Radiation inactivation of proteins: temperature-dependent inter-protomeric energy transfer in ox liver catalase

Michel POTIER,* Josée-France VILLEMURE and Louise THAUVETTE

Service de Génétique Médicale, Hôpital Sainte-Justine, Département de Pédiatrie, Université de Montréal, Montréal, Québec, Canada H3T 1C5

The radiation-inactivation method is widely used to determine the oligomeric structure of enzymes without need for solubilization or purification. We have used purified ox liver catalase, a tetrameric enzyme in solution, to study energy transfer between associated protomers responsible for oligomer inactivation. However, after freeze-drying the tetramer dissociates into an asymmetric dimer. In the present paper we compare both the radiation-inactivation size (obtained by following the activity decay) and the target size (obtained by measuring the amount of remaining protein by SDS/PAGE) of catalase under various states of aggregation and temperature. At -78°C , only one protomer was fragmented after being hit by a γ -ray and, as expected, this protomer was also inactivated. This result was obtained when

either catalase was in tetrameric or in dimeric forms. However, at 38°C , even though a single monomer was fragmented as at -78°C , the whole dimer was inactivated. This result suggests that, at the higher temperature, there is a transfer of energy from the fragmented protomer to the other associated protomer, causing inactivation of the whole dimer. The inactivation of oligomeric enzymes is a two-step mechanism involving: (1) fragmentation of the hit monomer, followed by (2) temperature-dependent energy transfer from the fragmented towards the associated protomer. Thus we conclude that the radiation-inactivation size reflects the transfer of absorbed energy inside the oligomer which causes inactivation of one or several monomers.

INTRODUCTION

The radiation-inactivation method is widely used to determine the molecular size of proteins *in situ* (Kempner and Schlegel, 1979; Beauregard et al., 1987; Kempner and Fleisher, 1989). The method is based on the target theory, which relates the rate of loss of enzyme activity under radiation exposure to molecular mass (Lea, 1955). The main advantage of the method is that membrane-bound or aggregated proteins are studied directly without solubilization or purification and that the molecular size of the catalytic unit, either monomer or oligomer, is obtained directly. However, the applicability of the method is limited because, in some oligomers, radiation energy is transferred between subunits causing inactivation of the whole oligomer (Saccomani et al., 1981). In the presence of inter-protomeric energy transfer, no conclusion can be obtained on the size of the active enzyme either monomer or oligomer (Beauregard et al., 1987). Thus it appears necessary to understand the physical conditions which determine energy transfer between subunits so that the radiation-inactivation method would be more general and useful for the study of oligomeric proteins.

When an ionizing radiation interacts with a protein molecule, a relatively large amount of energy (around 66 eV) is deposited (Kepner and Macey, 1968). This absorbed energy is dissipated in the molecule by causing permanent damage in the form of covalent-bond breaks or conformational changes. Two types of energy transfer have been characterized in proteins. In type I, the quantity of energy transferred is sufficient to cause breaks in several protomers, as previously suggested for several proteins and, principally, the ATPases [reviewed by Beauregard et al. (1987)]. However, this interpretation has been challenged by le Maire et al. (1990) on the basis of a study of 12 well-characterized soluble oligomeric proteins where the fragmentation of proto-

mers under radiation exposure occurred independently. Those authors found no evidence of energy transfer of a magnitude sufficient to cause simultaneous fragmentation of several protomers from a single hit. In type II energy transfer, recently documented in tetrameric *Escherichia coli* β -galactosidase (Potier et al., 1991), the mechanism of inactivation involves fragmentation of the protomer directly hit by the γ -ray and temperature-dependent conformational changes in associated protomers. This denaturation is due to energy transfer at higher temperatures.

In the present paper we study the enzyme catalase, which exists as a tetramer in solution with 222 symmetry and as a highly asymmetric dimer after freeze-drying (Tanford and Lovrien, 1962). We report that catalase also shows type II energy transfer, reacting similarly to β -galactosidase when irradiated at low and high temperatures.

MATERIALS AND METHODS

Materials

Ox liver catalase and SDS were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Before use for irradiation experiments, the purity of the preparation was tested by SDS/PAGE as described by Laemmli (1970).

Irradiation conditions

Catalase solutions (0.1 mg/ml) in 10 mM Tris, pH 8, were either freeze-dried overnight or frozen rapidly in 1.5 ml Eppendorf microcentrifuge tubes. The freeze-dried powders were flushed with dry nitrogen and capped before being irradiated in a Gammacell 220 ^{60}Co source (Atomic Energy of Canada, Ottawa, Canada) at a dose rate of about 1.2 Mrad/h (Beauregard et al.,

1987). The temperature of the frozen samples was kept at -78°C during irradiation with crushed solid CO_2 . The freeze-dried samples were irradiated either in solid CO_2 at -78°C or at the irradiation chamber temperature (38°C). Enzyme activity was measured by a spectrometric procedure (Beers and Sizer, 1952), and a linear relationship was observed with a quantity of enzyme up to $0.4\text{ }\mu\text{g/ml}$.

Fragmentation of catalase protomers as a function of increasing radiation dose was monitored quantitatively by SDS/PAGE in an 11% (w/v)-polyacrylamide gel (Laemmli, 1970) as previously described by Karlish and Kempner (1984). The gel was stained with Coomassie Blue R-250, destained, and the band corresponding to the intact protein was excised from the gel. The Coomassie Blue stain was extracted overnight in 5% (w/v) SDS and the absorbance of the extract was determined at 605 nm. The relationship between absorbance at 605 nm and amount of protein was linear over the range $2\text{--}40\text{ }\mu\text{g}$ of catalase.

The logarithm of the amount of catalase activity or of protein stain intensity was plotted as a function of the radiation dose (expressed in Mrad) and the dose necessary to diminish catalase 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 catalase activity, and the target size (TS), measured by monitoring the stain intensity of the band corresponding to the native catalase protomer in an SDS/polyacrylamide gel, were computed from the equation (Beauregard et al., 1987):

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

where t is the irradiation temperature in $^{\circ}\text{C}$. The RIS is defined as the mass of 1 mol of protein inactivated by a hit, and TS as the mass of 1 mol of protein whose polypeptide chain has been fragmented by a hit (Beauregard et al., 1987).

RESULTS

Radiation fragmentation of catalase

SDS/PAGE analysis of catalase under increasing doses of γ -rays at -78°C showed progressive decay of the 57.5 kDa protomer band into defined radiolysis fragments of about 29–53 kDa (Figure 1). Identical fragments were obtained when the enzyme was irradiated either at 38 or -78°C , suggesting that the fragmentation pattern is independent of irradiation temperature (only data at -78°C are shown in Figure 1). A semi-logarithmic plot of the band intensity corresponding to intact catalase protomer versus radiation dose at -78°C yielded a D_{37} of 50 Mrad, corresponding to a TS of 25 kDa according to the usual equation (eqn. 1), but gave a value of 35 kDa according to the calibration curve of le Maire et al. (1990). A similar TS value was obtained when irradiation was carried out at 38°C (Table 1). However, at both temperatures the TS values were lower than those expected for the monomer (57.5 kDa), even when the calibration curve of le Maire et al. (1990) was used. As shown on the SDS/PAGE gel, several radiolysis fragments migrated close to the band corresponding to the monomer (Figure 1). These fragments may interfere with the determination of the residual quantity of intact protomers, yielding an underestimated protein decay with dose and consequently a lower apparent TS value.

Radiation inactivation of catalase

The freeze-drying procedure which caused dissociation of tetra-

meric catalase into dimers was accompanied by a loss of about 70% of its activity. Even freezing by itself caused the loss of 46% of catalase activity. Figure 2 shows the decay of catalase activity as a function of radiation dose at -78 and 38°C . The corresponding RIS values are given in Table 1. We found similar RIS values for catalase irradiation at -78°C , either in frozen solution or in freeze-dried form, which corresponded approximately to the catalase protomer. The RIS values at -78°C for the frozen and freeze-dried catalase preparations (45 and 55 kDa respectively) were higher than the corresponding TS values and closer to the expected value of 57.5 kDa. However, the difference between RIS and TS values were not statistically significant (Student's t test: $P < 0.01$). In contrast, when the irradiation was carried out at 38°C , a much larger RIS value of 133 kDa was obtained (Table 1). This value corresponded approximately, considering the experimental error, to that expected for a dimeric arrangement of catalase protomers (115 kDa). Thus we conclude that, at 38°C , each hit causes the fragmentation of a single protomer, but that the associated protomer in the dimer is also inactivated. The inactivation of the

Figure 1 SDS/PAGE of radiolysis fragments of catalase on a 11% (w/v) gel

Samples containing $25\text{ }\mu\text{g}$ of catalase were applied in each lane after irradiation at -78°C with the following doses: lane 1, unirradiated control; lane 2, 2.01 Mrad; lane 3, 4.02 Mrad; lane 4, 6.03 Mrad; lane 5, 8.03 Mrad; lane 6, 11.2 Mrad; lane 7, 14.2 Mrad; lane 8, 18.1 Mrad; lane 9, 24.1 Mrad. The D_{37} value was 41.8 Mrad.

Table 1 Radiation inactivation size and target size of ox liver catalase

Results are means $\pm$ S.D., with the number of determinations indicated in parentheses. Abbreviations: Fro, frozen sample; FD, freeze-dried sample; tetra, tetramer.

Measured decay	Physical state of protein	RIS (kDa)	TS (kDa)
Irradiation at -78°C			
Coomassie Blue	Fro, tetra	—	25 ± 4 (10)
Activity	Fro, tetra	45 ± 19 (3)	—
Coomassie Blue	FD, dimer	—	32 ± 15 (4)
Activity	FD, dimer	55 ± 13 (3)	—
Irradiation at 38°C			
Coomassie Blue	FD, dimer	—	30 ± 15 (5)
Activity	FD, dimer	133 ± 31 (5)	—

Figure 2 Radiation inactivation of catalase as a function of radiation dose

Inactivation of catalase at -78°C (●) and 38°C (○). The least-squares lines are shown, and the experimental points represent means of triplicate determinations. The SD from the mean did not exceed 20% of the mean value.

associated protomer does not occur at -78°C when catalase is irradiated either in frozen solution or in the freeze-dried form.

DISCUSSION

Catalase is a tetramer of identical protomers of 57.5 kDa organized in a 222 symmetry, each bearing an active site. Three types of subunit interfaces are found in the crystal X-ray structure corresponding to the *P*, *Q* and *R*-axes of symmetry in the catalase tetramer (Murthy et al., 1981). Freeze-drying caused both dissociation into dimer (Tanford and Lovrien, 1962) and the loss of about 70% of catalase activity. The dimer interface is due to *R*-axis interactions implicating 17 N-terminal and three C-terminal amino acid residues. At -78°C we found no evidence of energy transfer between the subunit interfaces when catalase was either in tetrameric or dimeric form. However, at 38°C we found that the whole dimer is inactivated after a hit by a γ -ray. The transfer of energy involved in this case likely occurred through the *R*-axis interface and was of sufficient magnitude to cause inactivation, but without fragmentation of the associated protomers. Only the subunit that has been directly hit by the γ -ray has been fragmented, since the TS value was similar to that of the monomer. It is interesting to note that, although freeze-dried catalase showed some heterogeneity by hydrodynamic methods (Tanford and Lovrien, 1962), this heterogeneity was not observed by radiation inactivation. That is, the inactivation of catalase as a function of dose is strictly first-order, indicating that a single molecular species is inactivated by irradiation (Figure 2).

The experimental evidence provided here suggests that inactivation of catalase 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, irreversible and involves a conformational change in the protomer associated to the fragmented protomer. This conformational change would be responsible for the loss of enzyme activity in the associated protomer of the dimer after a hit. This second process occurs

only at a higher temperature and results from energy transfer through the non-covalently linked *R*-axis protomer interface. Venter (1985) proposed that changes in the secondary and tertiary structures of proteins without fragmentation is sufficient to cause inactivation by radiation exposure. However, the present study provides direct experimental evidence in favour of such an inactivation mechanism in catalase. Similar findings were also reported for the tetrameric enzyme β -galactosidase from *Escherichia coli*, where energy transfer occurred only at 38°C and the whole tetramer was inactivated following a hit. Similarly to catalase, the associated subunits were not fragmented but, by gel-filtration h.p.l.c. and differential u.v., spectroscopy, Potier et al. (1991) provided evidence of a conformational change in these denatured subunits.

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 inter-molecular energy transfer in theoretical chemistry (Vaidhyanathan and Jung, 1987). Under our experimental conditions, where proteins are irradiated in frozen solution or in freeze-dried form, the indirect action of free radicals on the protein is believed to be limited (Kempner and Schlegel, 1979). That is, under our experimental conditions (frozen solution or freeze-dried powder), most free radicals formed by irradiation of water are immobilized in the sample and are de-activated before reacting with a protein. The direct effect of an ionizing radiation is due to primary ionization in a sphere of about 2 nm (20 Å) around a protein molecule which will release an enormous amount of energy (66 eV), enough, in fact, to break the equivalent of 15 C-H or C-C bonds. Ultimately, electron-excited states of about a few electronvolts are obtained and the mode of de-excitation 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) phonon 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 overlap at higher temperature (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 catalase, β -galactosidase (Potier et al, 1991) 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. More work is needed to identify the structural bases of inter-protomer energy migration and how it is affected by temperature or other experimental conditions.

We acknowledge the financial support of the Medical Research Council of Canada.

REFERENCES

- Beauregard, G., Maret, A., Salvayre, R. and Potier, M. (1987) *Methods Biochem. Anal.* **32**, 313–343
- Beers, R. F. and Sizer, I. W. (1952) *J. Biol. Chem.* **195**, 133–140
- Davydov, A. S. (1973) *J. Theor. Biol.* **38**, 559–568
- Karlish, S. J. D., and Kempner, E. S. (1984) *Biochem. Biophys. Acta* **776**, 288–298
- Kempner, E. S. and Fleisher, S. (1989) *Methods Enzymol.* **172**, 410–439

- Kempner, E. S. and Schlegel, W. (1979) *Anal. Biochem.* **92**, 2–10
- Kepner, G. R. and Macey, R. I. (1968) *Biochim. Biophys. Acta* **163**, 188–203
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- le Maire, M., Thauvette, L., de Foresta, B., Viel, A., Beauregard, G. and Potier, M. (1990) *Biochem. J.* **267**, 431–439
- Lea, D. E. (1955) *Actions of Radiations on Living Cells*, 2nd edn., pp. 69–99, Cambridge University Press, Cambridge
- Murthy, M. R. N., Reid, T. J., Sicignano, A., Tanaka, N. and Rossman, M. G. (1981) *J. Mol. Biol.* **152**, 465–499
- Potier, M., Thauvette, L., Michaud, L., Giroux, S., and Beauregard, G. (1991) *Biochemistry* **30**, 8151–8157
- Saccomani, G., Sachs, G., Cuppoletti, J. and Jung, C. Y. (1981) *J. Biol. Chem.* **256**, 7727–7729
- Tanford, C., and Lovrien, R. (1962) *J. Am. Chem. Soc.* **84**, 1892–1896
- Vaidyanathan, Y. S. and Jung, C. Y. (1987) *Receptor Biochemistry and Methodology*, vol. 10 (Venter, J. C. and Jung, C. Y., eds.), pp. 153–162, Alan R. Liss, New York
- Venter, J. C. (1985) *The Receptors*, vol. 2 (Conn, P. M., ed.), pp. 245–280, Academic Press, New York

Received 14 July 1993/14 September 1993; accepted 5 October 1993