Biochimica et Biophysica Acta, 302 (1973) 376–381

BBA 66873

EFFECTS OF γ-IRRADIATION ON THE ENZYMATIC PROPERTIES OF LYSOZYME

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(Received October 23rd, 1972)

SUMMARY

Dry irradiated lysozyme (mucopeptide *N*-acetylmuramylhydrolase, EC 3.2.1.17) and its fractions have been subjected to kinetic studies. The aggregation of heavily damaged products is delayed by substrates, indicating a possible involvement of the active site in the aggregation process. The activation energy, $E_{a,app}$, for γ-irradiated lysozyme, and the apparent affinity constant ($K_{a,app}$) for irradiated lysozyme and its salt-soluble fraction, F-II, are the same as the observed with native enzyme. However, the maximum velocity (V) decreases with irradiation. Heavily damaged fraction, F-I, or salt-insoluble products are almost inactive. Difference spectra of lysozyme–glycol chitin complex show similar characteristics for native, irradiated lysozyme and its F-II fraction. However, F-I fraction spectra present abnormal characteristics, indicating alterations in or around a tryptophan moiety. It is proposed that unstable damaged enzyme molecules suffer further modifications during fractionation resulting in enzyme inactivation.

INTRODUCTION

Irradiated enzymes have different enzymatic properties than native enzymes, a reduction in specific activity being noted most frequently. The inactivation by radiation of ribonuclease has been studied by several groups. Haskill and Hunt[1] have shown that the Michaelis constant (K_m) does not change on irradiation of the native enzyme whereas the maximum velocity (V) is lowered. Similar results have been observed by Riesz and White[2] for irradiated dry ribonuclease, and by Smith and Adelstein[3] for ribonuclease irradiated in aqueous solution. Mee[4] has reported that the K_m and V of chymotrypsin irradiated in solution with X-rays are different from the native enzyme and change with the dose administered. Shore *et al.*[5] working with trypsin have shown that in irradiated dry trypsin the esterase and protease activities are lost at different rates.

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Since the structure[6] and enzymatic mechanism of lysozyme (mucopeptide *N*-acetylmuramylhydrolase, EC 3.2.1.17)[7] have recently been elucidated and several components have been isolated and characterized[8,9] from γ-irradiated lysozyme, it is of interest to study its enzymatic properties. In the following report the effects of γ-radiation on the kinetic properties of lysozyme are examined and related to radiation damage.

MATERIALS AND METHODS

Enzyme and substrates

Egg white lysozyme (3 × crystallized) from Sigma Chemical Co. was purified by chromatography[10]. Lysozyme (6 × crystallized) from Seikagaku Fine Biochemicals was used without further purification. *Micrococus lysodeikticus* (ATCC 4968) spray-dried cells, were obtained from Miles Laboratories. Glycol chitin was synthesized from purified chitin by the method of Senju and Okimasu[11] and fractionated following the procedure of Hayashi *et al.*[12].

Irradiation of solid lysozyme and its fractionation were carried out as previously described[9].

Enzyme assays

The enzyme activities were assayed using *M. lysodeikticus* as a substrate following the method described by Shinitzky *et al.*[13]. The course of lysis was followed recording the changes in absorbance of a cell suspension at 645 nm. Temperature effects on the enzyme activity were studied with a Gilford 2000 spectrophotometer equipped with thermospacers, and the temperature was measured with a thermocouple attached to the cuvette. The apparent activation energies ($E_{a,app.}$) for the enzymatic activities of native lysozyme and γ-irradiated lysozyme were calculated from the rate constants for cell lysis at different temperatures. The measurements were made within a short period of time, *i.e.* 90 s. The apparent affinity constants ($K_{a,app.}$) of the different samples of enzyme for *M. lysodeikticus* were measured following the method of Locquet *et al.*[14] using 0.01 M sodium phosphate–0.04 M NaCl, pH 6.2, as buffer[15]. The measurements were carried out at 25 °C in a Cary 16K spectrophotometer. Enzyme solutions were prepared in distilled water.

The spectroscopic characteristics of the enzyme–substrate complex between lysozyme and glycol chitin have been studied following the method of Hayashi *et al.*[12]. The difference spectra in the ultraviolet region (270–320 nm) were recorded in a Cary 16K spectrophotometer within 15–17 min after the substrate was added to the enzyme solution. Incubation and recording of the spectra were done at 25 °C.

RESULTS

Inactivation of lysozyme

Crystalline lysozyme when irradiated *in vacuo* with ^{137}Cs γ-rays was inactivated exponentially and the sensitive molecular weights[16] calculated from the D_{37} dose were dependent on the pH of the enzyme assay (Table I). The degree of enzyme inactivation depended also on the treatment previous to assay. Irradiated samples dissolved in water presented less inactivation than those samples dissolved in salt

TABLE I

VARIATION OF THE "SENSITIVE MOLECULAR WEIGHTS" AS A FUNCTION OF pH OF ASSAY FOR γ-IRRADIATED LYSOZYME

Assays were made on the water-soluble fraction using *M. lysodeikticus* cells as substrate in 0.12 M phosphate–0.06 M citrate–0.05 M NaCl buffer.

pH	D_{37} *(Mrad)**	$M \times 10^{-3}$**
3.0	40.5	17.8
4.0	46.0	15.6
5.0	31.0	23.2
6.0	46.0	15.6
7.0	41.5	17.3
8.0	39.5	18.2

* D_{37} = dose needed to inactivate 63% of the original enzyme activity.
** $M \times 10^{-3}$ = mass of the "target unit" expressed in terms of sensitive molecular weight; $M = \frac{0.72 \cdot 10^{12}}{D_{37}}$ (ref. 16).

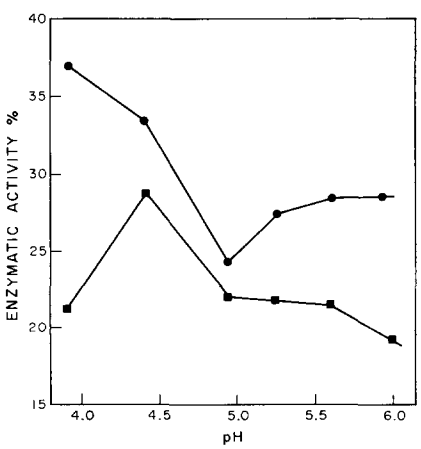


Fig. 1. Effect of pretreatment on the enzymatic activities at different pH values of γ-irradiated lysozyme (45.8 Mrad). Enzymatic activities of samples dissolved in water (●), and dissolved in buffer (■). The activities were assayed using *M. lysodeikticus* as substrate in 0.12 M phosphate–0.06 M citric acid–0.05 M NaCl buffers.

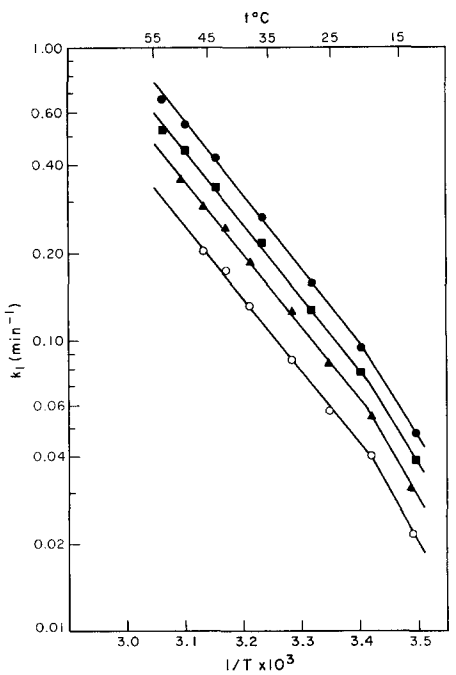


Fig. 2. Arrhenius plot for lysis of *M. lysodeikticus* by native lysozyme (●) and lysozyme irradiated with 10 Mrad (■), 20 Mrad (▲) and 30 Mrad (○) of γ-rays. For details, see text.

TABLE II

APPARENT ACTIVATION ENERGIES FOR LYTIC ACTIVITY OF γ-IRRADIATED LYSOZYME
The $E_{a.app.}$ were calculated from the slope of the line obtained on plotting the log of the apparent rate constant for lysis (k) against $1/T$[18]. The lytic activity measurements used were between 25 °C and 50 °C. Enzyme concentration 6.6 μg/ml, *M. lysodeikticus* cells: 0.3 mg/ml in 0.12 M Na_2HPO_4–0.06 M citric acid–0.05 M NaCl buffer.

Dose (Mrad)	*$E_{a.app.}$ (cal/mole)*		
	pH 4.62	*5.54*	*6.51*
0	7300	9360	11 550
10	7290	9340	11 490
20	7290	9280	11 390
30	7380	9400	11 650

solutions (Fig. 1). Samples dissolved in water showed at pH 5.00 their maximum radiation sensitivity (Table I) and also a minimum in solubility as reported by Maksimov *et al.*[17].

Kinetic studies

Due to the complexity of *M. lysodeikticus* lysis the kinetic parameters reported here cannot be interpreted in a mechanistic sense; therefore, they are being expressed as apparent values.

The apparent activation energies ($E_{a,app.}$) for irradiated lysozyme were calculated from Arrhenius plots for the lysis of *M. lysodeikticus* (Fig. 2). No significant differences between the $E_{a,app.}$ values for native and irradiated enzyme (Table II) could be observed. Native lysozyme, whole irradiated lysozyme (26 Mrad) and its salt-soluble fraction, F-II, had the same apparent affinity constant ($K_{a,app.}$), 159 mg·l^{-1} of *M. lysodeikticus* dry cells (Fig. 3). However, the maximum velocity (V) was lower in the irradiated samples (Table III). It was not possible to evaluate the $K_{a,app.}$ and V of the salt-soluble fraction from irradiated lysozyme, F-I, due to floculation of the damaged enzyme in the presence of bacteria cells.

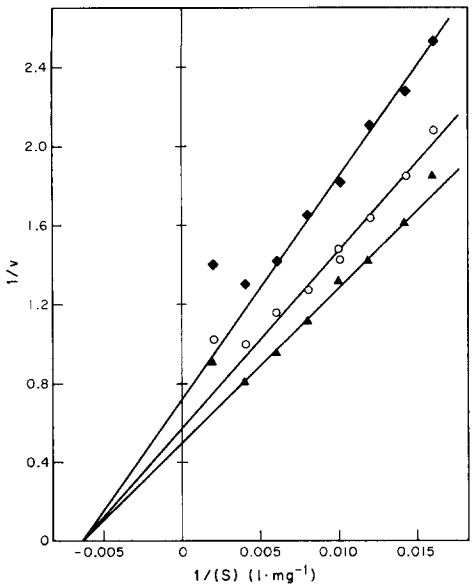
TABLE III

ENZYMATIC CHARACTERISTICS FOR NATIVE LYSOZYME: IRRADIATED LYSOZYME AND F-II FROM IRRADIATED LYSOZYME (DOSE = 26 Mrad)

Enzyme	*M. lysodeikticus**		*Glycol chitin†*		*% Enzymic activity (lysis)*
	V (mg/l per s)	*% Enzymic activity*	*$\Delta A_{292.5\ nm}$*	*% Enzymic activity*	*% Enzymic activity (diff. spect.)*
Native lysozyme	2.00	100.0	1100	100.0	1.000
Irradiated lysozyme	1.39	69.5	814	74.0	0.940
F-II fraction	1.72	86.0	930	84.5	1.018

* Suspension of *M. lysodeikticus* in 0.01 M sodium phosphate–0.04 M NaCl buffer, pH 6.2 ionic strength = 0.05.

† Glycol chitin 0.2% in 0.1 M sodium acetate buffer, pH 4.0.



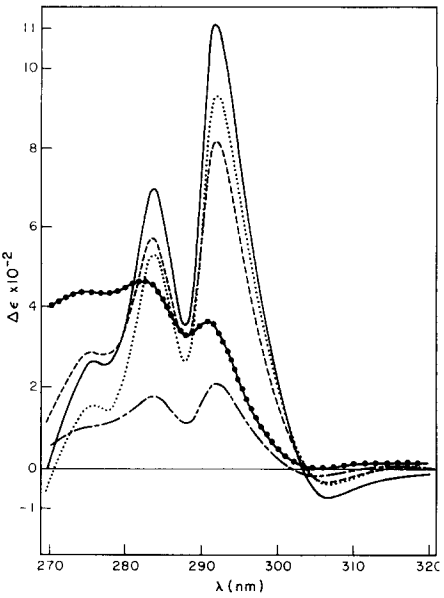


Fig. 3. Reciprocal velocity ($1/v$) *versus* reciprocal substrate concentration ($1/[S]$) plots for native (▲), irradiated lysozyme (●), and its salt-soluble fraction F-II (○). Radiation dose: 26 Mrad; the assays were carried as described in the text using *M. lysodeikticus* as substrate.

Fig. 4. Difference spectra of the enzyme–substrate complexes of glycol chitin with native (———), irradiated lysozyme (— — —), salt-soluble fraction F-II (· · · · · ·), salt-insoluble fraction components, F-IB (-.-.-.-.-) and F-IA (— - —). The buffer solution was 0.1 M sodium acetate, pH 4.0, and final concentration of enzyme was 0.1%. Concentration of glycol chitin, 0.2%.

Spectrophotometric study of the lysozyme–glycol chitin complex

The difference spectrum for the enzyme–substrate complex corresponding to native lysozyme presented the pattern described by Hayashi *et al.*[12], with maxima located at 276, 284 and 292.5 nm (Fig. 4). Whole irradiated lysozyme and the F-II enzyme–substrate complexes showed the same spectroscopic characteristics that were observed with the native enzyme complex. The difference absorbance (ΔA) at 292.5 nm has been shown to be proportional to the enzymatic activity[12]. In Table III the $\Delta A_{292.5\ nm}$ and the relative enzyme activities with respect to native lysozyme are shown. The difference spectra for the complexes corresponding to the F-I components presented patterns different from that corresponding to native lysozyme (Fig. 4).

DISCUSSION

The results presented here indicate that the substrates of lysozyme have some protective effect on the γ-irradiated enzyme when exposed, in the absence of oxygen to buffered solutions of polibasic acids. The binding of substrate to the active site of radiation-damaged lysozyme must shield some groups that are necessary for the initiation of interactions leading toward irreversible aggregation. The groups involved in the aggregation of irradiated lysozyme[19] seem to be similar to those involved in the reversible dimerization of lysozyme[20], which can be prevented by substrates[21], indicating the role of the active site as an association center. Enzyme molecules

presenting disulfide damage[9] are more prone to aggregation, perhaps due to the presence of new hydrophobic areas on their surfaces.

The fact that the values of $E_{a,app.}$ and $K_{a,app.}$ are not different for native, irradiated lysozyme, and F-II, together with the similarities of the spectra for their $E \cdot S$ complexes, imply little alterations in the active site and its surroundings. The good correlation between the activities determined from V measurements and $\Delta A_{295\ nm}$ (Table III), indicate the absence in irradiated lysozyme and F-II of non-productive complexes[22,23] that can bind substrate without hydrolyzing it.

The abnormal spectroscopic characteristics of the $E \cdot S$ complexes for F-I components are indicative of perturbations in chromophores other than tryptophan, like products from tryptophan radiolysis. Different from whole irradiated lysozyme, F-I releases free ammonia under mild alkaline conditions[9] and also presents destruction of the indole chromophore of tryptophan[24]. These characteristics and the low enzymatic activities for the F-I components point to the fractionation step as the source of further modifications.

These post-irradiation modifications would lead to the formation of radiation-damaged species with their properties depending on the fractionation procedure and not exclusively on the irradiation process.

ACKNOWLEDGEMENT

This work was supported by the U.S. Atomic Energy Commission under Contract AT(690)-11.

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