Inactivation of Secretory Phospholipase A_2 by Ionizing Radiation

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ABSTRACT The extracellular phospholipase A_2s (PLA_2) from cobra venom, rattlesnake venom, and porcine pancreas were analyzed by radiation inactivation to determine their functional aggregation states. The analysis was performed in the presence of the protein transferrin at two different concentrations of PLA_2: 5 μg/ml and 2500 μg/ml. The small size of these proteins necessitated the use of high radiation dosages. The catalytic activity of all samples decreased as a single exponential as a function of radiation dosage, to >97% inactivation. Target size analysis of these curves yielded sizes corresponding to dimers for all three PLA_2s, indicating that all three enzymes exist as dimers or larger aggregates under the conditions studied. An analysis of the amount of intact protein remaining by sodium dodecyl sulphate-polyacrylamide gel electrophoresis showed that the loss of protein also followed a dimeric size for all three PLA_2s. The loss of protein as a dimer indicates that transfer of radiation energy is occurring between polypeptides.

INTRODUCTION

Cobra venom phospholipase A_2 (PLA_2) is a 13.3 kDa enzyme which catalyzes the hydrolysis of fatty acids from the *sn*-2 position of membrane phospholipids (Reynolds and Dennis, 1991; Dennis, 1983). The enzyme displays a strong tendency to aggregate and forms dimers and higher order aggregates at high protein concentrations (Deems and Dennis, 1975). The recently solved x-ray crystal structure of cobra venom PLA_2 has revealed that it crystalizes as a trimer (Fremont et al., 1993). In fact, a trimeric association has been observed in three different crystal forms (B. Segelke, N.-H. Xuong, I.A. Wilson, and E.A. Dennis, manuscript in preparation). None of the methods employed to date (Deems and Dennis, 1975; Lewis et al., 1977; Plückthun and Dennis, 1985; Hazlett and Dennis, 1985b; Lombardo and Dennis, 1985; Hazlett and Dennis, 1988b) have been able to demonstrate a functional requirement for aggregation, and it is not known whether this aggregation is related to a catalytically active or inactive form of the enzyme.

One method that has often been employed to determine the size of catalytically active subunits is the technique of radiation inactivation (for review see Kempner, 1988). During radiation inactivation, proteins or other macromolecules are exposed to ionizing radiation at low temperature (Kempner et al., 1986) resulting in severe damage to the macromolecule. γ-rays and electrons interact randomly throughout the exposed sample, and the probability of an interaction ("primary ionization") with a given molecule is proportional to the mass of the macromolecule. In each interaction, a large amount of energy (1500 kcal/mol, on the average) is deposited in and absorbed by the target molecule, resulting in irreversible damage via the breakage of covalent bonds. This damage may be observed by a decrease in the number of molecules as well as by a loss in biological activity. Target analysis (Kempner and Miller, 1983; Horowits et al., 1986) of this loss as a function of radiation dosage permits calculation of the functional mass of the protein or macromolecule involved.

This technique has been successfully applied to the study of many enzymes (Kempner, 1988; Kempner and Fleischer, 1989) including lipases (Kempner et al., 1991), a Ca^{2+}-independent PLA_2 from $P388D_1$ cells (Ackermann et al., 1994), and the 85 kDa human cytosolic PLA_2 (Tremblay et al., 1992). Herein we apply this technique to study the smaller secretory PLA_2s from the cobra and other species.

MATERIALS AND METHODS

Materials

Cobra venom (*Naja naja naja*) was purchased from Miami Serpentarium (Punta Gorda, FL), and PLA_2 was purified as described previously (Reynolds and Dennis, 1991; Hazlett and Dennis, 1985a). The enzyme was dialyzed against water and lyophilized before use. PLA_2 from rattlesnake venom (*Crotalus adamanteus*) and from porcine pancreas were purchased from Worthington Biochemical Corp. (Freehold, NJ) and Boehringer Mannheim (Indianapolis, IN) respectively. Human transferrin (iron free) and Coomassie brilliant blue 250R were purchased from Sigma Chemical Co. (St. Louis, MO). *Staphylococcus aureus* nuclease, salmon sperm DNA, and Triton X-100 were from Calbiochem Corp. (San Diego, CA). 4,4′-Dithiodipyridine was obtained from Aldrich Chemical Co. (Milwaukee, WI). Dipalmitoyl-*sn*-glycero-3-phosphorylcholine (dipalmitoyl PC), dioctanoyl-*sn*-glycero-3-phosphorylcholine (dioctanoyl PC), and diheptanoyl-*sn*-glycero-3-phosphorylcholine (diheptanoyl PC) were purchased from Avanti Polar Lipids, Inc. (Birmingham, AL). 1,2-Bis(decanoylthio)-1,2-dideoxy-*rac*-glycero-3-phosphatidylcholine (thioPC) was synthesized as described previously (Hendrickson et al., 1983).

Sample preparation and irradiation

All enzyme samples for irradiation were prepared in 10 mM Tris-HCl buffer, pH 8.0, containing 1 mM EGTA. Samples contained PLA_2 from cobra,

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rattlesnake, or pancreas at concentrations of 5 or 2500 μg/ml. The samples also contained small amounts of staphylococcal nuclease at 25 μg/ml. Transferrin was added to bring the total protein concentration to 10 mg/ml. Any other additions are as described in the text. Aliquots (0.2–0.3 ml) of the prepared samples were placed in glass ampules (#12012, Kimble Glass, Inc., Vineland, NJ) and frozen at −70°C. The ampules were then sealed in an oxygen-gas flame. The frozen samples were held at −80°C except during irradiation, which was performed at −135°C (Kempner et al., 1986). Samples were irradiated with high energy electrons as described previously (Harmon et al., 1985). Radiation dosage was determined by thermoluminescent dosimetry. After irradiation, the frozen ampules were opened, purged with nitrogen, allowed to thaw at room temperature, and then placed on ice. After thorough mixing, aliquots were removed for assay.

Enzymatic assays

Cobra and rattlesnake PLA_2 activities were determined using a spectrophotometric thiol assay as described previously (Hendrickson and Dennis, 1984; Yu and Dennis, 1991) The thiol assay contained 1 mM thioPC and 4.25 mM Triton X-100 in 25 mM Tris-HCl buffer, pH 8.5, with 10 mM $CaCl_2$, 0.1 M KCl, and 0.83 mM dithiodipyridine. The reaction was initiated by the addition of 75 ng of PLA_2 and incubated at 30°C. The reaction was monitored by following the absorbance at 324 nm for 1–2 min. For some experiments, cobra PLA_2 activity was determined using a titrimetric pH stat assay (Deems and Dennis, 1981). The pH stat assay contained 5 mM dipalmitoyl PC, 20 mM Triton X-100, and 10 mM $CaCl_2$ at pH 8.0 and 40°C. This reaction was initiated by the addition of 50–100 ng PLA_2 and was monitored for 2–3 min by observing the titration of the enzymatic products with 5 mM KOH. The porcine pancreatic PLA_2 was also assayed titrimetrically using the method of De Haas et al. (1971). The assay mixture contained 10 mM dioctanoyl PC, 0.1 M NaCl, 10 mM $CaCl_2$, and 0.5 mM Tris-HCl at pH 8.0 and 40°C. The reaction was initiated by the addition of 50–100 ng of the enzyme, and the titration rate was recorded for 2–3 min.

Nuclease activity was measured by the method of Cuatrecasas et al. (1967), except that the buffer was 25 mM sodium glycinate, pH 9.5. The assay mixture also contained 50 μg salmon sperm DNA, 10 mM $CaCl_2$, and 0.1% bovine serum albumin at 30°C. The reaction was initiated by the addition of 25–100 ng nuclease and was monitored at 260 nm.

Electrophoresis

In addition to the enzymatic assays, the samples containing high concentrations of PLA_2 were also analyzed for the amount of intact protein remaining. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970). 18% gels were prepared using 16 × 18 cm plates, 1.4 mm spacers, and 10 well combs. Protein samples containing 30 μg of PLA_2 were prepared from equal volume aliquots of both irradiated vials and nonirradiated controls. One well of each gel contained Bio-Rad prestained low molecular weight protein standards (Bio-Rad Laboratories, Richmond, CA). After electrophoresis, gels were stained with Coomassie brilliant blue 250R. Color transparencies of the stained gels were obtained by photographing with 4 × 5-inch color slide film. The protein band on the transparencies corresponding to PLA_2 was scanned at 575 nm using a DU-8 spectrophotometer from Beckman Instruments, Inc. (Fullerton, CA). The area under each peak was determined by the internal computer or by cutting and weighing the printed scan. The amount of PLA_2 electrophoresed was determined to be in the linear range of the Coomassie response under these conditions. The electrophoresis of transferrin samples was determined by the same procedure using 12% gels.

Data analysis

Target analysis of the radiation inactivation data was performed as described previously (Harmon et al., 1985). Target sizes were determined from the slope of the inactivation curves by using both an unconstrained, least squares fit of the data as well as by a fit constrained to 1.0 at zero radiation dose. No significant difference between these two methods were observed, and the values from the unconstrained procedure are reported here. Target sizes are reported ±SD, which represents the variation between experiments.

RESULTS

Two proteins, staphylococcal nuclease and human transferrin, were added to all radiation inactivation samples as internal controls. The nuclease served as an internal control for enzymatic activity measurements and for the calculation of radiation dosage. Any deviations in radiation dosage from calculated values would be reflected by anomalous activities in both PLA_2 and nuclease assays. Nuclease was chosen based on the similarity of the protein monomer size to that of PLA_2. The inclusion of transferrin served two functions. First, transferrin acted as a control for the analysis of protein monomers by SDS-PAGE. Second, optimal results with this technique are observed using samples with a high protein content (Kempner and Miller, 1994), and the inclusion of transferrin served to increase the protein content of the samples. Transferrin has been used successfully in this capacity in other studies (Santos et al., 1988). Neither of these additions affected PLA_2 activity. No change in activity was noted upon freezing and thawing of samples.

After exposure to various doses of ionizing radiation, aliquots were removed from the thawed samples and assayed for both PLA_2 and nuclease activity. Samples containing high concentrations of cobra PLA_2 (2500 μg/ml) were also analyzed by SDS-PAGE. Data from a typical experiment are shown in Fig. 1. The PLA_2 enzymatic activity was observed to decrease as a simple exponential function of radiation dose. The surviving PLA_2 activity was used to calculate a target size for the active structure. In the experiment shown, a target size of 28.3 kDa was obtained for cobra PLA_2 and 39.6 kDa for nuclease. SDS-PAGE of the 2500 μg/ml PLA_2 samples revealed a Coomassie-stained PLA_2 band migrating at M_r = 13,000. Densitometric scanning of this band in several irradiated samples permitted quantitation of the surviving PLA_2 protein. The SDS-PAGE data shown in Fig. 1 yielded a target size of 24.7 kDa. For the high concentration cobra PLA_2 samples, four such SDS-PAGE and activity experiments were performed yielding an average target size of 29 ± 3 kDa for PLA_2 activity and 25 ± 2 kDa for the SDS-PAGE gel band. The average target sizes obtained from PLA_2 and nuclease activities and from SDS-PAGE analysis are summarized in Table 1.

Radiation inactivation experiments were also conducted with the low concentration cobra PLA_2 samples (5 μg/ml). At this concentration, there is too little protein to analyze by SDS-PAGE. Thus, with these samples, only enzyme activity was measured. Fig. 2 shows the loss of PLA_2 activity in five different experiments. These data also demonstrate the reproducibility between experiments. The average target size for the loss of PLA_2 activity in these experiments was 27 ± 4 kDa. The measurements were indifferent to alterations in assay conditions, because data obtained using the pH stat assay were comparable to that obtained using the spectrophotometric thiol assay. In two experiments, the amount of

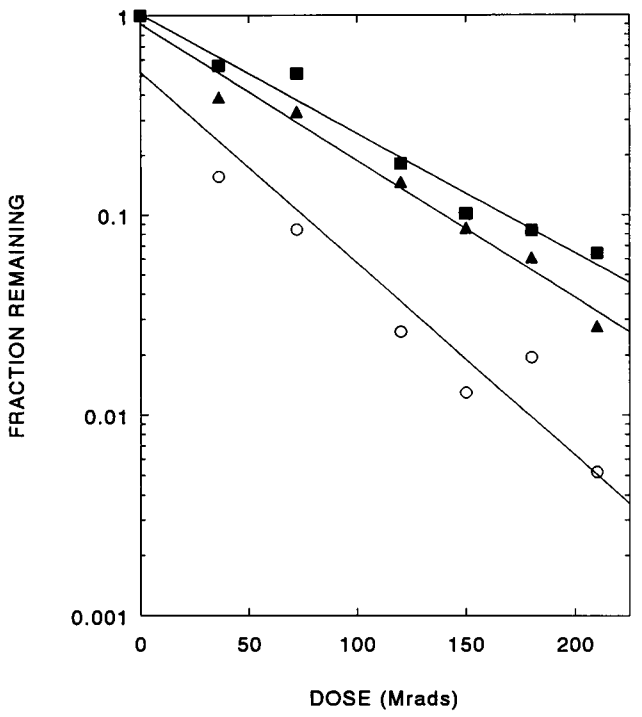

FIGURE 1 Radiation Inactivation of samples containing cobra PLA_2 at high concentration. Irradiated samples containing 2500 μg/ml cobra PLA_2 were assayed for surviving PLA_2 activity (▲), surviving PLA_2 protein from SDS-PAGE (■), and surviving staphylococcal nuclease activity (○), as described in the text. The fraction of remaining activity is plotted as a function of radiation exposure at −135°C. The lines shown are a least-squares fit to the data.

transferrin remaining in irradiated samples was measured by SDS-PAGE. A rapid loss of transferrin protein with radiation exposure was observed, which led to an average target size of 78 kDa. Table 1 gives the average target sizes determined for these parameters.

Cobra PLA_2 was also irradiated in the presence of diheptanoyl PC, yielding a target size of 33 ± 6 kDa, which is not statistically different from the target size obtained in its absence (see Table 1). In a few experiments cobra PLA_2 was irradiated in the presence of 10 mM $CaCl_2$ (see Fig. 2), dioctanoyl PC, or dipalmitoyl PC (data not shown). None of these compounds had any significant effect on the target size of PLA_2.

In addition to radiation inactivation of the cobra PLA_2, we also measured the target sizes of the rattlesnake and pancreatic enzymes. As above, these PLA_2s were studied at high and low concentrations of PLA_2 and were measured for remaining PLA_2 and nuclease activities and for radiation destruction of PLA_2 protein. The target sizes for these parameters are summarized in Table 1. The data for the loss of PLA_2 activity in samples containing a low concentration of rattlesnake PLA_2 are shown in Fig. 3, while the loss of PLA_2 activity in low concentration pancreatic PLA_2 samples is shown in Fig. 4. The results with these two enzymes show a striking similarity to the results obtained with the cobra PLA_2. There was one difference noted in the quality of the data, however, in that the pancreatic PLA_2 activity observed at extremely high radiation doses showed considerable scatter in comparison with that observed with either the cobra or rattlesnake enzymes. Nevertheless, the inactivation curves show that in all three species at least 97% of the PLA_2 activity is due to a single-sized target whose mass corresponds to two monomers.

Several observations can be made by comparison of the data presented in Table 1. 1) It is clear that the target sizes obtained from PLA_2 and nuclease activities were not affected by the concentration of PLA_2 protein. These results are to be expected, given that the total protein in these samples was held constant at 2 mg/vial. 2) The target sizes for PLA_2 activity were essentially the same for cobra, rattlesnake, and pancreatic PLA_2. 3) Independent of the source of the enzyme, the target size determined from the loss of PLA_2 protein was the same as that from the loss of PLA_2 activity. 4) Staphylococcal nuclease behaved the same in all samples, regardless of the composition of the sample, with an average target size of 31 ± 6 kDa for the 16 experiments that were assayed. 5) For all of these enzymes, the measurements yielded target sizes very close to double the known monomer sizes of 13,300 Da for cobra PLA_2 (Davidson and Dennis, 1990), 13,800 Da for pancreatic PLA_2 (Volwerk and De Haas, 1982), 15,000 Da for rattlesnake PLA_2 (Wells, 1971), and 16,800 Da for nuclease (Taniuchi et al., 1967). 6) Unlike these smaller proteins, the loss of transferrin protein indicated a target size of 78 kDa, in good agreement with the known monomeric size of transferrin, 79,570 Da (MacGillivray et al., 1983) and with the previously reported target size of 75 kDa (Santos et al., 1988).

DISCUSSION

Cobra venom PLA_2 has been shown by several different techniques, including gel permeation chromatography (Deems and Dennis, 1975), ultracentrifugation (Deems and Dennis, 1975), fluorescence polarization (Hazlett and Dennis, 1985b, 1988a), suberimidate crosslinking (Roberts et al., 1977), and photoactivatable crosslinking (Lewis et al., 1977) to exhibit a concentration-dependent aggregation. Below 50 μg/ml, the enzyme exists as a monomer in solution. Between 0.1 and 2 mg/ml it exists predominantly as a dimer, and above about 5 mg/ml very large aggregates of enzyme appear (Deems and Dennis, 1975). Moreover, the enzyme is found as a trimer in x-ray crystal structures (Fremont et al., 1993). Unlike the cobra enzyme, the rattlesnake enzyme is generally found as a dimer in solution (Smith and Wells, 1981), whereas the pancreatic enzyme is generally found as a monomer (Volwerk and De Haas, 1982; Soares de Araujo et al., 1979; Hazlett et al., 1990). The relationship of these aggregation states to catalytic activity is unclear. Some researchers maintain that the extracellular PLA_2s are active as monomers (Jain et al., 1991) whereas others maintain that they are active as dimers (Romero et al., 1987). Because of the concentration-dependent aggregation of the cobra PLA_2, experiments were performed at two different concentrations: at 5 μg/ml, where

TABLE 1 Radiation inactivation target sizes

Sample	PLA_2 ($\mu g\ ml^{-1}$)	N	Target size* (kDa)			
			Activity		Gel band	
			PLA_2	Nuclease‡	PLA_2	Transferrin‡
Cobra	2,500	4	29 ± 3	35 ± 4	25 ± 2	
	5	5	27 ± 4	29 ± 4§		78¶
Cobra + diheptanoyl PC	5	3	33 ± 6			
Rattlesnake	2,500	3	36 ± 6	34‖	30 ± 4	
	5	3	28 ± 2	32 ± 10		
Pancreas	2,500	2	28		27	
	5	4	27 ± 8	30 ± 7		

*Target sizes are reported as the average of N runs ± the standard deviation.
‡Nuclease and transferrin were present, but not always assayed, in all samples.
§$N = 4$
¶$N = 2$
‖$N = 1$

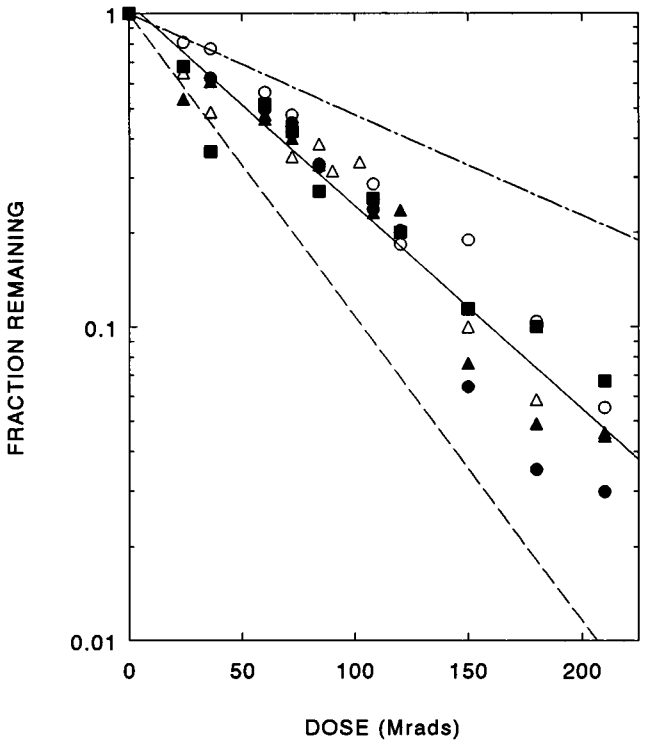

FIGURE 2 Radiation inactivation of cobra PLA_2 at low concentration. The fraction of remaining PLA_2 activity from five independent irradiation experiments is shown plotted as a function of radiation exposure at −135°C. The different symbols denote different experiments. Each sample contained 5 μg/ml cobra PLA_2. (■) Experiment containing 10 mM $CaCl_2$, assayed by pH stat. All other experiments were run in the absence of added Ca^{2+} and were assayed using the thiol assay. The solid line is the least-squares fit to the data as described in Materials and Methods. The upper broken line is the theoretical line expected for a monomer; the lower dashed line represents that of a trimer.

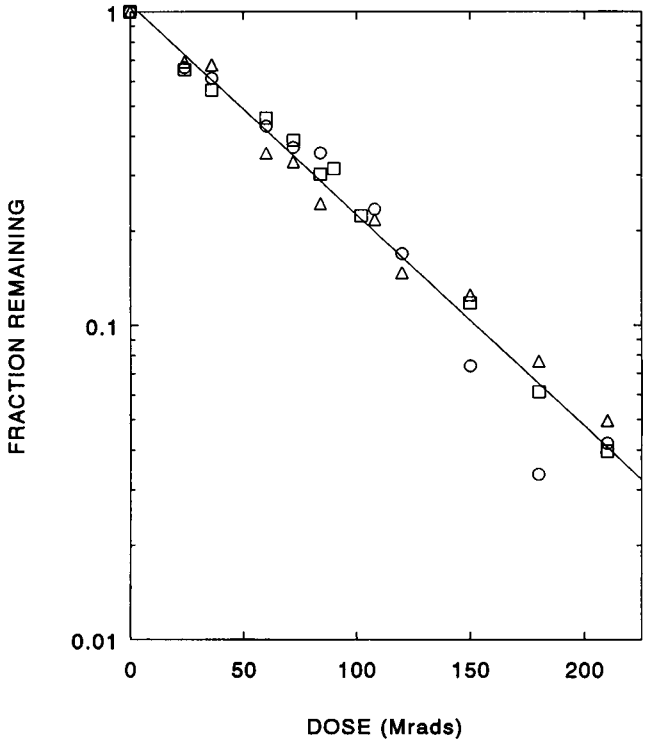

FIGURE 3 Radiation inactivation of rattlesnake PLA_2. The fraction of remaining PLA_2 activity from three independent irradiation experiments is shown plotted as a function of radiation exposure at −135°C. The different symbols denote different experiments. Each sample contained 5 μg/ml rattlesnake PLA_2.

the enzyme should be a monomer in solution, and at 2.5 mg/ml, where the enzyme should be a dimer or higher order aggregate. For comparison, rattlesnake and pancreatic PLA_2s were also investigated at both concentrations.

Radiation inactivation of cobra PLA_2 yielded a single exponential, which implies that only a single-sized structure is required for activity. In experiments performed at high and low concentrations of enzyme, the calculated target sizes were statistically indistinguishable. The best estimate of the target size for cobra PLA_2 activity in these experiments is 28 kDa. The known amino acid sequence of the single PLA_2 peptide is 13,348 Da (Davidson and Dennis, 1990). Therefore, the radiation target must correspond to a dimer. The same results were obtained for the rattlesnake and pancreatic enzymes.

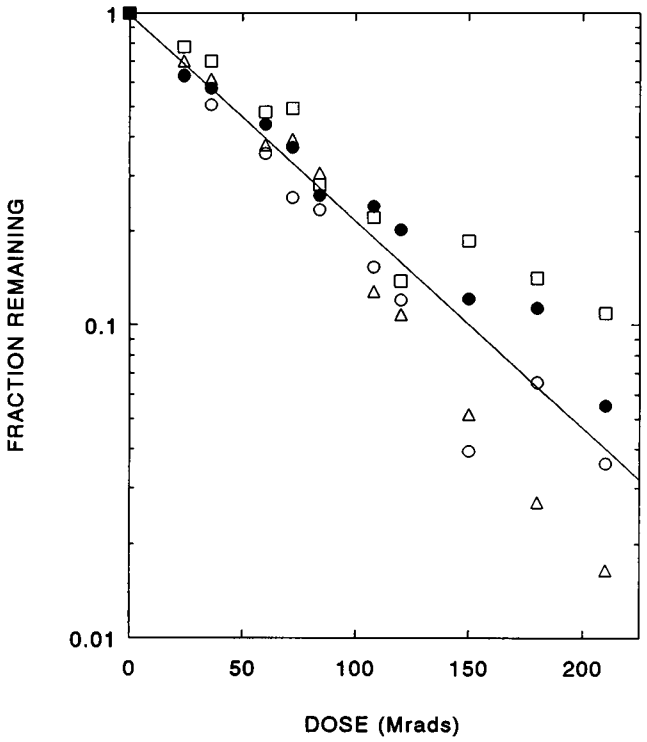

FIGURE 4 Radiation Inactivation of pancreatic PLA_2. The fraction of remaining PLA_2 activity from four independent irradiation experiments is shown plotted as a function of radiation exposure at −135°C. The different symbols denote different experiments. Each sample contained 5 μg/ml pancreatic PLA_2.

The results of these experiments clearly indicate that the trimeric form is not necessary for cobra PLA_2 to be enzymatically functional. Thus, the trimeric association seen in x-ray crystal structures is not catalytically relevant. This trimeric association could, however, still play a role in the concentration dependent aggregation of cobra PLA_2 observed in solution. This also raises an interesting question as to whether there is an inactive, trimeric storage form of the enzyme under physiological conditions (Fremont et al., 1993).

The loss of activity in an irradiated polypeptide follows from massive structural damage such as chain scission, and leads to a target size of that structure. Oligomeric target sizes for activity may arise for two different reasons, which can be experimentally distinguished (Kempner and Fleischer, 1989). The first is that several polypeptides are required to express enzymatic activity, either because the substrate (or cofactor) binds simultaneously to two (or more) polypeptides (such as in thymidylate synthase (Hardy et al., 1987)); the second reason is that the conformation of a peptide is influenced by interaction with one or more others (as in *Neurospora* glutamate dehydrogenase (Coddington et al., 1966)). In both of these cases, the structure of only one subunit would be destroyed by radiation, and the target size determined from the monomer band on SDS-PAGE would be that of the subunit. However, the target size for activity would be an oligomer. There is a second situation that can result in an oligomeric target size for activity; in these cases, the destruction of a polypeptide results not only from a hit occurring in that chain but also from the transfer of radiation energy from a geminate strand that suffered a primary ionization. The geminate polypeptide is destroyed in the process, and so is the adjacent chain. In such a case of energy transfer, subunit monomers resolved by SDS-PAGE will decrease with an oligomeric target size.

In the case of the cobra, rattlesnake, and pancreatic PLA_2s, the dimer target size determined from the loss of protein on SDS-PAGE gels clearly shows significant energy transfer between monomers. Whether one or two subunits are required for enzymatic activity is thus indeterminate, but certainly not more than two are involved.

The internal standard, transferrin, was the only protein that gave a monomeric target size. This protein, at 79.6 kDa, is considerably larger than the phospholipases tested in this study. The other internal standard, staphylococcal nuclease, decayed at a target size of 31 kDa, close to double that of the monomeric size of 16.8 kDa. The oligomeric target for nuclease activity is due either to energy transfer between monomers or to a functional requirement of two subunits for expression of catalytic activity. One study using NMR techniques reported dimers of staphylococcal nuclease (Alexandrescu et al., 1989). However, a concentration-dependent dimerization was observed at enzyme concentrations much higher than those employed in the present study.

As discussed elsewhere (Vaidhyanathan and Jung, 1987; Kempner and Fleischer, 1989; Jhun et al., 1991), the mechanism of energy transfer between proteins is not well understood. While energy transfer is known to occur between polypeptides that are linked by a disulfide bond (Haigler et al., 1985), the occurrence of energy transfer between noncovalently linked polypeptides is uncommon. Nonetheless, several such examples of energy transfer have been documented including two calcium pump proteins (Hymel et al., 1984; Chamberlain et al., 1983), the proton pump of *Neurospora* plasma membrane (Bowman et al., 1985), the H^+/K^+ pump of gastric mucosa (Saccomani et al., 1981), D-β-hydroxybutyrate dehydrogenase (McIntyre et al., 1983), and the 12 transmembrane α-helices of the human erythrocyte glucose transporter (Jhun et al., 1991). It is interesting to note that all of these examples are of proteins that interact with membranes or phospholipids as does PLA_2. Further work will be needed to understand what physical or structural conditions lead to energy transfer and to find conditions that allow the analysis of the extracellular PLA_2s without this complication.

The observation of dimeric target sizes for all three PLA_2s means that under the conditions studied (frozen, in the presence of 10 mg/ml transferrin, etc.), these enzymes are all aggregated at least as dimers. They clearly differ from the monomeric target size reported for the human 85 kDa cytosolic PLA_2 (Tremblay et al., 1992) and the tetrameric/oligomeric target size found for the ATP-activated, Ca^{2+}-independent PLA_2 enzyme from the $P388D_1$ macrophage-like cell line (Ackermann et al., 1994). While dimeric states

have previously been reported for the low molecular weight PLA_2s (Smith and Wells, 1981; Hazlett et al., 1990; Romero et al., 1987; Bukowski and Teller, 1986; Myatt et al., 1991), the physiological significance of dimer formation is not understood, and the functional necessity of a dimer for catalytic activity has never been directly demonstrated. The occurrence of energy transfer in these samples prevents us from distinguishing between a monomer and a dimer as the functional form of the enzyme.

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