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MASTER

RADIATION EFFECTS IN BIOCHEMISTRY AND ORGANIC CHEMISTRY

Mancourt Downing, Project Director

The purpose of this project is to study effects of ionizing radiation on purified compounds found in biological systems. The experimental work of the past year and the work currently being conducted concerns the effects of radiation on protein in the solid state.

This report concerns five projects in various stages of completion as follows:

- I. The Effects of Gamma Radiation on Solid State Aspartate Transcarbamylase from Escherichia coli
- II. Protein Association of Aspartate Transcarbamylase
- III. Radical Scavengers in Irradiated Lysozyme
- IV. Denaturation of Irradiated Lysozyme
- V. Hydrogen Exchange and Perturbation Spectra of Irradiated Lysozyme
- VI. Publications

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THE EFFECTS OF GAMMA RADIATION ON SOLID STATE
ASPARTATE TRANSCARBAMYLASE FROM ESCHERICHIA COLI*

by

William N. Bigler

INTRODUCTION

Aspartate transcarbamylase (ATCase)¹ (EC 2.1.3.2) from Escherichia coli has been studied as a model system for the examination of the direct effects of ionizing radiation on regulatory subunit enzymes. The subunit structure of ATCase has been described as an oligomer, 3.1×10^5 molecular weight, of four protomers, each consisting of one "regulatory" and one "catalytic" protein chain, 2.7×10^4 and 5.0×10^4 molecular weight respectively (Changeux, J.P., et al, 1967). Mercurials cause the oligomer to dissociate to four sulfhydryl rich regulatory subunits and two dimeric catalytically active subunits, 1.0×10^5 molecular weight (Gerhart and Schachman, 1965). The oligomer is characterized by feedback inhibibility and a sigmoid dependence of activity upon aspartate concentration. The catalytic subunit has an affinity for substrate that is greater than the oligomer and hyperbolic dependence of activity upon aspartate concentration (Gerhart and Pardee, 1962). Association of the oligomer with various ligand molecules is believed to cause specific protein conformation changes (Dratz and Calvin, 1966; Gerhart and Schachman, 1968). The sensitivity of catalytic activity of many enzymes to the direct effects of ionizing radiation has been shown to be roughly related to the enzymes' molecular weights (Hutchinson and Pollard, 1961). Comparison of the distributions of radiation induced free radicals among the amino acids of dry enzymes and their carboxymethylated derivatives has established that the radiation sensitivity of individual residues is dependent upon each enzyme's conformation (White, et al, 1967).

In the present paper we report that a functional conformation change of a regulatory enzyme can significantly alter the radiation sensitivity of the enzyme's catalytic activity. We also report data on the effect of megarad doses of gamma radiation on the quaternary structure of this model subunit enzyme.

¹Abbreviations used: ATCase, aspartate transcarbamylase; CTP, cytidine triphosphate; ATP, adenosine triphosphate; D₃₇, dose required to reduce the activity by $1/e$.

MATERIALS AND EXPERIMENTAL PROCEDURES

All chemicals were obtained commercially. Dilithium carbamyl phosphate was purified by reprecipitation with cold ethanol (Gerhart and Pardee, 1962). L-leucine-4,5-³H was purchased from the New England Nuclear Corporation.

The partially diploid strain of Escherichia coli, its growth and derepression, and the purification of ATCase have been described previously (Gerhart and Holoubek, 1967). The enzyme product, carbamylaspartate, was assayed with the colorimetric method of Gerhart and Pardee (1962). Protein concentrations were determined by the Folin procedure (Lowry et al, 1951) or estimated from the 280 mμ absorption of purified enzyme preparations. Crystalline bovine serum albumin was the protein standard. The absorbance at 280 mμ of a 0.1% solution of ATCase was 0.61. This compares well with 0.59 reported previously (Gerhart and Schachman, 1965). The specific activity of our material was 1.2×10^4 units per hour, when assayed at pH 8.5, 2.5×10^{-2} M aspartic acid, 1.8×10^{-3} M dilithium carbamylphosphate, 5×10^{-2} M Tris-acetate.

Radioactively labeled ATCase was prepared as described in the accompanying publication (Bigler, et al, 1968). Labeled enzyme preparations contained approximately 65% oligomeric ATCase.

Optimal recovery of ATCase activity after lyophilization depends on the concentration of ATCase in the sample at the time it is frozen and the procedure for freezing. Pyrex tubes containing small volumes of enzyme solution were chilled to 0°, frozen in liquid nitrogen and stored in a salt-ice bath until evacuated. Evacuation to dryness was complete after three hours of lyophilization. During approximately 1/2 of the time the pressure was constant at 4-10 microns. Each dry sample was sealed in vacuo and stored at -10° until irradiated and/or redissolved. Recoverable enzyme activity decreased if the ATCase concentration was greater than 0.5 mg per ml or if the solution wasn't chilled before freezing. The described conditions yielded at least 97% recovery of activity and no change in the enzyme's response to CTP, ATP or maleate.

Lyophilized protein samples were irradiated in vacuo at $25 \pm 1^\circ$ in the kilocurie ¹³⁷Cs source. The dose rate, 0.388 ± 0.003 megarads per hour, was determined with Fricke chemical dosimeters (Fricke and Hart, 1966).

Five to 20% sucrose density gradients were prepared and fractionated as previously described (Martin and Ames, 1961). Each sedimentation was run for the time indicated at 38,000 rpm with a SW39 rotor in a Beckman Model L preparative ultracentrifuge. Fractions were collected directly in vials containing a Toluene-Biosolve I (Beckman Instruments) liquid scintillation cocktail and radioactivity was determined in a Packard Tricarb or Beckman LS-250 liquid scintillation counter.

RESULTS AND DISCUSSION

The dependence of ATCase activity on gamma-radiation dose absorbed by evacuated lyophilized ATCase was determined. Observed dose dependence varied with the aspartate concentration used to measure enzyme activity. The data graphed in Figure 1 were obtained with one set of irradiated samples assayed in the presence of a saturating concentration of aspartate, 25mM. These same samples when assayed with a non-saturating concentration of aspartate, 5mM, appear to have a biphasic dependence of activity upon dose as seen in the upper curve in Figure 2. The dose dependence of ATCase's inhibitability by CTP is represented by the decrease in the difference between the upper and the lower curve in Figure 2. The lower curve assays were performed with 1mM CTP present in the assay solutions.

Observed logarithmic dependence of an activity upon absorbed dose is frequently recorded in terms of the D_{37} . The average D_{37} values and the range of D_{37} values from such experiments as those reported in Figures 1 and 2 are recorded in Table 1. As an aid during the discussion "radiation molecular weights" estimated from the average D_{37} values are also listed in Table 1. The theoretical equation,

$$D_{37} \text{ (rads)} \times \text{Molecular weight (g per mole)} = 0.72 \times 10^{12},$$

(Hutchinson and Pollard, 1961) relating the D_{37} of dry spherical proteins to their "target size" was used to estimate these "radiation molecular weights".

The D_{37} for CTP inhibitability listed in Table 1 was determined for radiation doses to 2.2 megarads. Enzyme samples which had absorbed doses larger than 2.2 megarads were characterized by a relatively constant weak inhibition by CTP which we presume is due to interactions other than the

usual binding at the regulatory subunit. A similar low constant inhibition by CTP is characteristic of heavily irradiated ATCase solutions (Kleppe and Spaeren, 1967) and persistent but decreased affinity of heat treated ATCase for CTP has been reported (Weitzmann and Wilson, 1966). The "radiation molecular weight" calculated from D_{37} values obtained by measuring the sharp decrease in inhibitability by CTP is equal to the physical chemical molecular weight of ATCase, 3.1×10^5 (Gerhart and Schachman, 1965). This value is markedly different than the "radiation molecular weight" calculated from D_{37} values obtained by measuring maximal enzyme activity, 1.7×10^5 . The simplest, but not exclusive, interpretation of these data is that an ionizing event in a native ATCase molecule simultaneously inactivates all regulatory properties but only 1/2 of the catalytic sites on the enzyme. The dose dependent rise in observed enzyme activity, with non-saturating substrate assays, Figure 2, may be due to the release of inhibitory interactions between undamaged catalytic sites and regulatory subunits. Data in agreement with this interpretation were forthcoming from experiments designed to determine ATCase's radiosensitivity dependence on enzyme's conformation.

Replicate enzyme samples to be irradiated were lyophilized from solutions containing saturating concentrations of ligands known to alter ATCase's catalytic and conformational properties. The presence of substrate, carbamylphosphate, and substrate analogue, maleate, did not alter the D_{37} for maximal ATCase activity (Table 1). Association of enzyme with the feedback inhibitor, CTP, resulted in a decreased D_{37} , i.e., a greater radiosensitivity, for maximal ATCase activity (Table 1). In these experiments CTP present in the dry irradiated mixture was diluted to $10^{-7}M$ before the enzyme was assayed. The "radiation molecular weight" estimated for the CTP associated enzyme appears to correspond to a "target molecule" 50% larger than that estimated for the substrate associated enzyme. It is difficult to imagine a functional alteration, as the binding of CTP is believed to be, involving a 50% size change. Sedimentation studies (Gerhart and Schachman, 1968) have revealed that CTP associated ATCase has a 3.5% greater sedimentation coefficient than substrate associated ATCase. Thus, in solution, the inhibited ATCase conformation should be slightly more compact rather than larger. The large "radiation molecular

weight" and increased radiosensitivity, of dry ATCase in the presence of CTP is in our view not due to direct interactions between the radiation and the CTP molecules. This belief is based upon the idea that gamma radiation is not selectively absorbed by nucleotides and the fact that during X-irradiation of ATCase solutions CTP protects, probably by scavenging free radicals, rather than sensitizes the enzyme (Kleppe and Spaeren, 1967).

Our tentative explanation of these data incorporates portions of a previously proposed schematic model for ATCase's conformational change (Gerhart and Pardee, 1964; Gerhart and Schachman, 1968) and the possibility of transfer of absorbed radiation energy between the dry enzyme's non-covalently bonded proteins subunits. In the "loose" or substrate associated conformation an ionizing event may inactivate only one-half of the catalytic sites; i.e., transfer of absorbed radiation energy between the two catalytic subunits is inhibited. In the "tight" or enzymically less active conformation ATCase appears to have a greater radiosensitivity because more of the active sites are damaged by a single ionizing event, i.e., transfer of absorbed energy between the catalytic subunits is more likely in the "tight" conformation. This explanation of our radiosensitivity data is in agreement with the concept, within the cited model, that the subunits, particularly the catalytic subunits, are strongly interacting, closely associated, in the inhibited form of the enzyme but are weakly interacting, separated, in the conformation with the higher affinity for substrates. The suggested energy transfer barrier between the catalytic subunits in the "loose", more slowly sedimenting, conformation might be a "gap", bridged by regulatory subunits, which could close in the "tight", radiosensitive, conformation. The barrier might also be a free radical trap composed of portions of the sulfhydryl rich regulatory subunits. A "crease" between the catalytic subunits has been deduced from dissociation and kinetic data (Changeux, et al, 1967).

Since the direct effects of ionizing radiation do not result in dissociation of ATCase's subunits (see below) the simplest explanation of the rapid loss of inhibitory properties is that there is preferential transfer of absorbed radiation energy to the regulatory subunits. Preliminary data obtained with assays for the ability of low concentrations of maleate to

activate ATCase at low aspartate concentration (Gerhart and Pardee, 1964) indicate that this activation has a D_{37} similar to the value for CTP inhibitability. This result, together with the failure to find dissociation of the oligomer induced by radiation, may be interpreted as evidence that the homotropic active site interactions are not dependent on the aggregation of the catalytic and regulatory subunits but are actually mediated by the regulatory subunits.

Whole bacteria derepressed for ATCase were lyophilized and irradiated in an attempt to obtain material for comparison of the radiosensitivities of the purified enzyme and the enzyme in a state nearer to the in vivo environment. Resuspension and centrifugation of exposed cells usually released less than 10% of the total ATCase into supernatant fractions. Samples from these supernatants and enzyme released by rupturing the pelleted bacteria with a French pressure cell both provided radiosensitivity data similar to that obtained with the ligand-free purified enzyme (Table 1). Enzyme from the irradiated bacteria has the same characteristics, when assayed at non-saturating aspartate concentrations, as the purified enzyme was shown to possess (Figure 2). We feel that these results are direct evidence that the aggregation state of the purified enzyme is the same as that of the enzyme in the derepressed bacteria.

A possible explanation for the rise in enzyme activity, seen as a function of dosage in Figure 2, was that catalytic subunits were being dissociated from the radiation altered ATCase molecules. Radioactively labeled purified ATCase was lyophilized, irradiated (8.5 megarads), redissolved and fractionated by sucrose density gradient centrifugation as shown in Figure 3. A low dose, e.g., 2.2 megarads, of gamma radiation did not cause appreciable redistribution of labeled protein in the sedimentation pattern.

A dose of 8.5 megarads, which inactivates more than 85% of enzyme activity apparent with saturating substrate assays, was absorbed by all samples. The portion of labeled protein sedimenting at the position of native ATCase was not grossly altered by 8.5 megarads, Figure 3 and Table 2. Irradiated samples did not have an increase over non-irradiated samples of labeled protein in either high molecular weight fractions or fractions corresponding to catalytic subunit. Thus it appears that irradiation does not cause dissociation. The exchange of radioactive catalytic subunit

of irradiated ATCase with purified non-radioactive catalytic subunit (open circles Figure 2) rule out covalent crosslinking of ATCase as a mechanism of irradiation damage. The conclusion that ATCase's quaternary structure is not destroyed during radiation denaturation, is in partial disagreement with a conclusion reached in a report on effects of radiation on ATCase solutions (Kleppe and Spaeren, 1967). The latter conclusion was based on the inability of catalase, in the irradiated ATCase solutions, to prevent radiation induced release of catalytic subunits. Although catalase can remove peroxide from irradiated solutions it apparently cannot circumvent all indirect effects of radiation on dissolved proteins.

Enzyme samples lyophilized from relatively concentrated ATCase solutions contained aggregates of oligomeric ATCase molecules (Bigler, et al., 1968). The rapidly sedimentary aggregate seen in Figure 4 is not apparent in the similar but irradiated sample. Although we do not know the types of bonds holding either the subunits of ATCase together in the oligomer or the oligomers together in the aggregates it is apparent that the latter are more radiation sensitive.

Irradiated labeled ATCase that was treated with a dissociating concentration of mercurial (Gerhart and Holoubek, 1967) sedimented in a gradient buffered with 4.0×10^{-2} M potassium phosphate, pH 7.0, 5×10^{-5} M p-hydroxymercuribenzoate as a single component in the low molecular weight region of the gradient. Conditions were used which resolved the catalytic and regulatory subunits of unirradiated ATCase.

A small amount of mercurial has been shown to allow the exchange of unlabeled catalytic subunits free in solution with labeled catalytic subunits aggregated in native unirradiated ATCase (Bigler and Tolbert, 1968). The exchanging aggregates did not release any labeled protein which sedimented as regulatory subunits, therefore, no net dissociation occurs with the conditions used. Identical conditions caused irradiated labeled ATCase to dissociate into protein which sedimented as catalytic and regulatory subunits, Figure 3. Released irradiated catalytic subunits apparently retained their quaternary structure, an aggregate of two protein chains, unless larger mercurial concentrations were present. These dissociation data are evidence that direct radiation inactivation of ATCase

does not involve the formation of inter-subunit covalent bonds and does decrease the integrity of the quaternary structure of both the native oligomer and the dimeric catalytic subunit.

All of the above data tend to indicate that structural alterations of ATCase caused by inactivating ionizing radiation do not destroy but do weaken the quaternary structure. Radiation inactivation of ATCase must involve small conformation changes which allow the quaternary structure to persist. At non-saturating substrate concentrations the enzyme appears to be naturally radiation protection for the activity of the first enzyme of the pyrimidine pathway or the protection may be an adventitious result of the molecule's structure. Data on the radiation sensitivity of other regulatory enzymes, particularly those with dissimilar subunits, should determine if evolution has built similar protection into other enzymes.

FIGURE LEGENDS:

FIGURE 1: Gamma radiation dose dependence of maximal enzyme activity of lyophilized oligomeric ATCase. Fifty μ l samples containing 20 μ g of enzyme, 0.01 M imidazole, 2×10^{-3} M 2-mercaptoethanol, 2×10^{-4} M disodium EDTA and 0.35 M KCl, final pH 7.0, were lyophilized and sealed as described in experimental methods section. After irradiation and storage the samples were opened in a nitrogen atmosphere and dissolved in 1 ml of 3.5×10^{-2} M Tris-acetate, pH 8.5, that had been purged with nitrogen. The activity and inhibitability by CTP of enzyme solutions stored at 4° remained the same for several weeks. Enzyme concentration in each assay solution was adjusted to yield approximately 0.1 μ mole of carbamylaspartate after 25 minutes of reaction. Assay solutions contained 50×10^{-3} M Tris-acetate, 25×10^{-3} M aspartate, 1.8×10^{-3} M carbamyl phosphate.

FIGURE 2: Gamma radiation dose dependence of enzyme activity of lyophilized oligomeric ATCase determined with non-saturating levels of substrate. The same enzyme samples described in Figure 1 were assayed as before except with 5×10^{-3} M aspartate, 0-0, and with 5×10^{-3} M aspartate and 1×10^{-3} M CTP, X-X.

FIGURE 3: Effect of 8.5 megarads of gamma radiation on the sedimentation properties of ATCase. The cpm data graphed were obtained after fractionation of 5 ml 5-20% sucrose gradients that had been centrifuged for 12 hours at 38,000 rpm. The buffer in the gradient contained 4.0×10^{-2} M potassium phosphate, pH 7.0, 2×10^{-3} M 2-mercaptoethanol, and 2×10^{-4} M disodium EDTA. A sample of ATCase, containing tritium labeled leucine, was lyophilized from 0.4 mg protein per ml solution, irradiated in vacuo, redissolved in the same buffer contained in the gradients. One portion of the sample was allowed to stand for four hours at room temperature then layered on a cold gradient, X-X. Another portion of the sample, containing 0.3 mg of oligomeric ATCase, was mixed with .5 mg sample of unlabeled catalytic subunit and allowed to equilibrate at room temperature for four hours before layering on the second gradient, 0-0. Other components of the latter mixture were 4×10^{-2} M potassium phosphate, 9×10^{-3} M imidazole, pH 7.0, 2×10^{-4} M disodium EDTA, 2×10^{-3} M 2-mercaptoethanol, 0.45 M KCl, and 0.45×10^{-4} M p-hydroxymercuribenzoate. The mercurial was a portion of the catalytic

subunit preparation. The phosphate, EDTA, mercaptoethanol buffer was allowed to react with the subunit preparation for 20 minutes at room temperature before the labeled enzyme was added.

FIGURE 4: Effect of 8.5 megarads of gamma radiation on the sedimentation properties of oligomers of ATCase and aggregates of ATCase oligomers. Two tritium labeled ATCase samples, 100 μ l containing 0.8 mg of protein in pH 7.0 buffer, were lyophilized. One was exposed to 8.5 megarads of radiation, O-O. The other, X-X, was not irradiated. Both samples were dissolved, centrifuged for 16 hours and fractionated as described in Figure 3.

FIGURE 1

FIGURE 2

FIGURE 3

Fig 3

FIGURE 4

Fig 4

TABLE I

RADIATION INACTIVATION OF ATCase ACTIVITY AND INHIBITABILITY

Lyophilized ATCase Preparation and Assayed Property	Average D_{37} (megarads)	Range of D_{37} Observed (megarads)	Radiation Molecular Weight Calculated
Oligomeric ATCase -- activity	4.1 ^a	3.4 - 5.0 ^a	1.7×10^5
Oligomeric ATCase, 50 mM maleate, 3.6 mM carbamylphosphate -- activity	3.9 ^a	3.1 - 4.6 ^a	1.8×10^5
Oligomeric ATCase, 1 mM CTP -- activity	2.9 ^a	2.8 - 3.2 ^a	2.5×10^5
Depressed Bacteria -- activity	4.0 ^a	3.4 - 4.2 ^a	1.8×10^5
Oligomeric ATC -- inhibitability	2.3 ^{b,c}	1.5 - 3.0 ^{b,c}	3.1×10^5
Catalytic Subunit -- activity	8.8 ^{b,d}	7.4 - 9.8 ^{b,d}	8.3×10^4

a. Assay solution described in Figure 1.

b. Assay solution described in Figure 2.

c. Estimated with samples which absorbed less than 2.2 megarads.

d. Estimated with samples which absorbed between 2.5 and 8.5 megarads.

TABLE II
EFFECT OF 8.5 MEGARADS OF GAMMA RADIATION ON THE
SEDIMENTATION PROPERTIES OF ATCase

Enzyme Preparation	<u>Percent of Total ³H in Pooled Fractions</u>			<u>CPM Recovered</u> <u>CPM Lyophilized</u> Percent
	Sedimentation rate compared to Oligomeric ATCase			
	Faster	ATCase	Slower	
Unlyophilized, unirradiated	2	70	28	100
Lyophilized, unirradiated	5	72	23	100
Lyophilized, irradiated	4	60	36	90
Lyophilized, unirradiated (8 mg protein per ml, pH 7.0)	11	57	32	95
Lyophilized, irradiated (8 mg protein per ml, pH 7.0)	4	48	48	86

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PROTEIN ASSOCIATION OF ASPARTATE TRANSCARBAMYLASE

(PUBLICATION IS IN PREPARATION.)

RADICAL SCAVENGERS IN IRRADIATED LYSOZYME

by

Sarah Herbert

INTRODUCTION

The original object of our program was to study the formation of long-lived free radicals in γ -irradiated lysozyme using the method of Reisz and co-workers (1) which employs tritiated hydrogen sulfide (HTS) as a radical scavenger. This objective has been expanded to include use of different chemical and physical methods to study the nature of the distinct damaged molecules formed upon irradiation.

Following the two primary processes of radiation damage to biological macromolecules, ionization and excitation, secondary processes take place which cause the disruption of the macromolecule. One of these secondary processes may involve the formation of free radicals. Tritiated hydrogen sulfide has been used successfully as a radical scavenger for several proteins (2,3,4) including lysozyme. However, in these experiments the proteins were hydrolyzed to amino acids and the radical content of each kind of amino acid was analyzed. In the latest work using this method (5) the chymotryptic peptides of lysozyme were analyzed for the distribution of radicals, and it was found that the most heavily labeled peptides were in close proximity, and associated with the "hydrophobic box" of the molecule. In our work we are analyzing the labeling pattern of the whole protein species formed upon γ -irradiation of lysozyme. We hope that these results will correlate with previous work on amino acids and peptides and result in partial elucidation of the mechanism of radiation damage to biological macromolecules. In addition, our plan includes experiments designed to test the possibility of damage to the protein at the secondary or tertiary level. Irradiation gives rise to three major peaks on fractionation with an ion exchange column. These peaks will be characterized by disc gel electrophoresis, enzyme assays, titration curves, difference spectra, peptide maps, and possibly sedimentation velocity measurements. In addition, each of the species will be heat denatured and then re-examined as to their behavior on the

ion exchange column as well as the other criteria mentioned.

EXPERIMENTAL

Purification of Lysozyme - It has been reported by Stevens and Bergstrom (7) that commercial crystalline lysozyme is heterogeneous as determined by column chromatography using the cationic ion exchange resin Bio Rex 70. However, employing this method we have found that the lot of Pentex lysozyme we are using is relatively homogeneous, showing only one small peak in addition to the major peak (Figure 1). For this reason the lysozyme used in all of our experiments is Pentex crystallized egg white lysozyme, lot 19, without further purification.

Column Chromatography - The elution pattern of the protein from a Bio Rex 70 column is used as a method of identifying native lysozyme in relation to the different irradiated species. The same method is employed as that of Stevens and Bergstrom (7) with the exception of the method used to change the resin from the sodium to the hydrogen form. This is done by equilibrating the resin in the same potassium phosphate buffer that is used for elution, 0.2 M, pH 7.09.

Desalting - The lysozyme was removed from phosphate buffer and transferred into acetic acid-pyridine buffer pH 5.5 by passing through Bio Gel P2. The acetic acid-pyridine buffer can be removed by lyophilization.

Disc Gel Electrophoresis - Disc gel electrophoresis was performed according to the method of Reisfeld et al. (8) for basic proteins except that 30.0 mg $K_3Fe(CN)_6$ was added to Solution C to retard polymerization and prevent the formation of bubbles in the gels. A saturated sucrose solution was used for the sample, rather than a stacking gel. Coomassie brilliant blue (0.25%) in 10% acetic acid was used for staining. Destaining was accomplished by leaving the gels overnight in 7% acetic acid.

Enzyme Assay - The enzyme assay was performed using Sigma *Micrococcus lysodeikticus* cells and a Gilford recording spectrophotometer. The method used for the assay was that of Prasad and Litwak (9). A calibration curve was run each time the assay was performed.

FIGURE 1: Pentex lysozyme on Bio Rex 70

Irradiation of Enzyme Samples - Irradiations of the evacuated lysozyme samples were performed at 22-24°C in the kilocurie ^{137}Cs source at the University of Colorado. Each dose was approximately 22.5 Mrads, delivered in 59 hours.

Reaction with HTS - A special glass vessel was designed for the reaction of the irradiated dry protein with tritiated hydrogen sulfide in vacuo, so that the partial pressure of HTS would be 0.2 atm. A drawing of the apparatus is shown in Figure 2. The lysozyme and the HTS vessels are connected to the Y-tube and the break seals are broken. The HTS gas is drawn down into lysozyme by putting liquid nitrogen around the vessel. After ten minutes the HTS vessel is sealed off from the system and the liquid nitrogen is removed. The reaction is allowed to proceed for four hours. After this time any unreacted HTS is removed by putting liquid nitrogen around the Y-tube thereby drawing down the unreacted gas. This is sealed off and discarded. The lysozyme is then dissolved up in distilled water, allowed to stand, lyophilized, and redissolved in water until the specific activity of the lysozyme is constant.

RESULTS

Gamma-irradiation of native lysozyme produces three distinct protein species which can be separated by column chromatography. This is shown in Figure 3. The largest of the peaks, called P3-I, is eluted from the column at approximately the same position as the native protein (see Figure 1). This could indicate that this is the native species so it will be examined further with this possibility in mind. Each of the three peaks obtained from several experiments were desalted, re-dissolved in phosphate buffer, and re-chromatographed. As is shown in Figures 4, 5, and 6 the peaks elute in the same position as in the initial fractionation. There is a small overlap between P1-I and P2-I since in the original chromatography they are not cleanly separated. Thus it appears that the three peaks represent different molecular species.

The enzyme activity of each of the three protein species resulting from irradiation was determined. The activities of P1-I and P2-I were

FIGURE 2: Apparatus for HTS Reaction

63% and 65% of the original enzyme specific activity respectively from one irradiated enzyme preparation and 52% and 65% from another. The assay is extremely difficult to quantitate since it measures decrease in turbidity, so these results must be interpreted only qualitatively. The third protein species, P3-I, has the activity of native lysozyme.

Each of the irradiated species was subjected to disc gel electrophoresis. Figure 7 shows the results with 7.5% cross linked gels and Figure 8 for gels of 5 and 10% cross linkages.

These species cannot be distinguished by the disc gel techniques employed in these experiments.

The reactions of irradiated and un-irradiated lysozyme samples with HTS are in progress.

FIGURE 3: Irradiated lysozyme (22.5 Mrads) on Bio Rex 70

FIGURE 4: P1-I Rechromatographed on Bio Rex 70

FIGURE 5: P2-I Rechromato-
graphed on Bio Rex 70

FIGURE 6: P3-I Rechromatographed on Bio Rex 70

FIGURE 7: Native and Irradiated Species on 7.5% Gels

1. Native Lysozyme, 2. P1-I, 3. P2-I, 4. P3-I.

FIGURE 8: Native and Irradiated Species on 5% and 10% Gels

5% Gels

1. Native lysozyme, 2. P1-I, 3. P2-I, 4. P3-I.

10% Gels

1. Native lysozyme, 2. P1-I, 3. P2-I, 4. P3-I.

DISCUSSION

Since current theory postulates that the primary structure of a protein is responsible for its conformation it will be extremely interesting to determine what perturbations are present in the three species resulting from γ -irradiation. If the primary structure of the three species is identical then the fact that they can be separated by chromatography would indicate that there are changes in secondary and/or tertiary structure. The separation of the species on chromatography using an ion exchange column indicates that there may be differences in charge. Charge differences can be further investigated by running the titration curves of each of the protein species.

Since the three species all show enzyme activity there must be a certain amount of well defined tertiary structure in these molecules after irradiation. P3-I may have the initial tertiary structure since enzyme activity is identical with the native molecule. Disc gel electrophoresis indicates that these molecules are all the same size since it has been postulated that in this method the molecular sieving effect is predominant over any separation by charge. These data support the concept that the tertiary structure is perturbed by irradiation.

The reaction with HTS and subsequent submission of the protein to fractionation, and to the other tests should give further indication as to the nature of the damage. It will be particularly interesting to see if P3-I, which seems to be native lysozyme, shows covalent tritium labeling. This would indicate the existence of radicals in molecules which were then returned to their original conformation. It will also be interesting to see if decrease in specific enzyme activity of altered molecules can be correlated with initial radical concentrations.

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DENATURATURATION OF IRRADIATED LYSOZYME

by

Sarah Herbert

INTRODUCTION

The supposition that lysozyme is "damaged" at the secondary or tertiary level is suggested from the observation that the amino acid composition of the three fractionable species appears to be identical and the observation that denaturation of lysozyme is a reversible process. Current theory postulates that the primary structure is responsible for the conformation of a protein when going from a denatured to a native state. If the primary structure of the three major fractions is identical then the ability to fractionate must depend on differences in conformation which may be detected. One method of determining conformation damage is to examine the heat denaturation and then renaturation of each of the three lysozyme species. If the irradiated proteins return to the native conformation upon heat denaturation and subsequent renaturation this would indicate that irradiation brings about the formation of a "metastable" state in which these molecules can exist. However, if upon renaturation the molecules return to the same conformation as before denaturation this would indicate the existence of a new, thermodynamically stable state for these molecules. The primary structure of the molecules will then be investigated using the technique of peptide mapping. Provided with evidence about the change or lack of change in primary structure the heat denaturation data would be interesting in view of the theory that primary structure determines tertiary structure.

EXPERIMENTAL AND RESULTS

The heat denaturation and renaturation of native lysozyme as well as the different irradiated species was carried out in phosphate buffer (0.2 M, pH 7.09) using a Northeast Scientific temperature programmer and a Gilford recording spectrophotometer. The change in absorbance at 292 mμ is followed as a function of temperature. The temperature programmer is

set so that the increase in temperature will be approximately one degree every two minutes. This allows for the samples to reach equilibrium before a reading is taken. The concentration of enzyme used is determined by the lowest concentration of irradiated species (P2-I) eluted from the Bio Rex 70 columns. The other enzyme species are diluted to this concentration so there will not be any discrepancies in the data due to concentration effects.

The results of the heat denaturation of native lysozyme and P1-I are shown in Figure 1 and those of P2-I and P3-I in Figure 2. The forward and reverse curves are indicated in the figures. Examination of these two figures shows that the forward curves, i.e. heat denaturation, are quite different in shape indicating different protein conformations. This also holds for the native enzyme and P3-I which were originally believed to be the same (see preceding report). However, when one examines the reverse curves, i.e. renaturation, one sees that they are almost identical for all four protein species. Since this was the case it was thought that we should examine the denaturation of the previously denatured-renatured material. These results are shown in Figure 3 for native and P1-I, and in Figure 4 for P2-I and P3-I. These results also are very similar as to the shape of the curves. There is one difference with P1-I, namely that the reverse curve crosses over the forward curve and ends up above it. This, however, cannot be considered significant at this time since each experiment has only been performed once. Duplicate data must be obtained before any conclusions can be drawn from them.

FIGURE 1: Heat denaturation and renaturation of native lysozyme and P1-I

FIGURE 2: Heat denaturation and renaturation of P2-I and P3-I

FIGURE 3: Heat denaturation and renaturation of denatured-renatured native lysozyme and P1-I

FIGURE 4: Heat denaturation and renaturation of denatured-renatured P2-I and P3-I

DISCUSSION

The results of the heat denaturation and renaturation of native lysozyme and the three irradiated species are extremely interesting since each of the four proteins exhibits a unique denaturation curve but similar, if not identical, renaturation curves. This could indicate that the species formed upon irradiation of lysozyme differ only in conformation. These data are difficult to explain in terms of any chemical changes in the molecule. The results of the subsequent denaturation of the denatured-renatured protein species further support the theory of conformational changes alone. This is interesting from the standpoint of radiation chemistry since to my knowledge the conformational and chemical changes induced by γ -irradiation have never been separated. Further experiments will be performed examining the irradiated species from both a physical and a chemical aspect to see if more information can be obtained as to the nature of the changes. An important experiment to perform is to determine the enzyme activity on each of the denatured-renatured species. These experiments are in progress.

HYDROGEN EXCHANGE AND PERTURBATION
SPECTRA OF IRRADIATED LYSOZYME

by

Dante Marciani

INTRODUCTION

Different mechanisms have been proposed to explain the inactivation of enzymes by ionizing irradiation. The most commonly accepted mechanism today is that the inactivation is a result of chemical covalent damage. Conformational damage can arise from alterations in the sequence of amino acids or in the position of disulfide bonds.

We have followed the conformational alterations induced by ionizing irradiation using the hydrogen exchange technique (1). We have also measured perturbation spectra of irradiated lysozyme and compared it with native enzyme.

Hydrogen exchange rates are supposed to be related to alpha helix and hydrophobic region content. Studies interpreted in this way indicate that lysozyme (7) and ribonuclease (2) have decreased content of alpha helix and hydrophobic regions after exposure to gamma irradiation. The interpretation of perturbation spectra data obtained in our laboratory this year indicates that irradiation causes an increase in hydrophobic region content rather than a decrease suggested from rate of hydrogen exchange data.

EXPERIMENTAL PROCEDURE AND RESULTS

Preparation of the tritiated lysozyme.

Heated lysozyme (tritiated or not tritiated) was prepared as before (7). After the process of heating, the yield of non-denaturated lysozyme was 93.5% of the original, a yield that is very similar to the one obtained for P-6 during the process of fractionation of commercial lysozyme by chromatography on Bio-Rex 70. The amount of lysozyme that remains in solution after heating was measured with Folin-Ciocalteu reagent.

The heated lysozyme product upon chromatography on Bio-Rex 70 yields only one peak, the position of this peak is similar to the position of the

peak obtained with heated P-6 lysozyme. These two observations indicate that the purification of lysozyme by heating is possible.

Disc gel electrophoresis cannot differentiate between any of the six peaks present in commercial lysozyme, but shows the presence of small amounts of unaccounted for protein. Upon heating these minor fractions are absent.

Hydrogen exchange was carried as before (1), using the method of gel filtration through Bio Gel P2. To study the time effects of storage on hydrogen exchange, irradiated lysozyme was allowed to exchange at different times after removal from the irradiating source. Exchanges carried out with samples irradiated with 10M Rads, at times of two minutes, two weeks and three months showed that the portion of each rate class remains constant over this time period but was dependent on the dose level.

The exchange rate of hydrogen in non-irradiated lysozyme was carried out four times, and it was found that the spread in the data confirmed the results of Kanarek (8) i.e., the H-exchange rate in lysozyme is not easily reproduced. However, the average of the four experiments is very similar to results that we obtained before.

Experiments carried out by dissolving irradiated material in H_2O and HTO respectively showed that during the lyophilization process there was some loss of exchangeable hydrogens. The loss was approximately 10 hydrogens in each class (III and IV). Perhaps during the process of lyophilization some exchange took place that is hard to control or evaluate.

Approximately the same results that were obtained before were repeated, (Table I). The lack of reproducibility is presumably due to the lyophilization process necessary for this kind of experiment.

A decrease in Hydrogens Class III and IV is found after irradiation. The decrease in Hydrogens Class IV is attributed to a decrease of the hydrophobic regions.

Solvent perturbation difference spectra of irradiated lysozyme.

The bases of the solvent perturbation technique are the following: (6) Spectra of tryptophane or tyrosine residues located on the surface of the protein are usually perturbed by changes in the physical properties of the solvent in the immediate vicinity of these chromophores. On the other hand,

tyrosines and tryptophanes deeply buried in the interior folds of the protein are shielded from the perturbing effect of the solvent since they do not come freely in contact with it. Consequently their spectra are not affected by the change in solvent properties. This technique involves the addition of mild or inert substances to the enzyme solution. The conformation of the protein is not apparently effected but spectral shifts are induced.

The data obtained is presumed to be related to the fraction of tryptophyl residues that are exposed or in contact with the solvent. Changes in the difference spectra of native and irradiated lysozyme are thus related to alterations of the hydrophobic regions near the tryptophyl residues.

The experimental procedure was as follows: A model mixture containing N-acetyl-L-tyrosine 4×10^{-4} M and N-acetyl-L-tryptophan 8×10^{-4} M in buffer 0.1 M acetic acid-sodium acetate, pH 5.00 was used as a model solution of denaturated lysozyme. The perturbant employed was 20% of polyethylene glycol (PEG). The exposure of the chromophores in this mixture is taken as 100%. The difference spectra were taken between the model solution and model solution plus PEG. The spectra were corrected for the absorption of PEG. To avoid variations in absorption caused by changes in concentration all the data are normalized, and the value: $\Delta E_{290}/E_{279}$ is used as a measure of the degree of exposure of tryptophan to the solvent and in this case is taken as 100% or 1.

The difference spectra of native lysozyme dissolved in the buffer, and native lysozyme plus PEG was then determined. The degree of exposure was then calculated with the normalized data. The same procedure is followed with the irradiated samples.

All the spectras are measured at 25°C 20 to 60 minutes after the perturbant is added to the protein solution. Figure I is the difference spectra of the model mixture, Figure II is difference spectra of lysozyme and Figure III that of irradiated lysozyme. Table I gives the degree of exposure as a function of irradiation dose. The results obtained indicate that irradiation causes the degree of exposure of the tryptophyl groups to decrease. This is an indication that the tryptophyl residues are buried during the process of irradiation or maybe that there is a formation of new hydrophobic regions in the protein molecule. The decrease of degree of exposure and the doses are related in an exponential fashion.

FIGURE I

Solvent perturbation difference spectra of Lysozyme model mixture in 0.1 M AcONa-AcOH, pH 5.00. Perturbant: Polyethylene glycol 20% (v/v) [Polyethylene glycol: P.E.G. 400, d.p.: 6]

Model mixture:

N-Acetyl-L-tyrosine ethyl ester 2×10^{-4} M

N-Acetyl-L-tryptophan ethyl ester 4×10^{-4} M

$\Delta E_{290}/E_{279} = 0.082$

$\Delta E_{\lambda}/E_{279}$

.08

.06

.04

.02

.00

272

280

288

296

$\lambda m\mu$

45 J

FIGURE II

Solvent perturbation difference spectra of heated lysozyme in
0.1 M AcOH-AcONa, pH 5., Perturbant: Polyethylene glycol 20% (v/v)
[P.E.G. 400, d.p.: 6]

$$\Delta E_{291.5}/E_{281} = 0.044$$

$$[\Delta E/E]_{\text{rel.}} = 0.539$$

FIGURE III

Solvent perturbation difference spectra of irradiated lysozyme
in 0.1 M AcOH-AcONa, pH 5., Perturbant: P.E.G. 400, 20% (v/v).

Doses: 10 MRads.

$$\Delta E_{292}/E_{281} = 0.0385$$

$$[\Delta E/E]_{\text{rel.}} = 0.470$$

TABLE I

		Former Experiments	New Experiments
Lysozyme - T	H Class III	25	31
dissolved in HTO	H Class IV	53	60
Lysozyme - T	H Class III	14	38
dissolved in H ₂ O	H Class IV	39	68

TABLE II

<u>DOSES</u>	<u>EXPOSURE</u>
0 MRads	0.539
5 MRads	0.510
10 MRads	0.470
15 MRads	0.450
20 MRads	0.420

DISCUSSION

Our studies indicate that ionizing radiation causes a change in the rate with which hydrogen can be exchanged with lysozyme and a change in the ability of polyethylene glycol to modify the spectrum of the enzyme. The data that is obtained from these methods relates to the conformational state of the molecule, however, the commonly applied interpretation of the data as it relates to structure does not give a consistent structural model. The interpretation of hydrogen exchange data suggests that ionizing radiation cause an increase in amino acid residues exposed to solvent while spectral perturbation studies suggest a decrease in the portion of the molecule in contact with the solvent. Perhaps this conflict of interpretation is not serious since spectral perturbation is dependent on the location of chromophoric residues while hydrogen exchange is not dependent on light absorption. Thus it is possible to conceive of irradiation damaged molecules which are in general more exposed to the solvent but that at the same time have fewer of their tryptophyl groups exposed than the native molecules.

The reproducibility of spectral measurement is fairly good, however, further work is required to improve the reproducibility of hydrogen exchange data. The measurements made to date with these two methods have been conducted with a mixture of radiation damaged molecules. Some of these molecular species are fractionable and stable, thus it may be possible to examine a more cleanly defined system in the future.

The working hypothesis that is being used to correlate the results obtained is that irradiation causes a disulfide interchange which results in a modified conformation which is reflected by modified hydrogen exchange and spectral properties. Clearly, additional chemical and physical studies are required to further substantiate this idea.

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