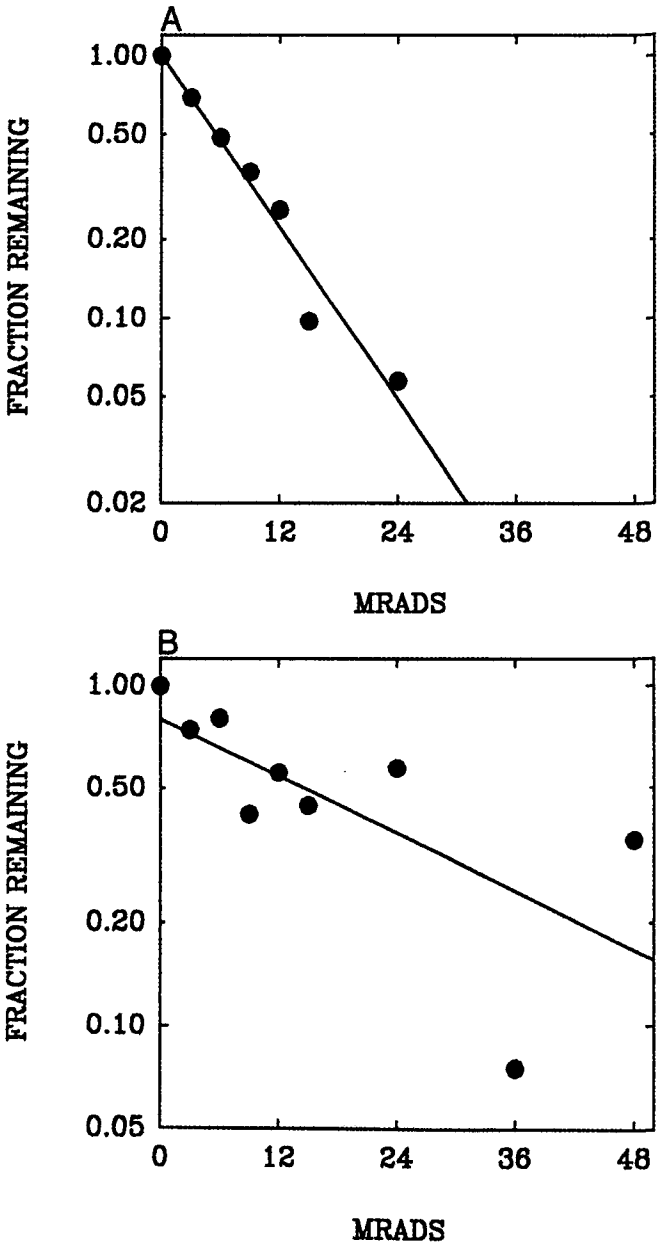

Figure 3. Radiation inactivation plots for Coomassie blue–stained glutamate dehydrogenase. Samples were irradiated in the absence (A) or presence (B) of 50 m*M* benzoic acid and mannitol. The target size in A was 228 kDa, with a correlation coefficient of –0.981. The target size in B was 59 kDa, with a correlation coefficient of –0.693. The target sizes were found to be statistically different ($F_{2,6} = 15.19$, $P = 0.0135$)

analysis. In the absence of free radical scavengers (benzoic acid/mannitol), a target size of 228 kDa (Figs. 2A and 3A) was observed. In the presence of free radical scavengers, the target size was about 59 kDa (Figs. 2B and 3B). If the 36 megarad point was deleted, the target size was 47 kDa. These values are very close to that of the monomer, which is 55 kDa (35). Previously, a target size of 72 kDa was observed for glutamate dehydrogenase irradiated in either the frozen or lyophilized states without free radical scavengers when detected by Coomassie blue staining of SDS PAGE gels (22). Thus, there does not appear to be energy transfer between the subunits during irradiation (22).

To assess whether these findings with glutamate dehydrogenase are more general, the target size of glucose-6-phosphate dehydrogenase was also determined both by activity measurements and by immunoblotting of the protein. As shown in Figure 4A and B, activity measurements of glucose-6-phosphate dehydrogenase revealed target sizes of 96 kDa and 52 kDa without and with 50 m*M* free radical scavengers, respectively. This agrees with our previous result of 120 and 57 kDa (26). Immunoblotting analysis (Figure 5A and B) gave target sizes of 84 and 60 kDa without and with free radical scavengers, respectively. Thus, the minimal unit for glucose-6-phosphate dehydrogenase activity appears to be that of the monomer. The data again demonstrate that smaller target sizes are observed when free radical scavengers are added to samples to be irradiated. In a previous study, increasing amounts of benzoic acid/mannitol were added to samples containing glucose-6-phosphate dehydrogenase before irradiation. The effect

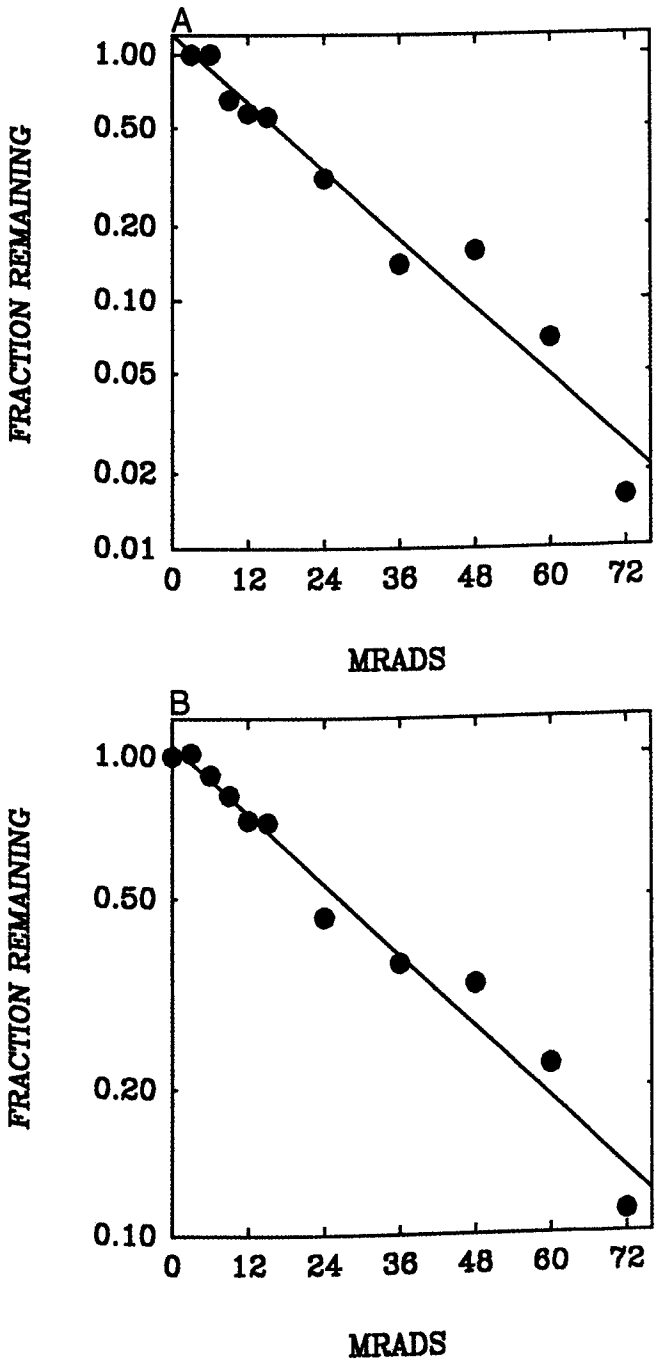

Figure 4. Radiation inactivation plots for glucose-6-phosphate dehydrogenase activity. Samples were irradiated in the absence (A) or presence (B) of 50 m*M* benzoic acid and mannitol. The initial Torula yeast glucose-6-phosphate dehydrogenase activity was 25 units/ml, 600 units/mg. The target size in A was 96 kDa, with a correlation coefficient of –0.976. The target size in B was 52 kDa, with a correlation coefficient of –0.985. The target sizes were found to be statistically different ($F_{2,9} = 90.17$, $P < 0.0001$).

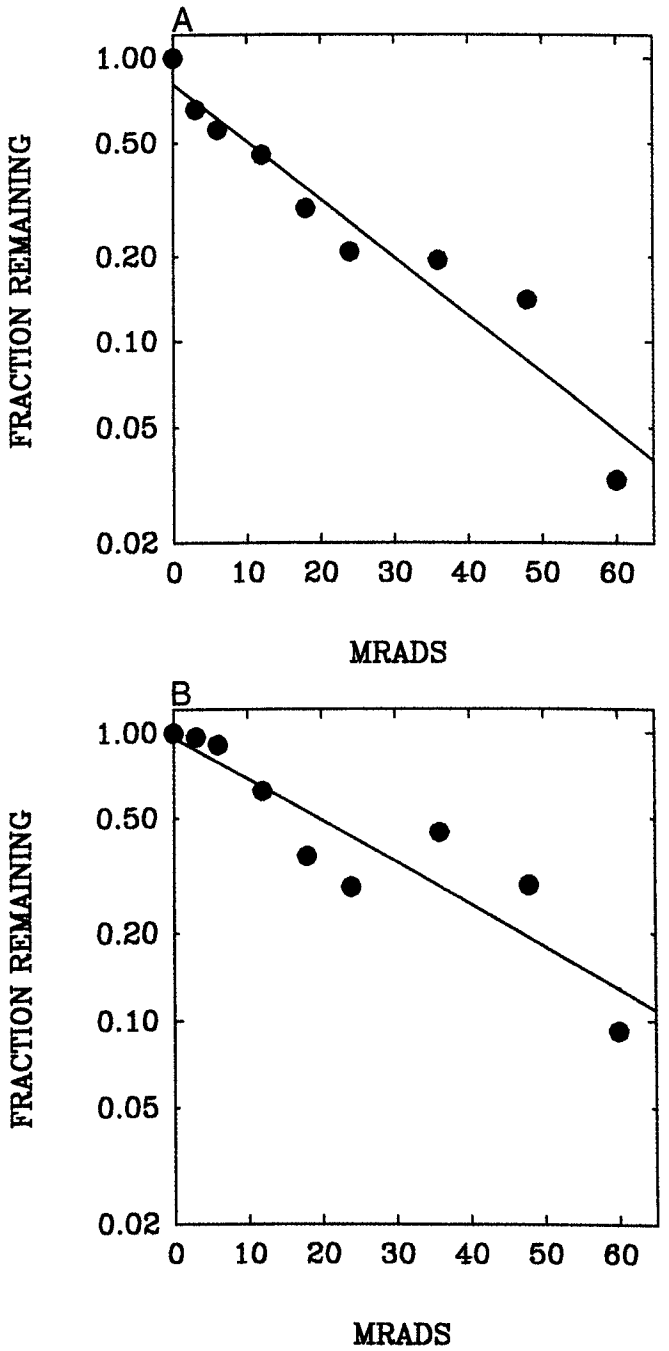

Figure 5. Radiation inactivation plots of glucose-6-phosphate dehydrogenase protein. Samples were irradiated in the absence (A) or presence (B) of 50 m*M* benzoic acid and mannitol. The target size in A was 84.3 kDa, with a correlation coefficient of –0.963. The target size in B was 60.7 kDa, with a correlation coefficient of –0.912. The target sizes were found to be statistically different ($F_{2,8} = 11.79$, $P = 0.0083$).

reached a maximum at 10 m*M* benzoic acid/mannitol and remained constant up to 100 m*M* (26). Thus, the minimum target size approximating the subunit was reached and did not get smaller with addition of more free radical scavenger.

To examine the question of whether long-lived free radicals are generated by the irradiation process, samples were irradiated with and without free radical scavengers. Glucose-6-phosphate dehydrogenase was added to the frozen samples after irradiation and shipment back to the University of South Florida. Adding glucose-6-phosphate dehydrogenase to irradiated solutions of only 0.25 *M* sucrose had no inhibitory effect (Fig. 6A). However, if the

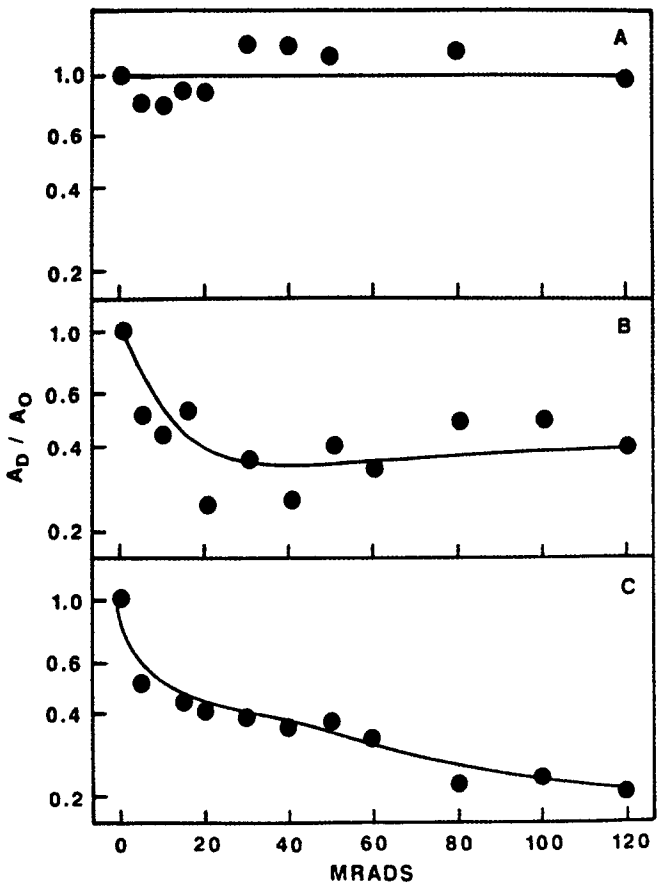

Figure 6. Effects of irradiated media on glucose-6-phosphate dehydrogenase activity. (A) 25 Units of glucose-6-phosphate dehydrogenase were added to 0.25 ml of 0.25 *M* sucrose irradiated up to 120 megarads. (B) Glucose-6-phosphate dehydrogenase was added to 0.25 ml of 0.25 *M* sucrose containing 50 m*M* benzoic acid and mannitol irradiated up to 120 megarads. (C) Glucose-6-phosphate dehydrogenase and 50 m*M* benzoic acid and mannitol were added to 0.25 ml of 0.25 *M* sucrose irradiated up to 120 megarads.

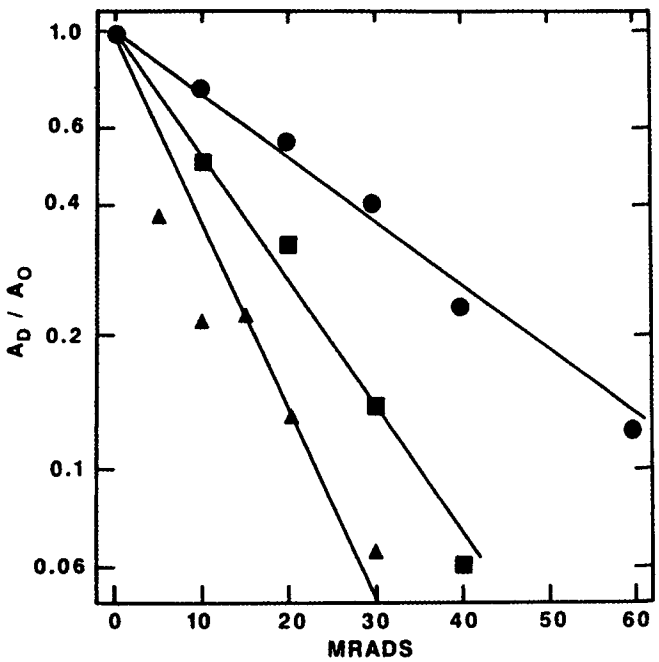

Figure 7. Effect of order of addition of 50 m*M* benzoic acid and mannitol on radiation inactivation of glucose-6-phosphate dehydrogenase. Irradiation was carried out with 50 m*M* benzoic acid and mannitol added before irradiation (●), without benzoic acid and mannitol (■), or with 50 m*M* benzoic acid and mannitol added after irradiation (▲). Activity remaining is given as A_D/A_0. The target sizes were 65 kDa (●), 124 kDa (■), and 192 kDa (▲).

free radical scavengers were included in the solution being irradiated, loss of over 50% of added glucose-6-phosphate dehydrogenase activity occurred at all doses of radiation ranging from 6 to 120 megarads (Fig. 6B). To determine whether the free radical scavengers had to be present during the irradiation, an experiment in which both the free radical scavengers and the glucose-6-phosphate dehydrogenase were added to irradiated sucrose samples was conducted. A dose-dependent loss of glucose-6-phosphate dehydrogenase activity was observed, in which case up to 80% of activity was destroyed (Fig. 6C). These experiments indicate that long-lived secondary radiolytic water products that can alter benzoic acid/mannitol into protein damaging molecules are generated. Yet when benzoic acid and mannitol are present during the irradiation of a protein, secondary damage to the protein is prevented.

The effects of long-lived radiolytic water products were further examined in the experiment depicted in Figure 7. When 50 m*M* benzoic acid and 50 m*M* mannitol were added to irradiated samples of glucose-6-phosphate dehydrogenase, apparent secondary damage was even more extensive than in samples without this added free radical scavenging system, giving a target size of 192 kDa, as compared with 65 and 124 kDa sizes for the sets irradiated with and without benzoic acid/mannitol, respectively.

Since we had previously shown that addition of 10 to 50 m*M* benzoic acid/mannitol could effectively protect DNA polymerase I, glucose-6-phosphate dehydrogenase, NADH:nitrate reductase, and sulfide oxidase from secondary free radical mediated damage during irradiation (26), the question of whether certain other compounds could also be protective was examined. This was done by irradiating several sets of samples containing glucose-6-phosphate dehydrogenase in 0.25 *M* sucrose with 42 megarads so that remaining enzyme activity was reduced to about 7% in the absence of potential free radical scavenger (Table 1). The benzoic acid/mannitol system was most effective. Maximal protection with these compounds is observed between 10 and 100 m*M* (26). Addition of dithiothreitol afforded partial protection, which plateaued between 20 and 100 m*M*. Glutathione addition had essentially no effect, whereas addition of L-cysteine was very inhibitory. The inhibitory effect of L-cysteine was even more pronounced at 5 m*M*. This inhibitory effect of L-cysteine may relate to production of the thiyl cysteine radical by reactive free radicals, such as hydroxyl radicals generated by the irradiation procedure (43). Addition of monothioglycerol or ascorbic acid produced small protective effects. Mercaptoethylamine,

Table 1. Effects of Addition of Free Radical Scavengers on Glucose-6-Phosphate Dehydrogenase Activity Remaining After 42 Megarads of Irradiation[a]

Addition	Activity remaining
None	0.074 ± 0.010
Benzoic acid, mannitol, 50 m*M*	0.350 ± 0.021
Glutathione, 100 m*M*	0.095 ± 0.006
Dithiothreitol, 100 m*M*	0.190 ± 0.015
L-Cysteine, 100 m*M*	0.022 ± 0.009
Monothioglycerol, 100 m*M*	0.138 ± 0.019
Mercaptoethylamine, 100 m*M*	0.053 ± 0.015
Ascorbic acid, 100 m*M*	0.123 ± 0.032
Diamide, 20 m*M*	0.066 ± 0.003
Hydrogen peroxide, 20 m*M*	0.052 ± 0.005
Catalase, 50 Kunits	0.169 ± 0.038
Superoxide dismutase, 1000 units	0.109 ± 0.010
Glutathione peroxidase, 2.5 units + 20 m*M* glutathione	0.016 ± 0.003

[a] Values are expressed as means ± SD relative to the unirradiated control.

diamide, and hydrogen peroxide additions were ineffective. Addition of a huge amount of catalase, 50 Kunits, was partially effective. Lesser amounts were ineffective. Addition of superoxide dismutase produced a small effect, while addition of glutathione peroxidase/glutathione was actually inhibitory.

Discussion

Reports have appeared that question the validity of radiation inactivation analysis for the determination of the functional size of a protein (39, 40). Some proteins exhibit higher mass sizes than expected. The specific biochemical properties of each enzyme, such as highly sensitive sulfhydryl groups, may make a significant contribution to its radiation sensitivity (40, 42). Reported functional unit sizes for enzymes and proteins determined by radiation inactivation analysis range from monomer to hexamer (5, 8, 18–25). We show that addition of the free radical scavenging system of benzoic acid/mannitol reduced the apparent functional unit size of bovine glutamate dehydrogenase from a hexamer to a trimer based on enzyme activity measurements (Fig. 1). The trimer minimal functional unit agrees with the minimal unit determined by denaturant inhibition of catalysis, coenzyme binding, and effector binding studies (34). When the determination of residual glutamate dehydrogenase protein was made by SDS PAGE, the addition of the free radical scavengers decreased the target size from 228 kDa to 59 kDa, a result that is in agreement with the subunit mass (Figs. 2 and 3). This result agrees with those of an earlier report (23), which concluded that radiation energy is not transferred from one subunit to another. With glucose-6-phosphate dehydrogenase, inclusion of the free radical scavenging system yielded the monomer as the functional unit, regardless of whether the determination was made by enzyme activity or immunoblotting of the protein (Figs. 4 and 5). This is critical, because glucose-6-phosphate dehydrogenase is widely used as an internal standard in radiation inactivation analysis and it has been thought that the dimer is the functional unit (31).

Irradiation of media with or without benzoic acid/mannitol demonstrated the formation of long-lived radiolytic products. Addition of benzoic acid/mannitol to irradiated media produced protein-damaging secondary radiation products (Fig. 7). Clearly, free radicals remained in the frozen state long after irradiation, and addition of benzoic acid/mannitol then produced extensive secondary damage to the target protein. The data indicate that the benzoic acid/mannitol free radical scavenging system may exert its protective effects by competing with the target protein for free radicals.

When *in situ* radiation inactivation of HMG-CoA reductase in microsomes or liver pieces was carried out, addition of the free radical scavenging system was not needed (8, 15). This may reflect the protective effects of double bonds in fatty acid moieties of membrane phospholipids. The monomer minimal functional unit of HMG-CoA reductase was obtained for samples from rats fed colestipol and mevinolin, and the disulfide-linked dimer size was obtained from liver samples from normally fed rats. The functional unit sizes for both alkyl-dihydroxyacetonephosphate synthase and dihydroxyacetonephosphate acyl transferase determined by *in situ* radiation inactivation corresponded to their single polypeptide chains (41).

The generation of radiolytic water products during irradiation and the prevention of their formation by various small molecules have previously been examined (31, 42). Exposure of yeast alcohol dehydrogenase to water irradiated with 3 to 48 megarads resulted in a 70% loss of activity without affecting the Km (42). In Figure 6A, addition of glucose-6-phosphate dehydrogenase to 0.25 *M* sucrose irradiated with up to 120 megarads did not inhibit the enzyme. It was previously shown that irradiation in sucrose solutions protected glucose-6-phosphate dehydrogenase from secondary damage (31); however, the target size remained at 100 kDa, a value that is larger than the subunit size. Cysteamine and ascorbate solutions had the same effect (31). In this study, the combination of benzoic acid/mannitol reduced the target size of glucose-6-phosphate dehydrogenase to that of the subunit size (Figs. 4 and 5).

The possibility that compounds other than benzoic acid/mannitol (e.g., thiols) might serve as effective free radical scavengers and prevent secondary damage during irradiation was investigated. Addition of 10 m*M* thioglycerol to samples of yeast alcohol dehydrogenase during irradiation has been reported to reduce the target size from 128,000 to 67,000, a size that is closer to that of the monomer rather than the tetramer, which is usually assumed to be the minimal functional unit (42). As shown in Table 1, addition of dithiothreitol did afford some protection. However, none of the other compounds or systems was as effective as benzoic acid/mannitol at preventing secondary damage.

Taken together, the data demonstrate that elimination of secondary damage by free radical scavengers during the irradiation procedure allows an estimation of the minimal unit required for enzyme activity. For glutamate dehydrogenase, this appears to be the trimer, while for glucose-6-phosphate dehydrogenase it is the monomer. It also appears that energy is not transferred between subunits during irradiation. A reexamination of reports of various oligomers serving as the minimal functional units based upon radiation inactivation analysis is indicated.

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