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CONFORMATIONAL CHANGES IN SEVERAL ENZYMES
DURING PHOTODYNAMIC INACTIVATION

by

Thomas R. Hopkins

Technical Report

U.S. Atomic Energy Commission Contract No. AT(11-1)-875

Department of Molecular and Genetic Biology
University of Utah
Salt Lake City, Utah 84112

August 1967

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ABSTRACT

Most enzymes can be inactivated by light in the presence of appropriate sensitizing dyes and molecular oxygen, a phenomenon called photodynamic action. Certain types of amino acids in the polypeptide chains are oxidized in the process. The photooxidation rate of each susceptible amino acid depends upon the particular enzyme, type of dye, and the reaction condition. The photooxidation rate of the most rapidly destroyed amino acids, histidine and tryptophan, can approach the rate at which the enzyme is inactivated. Beyond this, little is known about the mechanism by which the enzyme loses its catalytic function. To date, no systematic study on the biophysical changes of photodynamically treated enzymes has been reported.

The present work is concerned chiefly with studies of conformational changes in ribonuclease, trypsin, chymotrypsin, and lysozyme during the course of photodynamic inactivation. Methylene blue, eosin Y, and in some experiments, flavin mononucleotide were used as sensitizing dyes. Conformation-sensitive parameters studied include fluorescence, the behavior on molecular sieve and ion-exchange chromatography, electrophoresis mobility on cellulose acetate, immunological reactivity, sensitivity to thermodenaturation, fluorescence polarization, viscosity, solubility, fluorescence transitions in urea and protein digestibility.

A secondary objective of this thesis was to determine whether altered forms of enzyme molecules still possessing catalytic activity

were produced as a result of photodynamic treatment. The altered characteristics examined were urea sensitivity, salt sensitivity, thermosensitivity, digestability, and low enzyme turnover. For comparative purposes, the enzymes were also photoinactivated in 8 M urea.

In general, all four enzymes showed some structural alteration. With ribonuclease and lysozyme, the rate of alterations correlated directly with the rate of loss of enzyme activity. This is evidence that at least some of the photoinactivation of these enzymes occur by changes in protein conformation. Chymotrypsin and trypsin showed slight conformational changes but the rate of these changes did not correlate directly with the loss of enzyme activity. When eosin Y was used as the sensitizer for all four enzymes, one or two tryptophan residues were rapidly destroyed at a rate similar to the rate of enzyme photoinactivation, indicating that a tryptophan residue may be involved in the maintenance of enzyme activity. Charge interactions of the respective proteins were disrupted; ion-exchange chromatography and electrophoresis patterns of the photoinactivated enzymes were characteristic of less basic proteins than the native enzymes. The rates of photooxidation of trypsin, chymotrypsin, and ribonuclease in 8 M urea were, in general, several times faster than the rates in buffer alone. The native enzyme structure protects some amino acid residues from destruction by photodynamic action.

During the photodynamic inactivation of all four enzymes, active but altered enzyme molecules were produced. Urea-sensitive enzyme

fractions were found with trypsin and ribonuclease using flavin mononucleotide but not methylene blue or eosin Y as the sensitizer. Thermosensitive enzyme fractions were produced during the photodynamic inactivation of ribonuclease and lysozyme with all three sensitizers. Because of their autolytic properties, trypsin and chymotrypsin could not be tested for thermosensitivity. A low turnover enzyme was detected during the photodynamic inactivation of chymotrypsin as sensitized by all three dyes. Partially photo-inactivated enzyme with unusual sensitivity to 1.2 N sodium chloride, was not found with any of the four enzymes.

In conclusion, we have demonstrated that photodynamic treatment can produce altered enzymes which still retain intact active sites. Furthermore, some enzymes apparently lose catalytic ability as a result of the destruction of secondary and tertiary structure.

This work was supported by the Biology Branch, Division of Biology and Medicine, U.S. Atomic Energy Commission, under Contract No. AT(11-1)-875.

LIST OF ABBREVIATIONS

<u>Abbreviation</u>	<u>Meaning</u>
μM	micromolar
μg	microgram
μl	microliter
nm	nanometer, millimicron
RNase	ribonuclease
RNase-S	ribonuclease-subtilisin treated
UMP	uridine monophosphate
DFP	diisopropyl fluorophosphate
ATEE	N-acetyl-L-tyrosine ethyl ester
BAEE	benzoylarginine ethyl ester
C23P	cytidine 2',3'-cyclic phosphate
FMN	flavine mononucleotide
MeB	methylene blue
EoY	eosin Y
DMABA	p-dimethyl-aminobenzaldehyde
U.V.	ultraviolet
TCA	trichloroacetic acid
HEAH	hard-to-exchange amide hydrogens
T_m	midpoint temperature of thermal transition
ΔA	change in absorbance

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1. INTRODUCTION

A. Photodynamic Action on Enzymes

Within three years of the discovery of the phenomenon of photodynamic action, Blum showed that sensitizing dyes could inactivate papain, invertase (sucrase) and diastase (amylase) in the sunlight (12). Studies have since been made on all categories of enzymes (hydrolases, lyases, isomerases, lipases, transferases and oxidoreductases) as well as other proteins such as insulin, antibodies, collagen, myoglobin, snake venoms, bacterial toxins, etc. These substrates are listed in detail in a review by Spikes and Livingston (132). The fundamental chemical change which occurs in enzymes inactivated by dye-sensitized photooxidation is the destruction of certain amino acid residues including histidine, tryptophan, tyrosine, methionine and cysteine. The first two amino acids are generally destroyed most rapidly. Peptide bonds are not broken by photodynamic action (144,156,42). Not all of the residues of a given amino acid are destroyed at the same rate in an enzyme. Because of protein folding, some susceptible amino acids presumably are buried in the interior of the enzyme and are less available for photooxidation. By analysis of peptide fragments from the protease digestion of previously photoinactivated enzyme, it should be possible to locate the position of the photooxidized amino acid residues. A beginning has been made with insulin (160) and ribonuclease (72).

The photodynamic alteration of amino acid residues in enzymes involves the uptake of molecular oxygen. Little is known about the oxidative products and even less is known about the chemical intermediates

probably involved in the photooxidative mechanism. It is apparent that much work must be done on the detection and measurement of short-lived chemical species and on the organic chemistry of the dye-sensitized photooxidation of the amino acid residues.

A number of biophysical changes have been measured during photodynamic inactivation of enzymes. Generally, the ultraviolet absorption decreases (155), solubility decreases (144, 155, 4), viscosity increases (155, 157, 112, 116, 7), sedimentation coefficient changes (39, 13, 145, 30), optical rotation changes (39, 109, 116), coenzyme and metal binding behavior change (88, 162), digestibility by proteolytic enzymes increases (39), and antigenic properties are lost (159). Altered but enzymatically active enzymes have been detected after photodynamic treatment of native enzymes. Among the changed properties of such active enzymes are increased sensitivity to urea (42), sensitivity to elevated temperatures (109, 31, 40), altered elution patterns in column chromatography (72), different mobilities on polyacrylamide gel electrophoresis (44), increased specific enzymatic activity (143, 62) and a change in the ratio of the rates of hydrolysis of ester and peptide substrates (143, 61).

Since this thesis is devoted to studies of photodynamic effects on ribonuclease (RNase), lysozyme, chymotrypsin and trypsin, a detailed survey of the effects of photodynamic action on these enzymes follows:

Photodynamic action on RNase

The first study of the dye-sensitized photooxidation of RNase was reported in 1951 by Shugar (119). Using sunlight or a properly filtered high-pressure mercury vapor lamp and FMN as the sensitizer, RNase

activity was not decreased after several hours of exposure in phosphate buffer, pH 7.1. In contrast, lysozyme sensitized with FMN was completely inactivated in a few hours. These two enzymes were selected because one of them, RNase, lacks tryptophan in its structure. Since Gorbach and Lerch (49) had postulated earlier that photodynamic inactivation sensitized with FMN was due to reaction with tryptophan residues in the enzyme molecule, Shugar felt that his negative results with RNase confirmed their postulate.

Four years later Weil and Seibles (157) reported in one of a series of articles investigating the effects of photodynamic action on purified enzymes, that RNase lost all of its enzymatic activity when 3 moles of oxygen were taken up per mole of enzyme. Analysis of the amino acid composition revealed that the only major change in amino acid content was the destruction of 3 of the 4 histidine residues. No alteration in tyrosine, phenylalanine, lysine and arginine was observed, but a small decrease in methionine was noted. The decrease of histidine was proportional to the decrease in enzymatic activity up to 50% inactivation of the RNase. Reaction conditions were 5 mg/ml RNase in 0.05 M phosphate buffer, pH 8.0 with a final concentration of 0.005% methylene blue.

Illumination was done at 37° C. As will be shown later, these reaction conditions are detailed because it has become obvious that the kinetics and type of amino acids destroyed by photodynamic action vary with temperature and pH (130,156). An increase in the ultraviolet (U.V.) absorbance of the treated enzyme in the 230-270 nm range was attributed to the photooxidation of histidine. The relative viscosity and optical rotation at 589 nm of photooxidized RNase remained unchanged compared to

thioglycolate (under their conditions methionine sulfoxide was completely reduced to methione) increased the binding constant by a factor of 10 over that of non-reduced, photooxidized peptide. Peptide maps of trypsin and chymotrypsin digests of RNase-S showed that histidyl residues 105 and 119 on RNase-S-Protein were photooxidized at comparable rates while histidine-12 was destroyed at a much lower rate. In these studies RNase was photooxidized in 0.2 M ammonium bicarbonate, pH 8, and irradiated at room temperature in the presence of 0.001% methylene blue. Studies on the free RNase-S-Peptide in solution showed that activity was lost at about the same rate as with RNase-S, however, the peptide was oxidized much more slowly when present as a complex with RNase-S-Protein. Thus, histidine-12 was protected in the combined form. When partially photooxidized RNase-S was chromatographed on Amberlite IRC-50 resin, three peaks in addition to the native enzyme peak were observed. The first peak to elute off the resin was identified as fully inactivated enzyme. The second peak was as active as the starting material and peptide map analysis showed the loss of histidine-105. The third was completely inactive and had lost histidine-119.

Thus, photooxidation of RNase-S-Peptide causes the oxidation of histidine-12, loss in activity and about a 1000 fold drop in the association constant between S-Peptide and its native partner. As mentioned earlier, methionine-13 is also photooxidized. It is involved in the associative process, but not with the activity of RNase-S. Although no experimental detail is given, Kenkare and Richards also report that the loss of histidine-119 in the RNase-Protein reduced the affinity of RNase-Protein for native RNase-Peptide.

The photooxidation of solid RNase-S suspended in 85% saturated ammonium sulfate adjusted to pH 7.7 was examined. Methylene blue was preferentially taken up into the crystals. The qualitative effects were identical with those in solution, although the overall rate was about 50 times slower. The comparison of X-ray diffraction patterns of native and photooxidized crystals of RNase-S as well as the above changes in association binding suggested that only small conformational changes may have occurred.

As mentioned earlier, the conditions at which the photooxidation occurs can influence the types of reactions that occur. DasGupta and Boroff (26), studying methylene blue sensitized photodynamic action on RNase at 23° C found that at the pH of 6.8, histidine, methionine, and tyrosine were partially oxidized. In contrast, photooxidation under identical conditions but in 0.1 M acetate buffer, pH 3.8, caused a slight decrease in methionine but no significant decrease in concentration of either tyrosine or histidine. Similar differences were also noted with photooxidized lysozyme at the same two pH values. It appears that photooxidation may be made more selective by varying the pH of the reaction mixture.

The temperature of the reaction system also influences the sensitivity. While comparisons with RNase have not been made, Koshland, et al. (74,105) have reported that photooxidation of chymotrypsin at neutral pH and 10° C resulted in destruction of histidine and methionine but not tryptophan. In contrast, Weil and Buchert (156) completely photoinactivated chymotrypsin at neutral pH and 37° C and

found that three tryptophan residues and one histidine residue were oxidized. Studies in the same laboratory (160) on the photooxidation of insulin sensitized with methylene blue showed that changes in amino acid composition were restricted to two histidine residues at pH 7.0 and 2° or 10°, while at 20° and 40° there was probably additional loss of tyrosine. Like RNase, insulin has no tryptophan residues.

It is well known that substrates and their analogues often protect enzymes against inactivation. In the presence of the substrate analogues, uridine 2' (or 3')-phosphate (RNase: UMP - 1:80 mole/mole) or cytidine 2' (or 3')-phosphate, Waku and Nakazawa (147) found that the rate of the methylene blue sensitized photoinactivation of RNase at pH 6.1 (0.08 M acetate) and 37° C was much slower than in the absence of substrate analogue. No change was observed in UMP itself by paper chromatography and ultraviolet absorption. The activity of RNase A was almost completely destroyed after absorption of 4 moles of O₂ per mole of enzyme. At pH 8.0 the rate of inactivation was slower and, because the analogues do not bind at the elevated pH, the rate was independent of the presence of substrate analogue. When samples of RNase photooxidized with and without substrate analogue were chromatographed on IRC-50 resin, both showed an inactive protein fraction eluting more rapidly than the native enzyme. The sample photooxidized in the presence of UMP, however, showed an additional peak which eluted slightly before the native enzyme and possessed the full activity of the parent RNase. Amino acid analysis of the new, active peak showed that two moles of histidine were oxidized in addition to traces of cystine, methionine and tyrosine residues. We have seen by the work of Kenkare and Richards, as well as others (24),

that histidine 12 and 119 are necessary for enzyme activity. It appears that histidine 48 and 105 are not concerned with the activity of RNase and that in the presence of substrate analogues the two essential histidines are protected from photooxidation. In conflict, Kenkare and Richards (72) reported that the presence of substrate had no effect on the rate of photooxidation of RNase-S. However, no experimental details were presented.

Another enzyme which hydrolyses RNA is the bacterial enzyme, RNase-T. It is quite different from pancreatic ribonuclease in that it has a low content of basic amino acids and, further, it contains tryptophan. Yamagata, et al. (164) photooxidized RNase-T in the presence of methylene blue or riboflavin in 0.1 M phosphate buffer, pH 8 or 7 at 37° C. Amino acid analysis revealed that only histidine and tryptophan residues were photooxidized. A kinetic analysis of the loss of these residues suggests that one or two histidine residues out of the three are essential for catalytic activity. The rate of photoinactivation sensitized by riboflavin was much slower, as would be expected, when a tungsten light source is used. They noted that the rate of inactivation increases somewhat when the pH of the reaction mixture is raised from 6.0 to 8.0. The only residue that was significantly photooxidized was histidine. Tryptophan remained almost unchanged. Thus, both dye sensitizers preferentially photooxidize histidine residues. Like pancreatic RNase, RNase-T seems to require one or two histidine residues for catalytic function.

Photodynamic action on lysozyme

In the early 1950's three laboratories reported that egg white lysozyme could be inactivated by photodynamic action. In an abstract, and, two years later, in a detailed paper (154,155) Weil's laboratory showed that the amino acid composition and activity of lysozyme were changed by MeB-sensitized photooxidation. The single histidine residue was completely destroyed when the enzyme had taken up 2 moles O_2 /mole enzyme. At this point 83% of the total tryptophan content (6 residues) and 30% of the enzymatic activity remained. Other amino acids such as tyrosine, methionine, and cysteine were unchanged. At 6 moles O_2 uptake/mole enzyme, all of the enzymatic activity was gone and the tryptophan and tyrosine content had decreased 50% and 19% respectively; cysteine and methionine were still not affected.

Lysozyme (2.5 or 5 mg/ml) was photooxidized in 0.05 M phosphate buffer, pH 8.7 and 0.005% methylene blue at 37° C. Preliminary experiments at several different pH values indicated that photooxidation of lysozyme increased with pH values up to a maximum at about pH 9.0. During the course of photooxidation CO_2 was evolved.

The extinction coefficient of the absorption maximum at 280 nm decreased progressively as lysozyme was photooxidized. At 5.3 mole O_2 uptake/mole enzyme (<5% activity remaining) the extinction coefficient was decreased 30% and upon further photooxidation, the peak eventually disappeared. Solubility of lysozyme in 0.1 M NaCl and pH 10.7 decreased and relative viscosity increased with photooxidation. Amino acid analysis indicated that histidine might participate in the enzymatic activity of lysozyme because the rate of decrease of this amino acid

on photodynamic treatment followed closely the rate of enzyme inactivation up to about 70% inactivation. However, since lysozyme retained about 30% of its activity even after the complete destruction of histidine it cannot be a participant in the catalytic center. Three or four of the tryptophan residues may also contribute to the activity of lysozyme.

Dickman and Proctor found that a solution of 7 μM lysozyme and 56 μM riboflavin could be inactivated under fluorescent lamps at room temperature (28).

At about the same time, Shugar (119) reported that lysozyme (0.5 mg/ml) in phosphate buffer, pH 7.1 and 50 μg riboflavin was completely inactivated in a few hours upon exposure to sunlight or monochromatic radiation of 365 nm wavelength from a filtered mercury vapor lamp. The inactivation was first order. The rate of photooxidation was retarded when tryptophan was added to the reaction mixture prior to illumination. Tyrosine also shows a protective effect, but to a lesser extent.

Using a colorimetric method to qualitatively determine the tryptophan content in lysozyme after inactivation, Shugar reported that a 50% inactivated sample had a "violet tint" slightly less than a non-irradiated control while a 100% inactivated sample was colorless. Thus tryptophan may be required for the maintenance of lysozyme activity.

Besides riboflavin and methylene blue, hematoporphyrin also sensitizes the photodynamic inactivation of lysozyme (18). Lysozyme (0.4 mg/ml) was irradiated with a filtered low pressure mercury lamp in the presence of phosphate buffer, pH 8, and hematoporphyrin (10^{-5} M).

First order inactivation was observed. Photodynamic inactivation of lysozyme was also studied in relation to enzyme and hematoporphyrin concentration. The inactivation increased with increasing concentration of enzyme or dye and then decreased. Optimum concentrations were 0.4 $\mu\text{g/ml}$ lysozyme and about 4×10^{-6} M hematoporphyrin. At pH 8.4, a solution of hematoporphyrin (10^{-6} M) and lysozyme (6 $\mu\text{g/ml}$) formed a pink precipitate. Changing this dye/lysozyme concentration ratio decreased the extent of precipitation. It was concluded that the photodynamic inactivation of lysozyme is dependent on a combination of the hematoporphyrin with the substrate.

A comparative study of the methylene blue sensitized photoinactivation of lysozyme and RNase at two different pH values was reported by DasGupta and Boroff (26). The photooxidation process was monitored with a recording polarograph and samples were analyzed for the effect of photodynamic action upon tryptophan, tyrosine, histidine and methionine residues. Lysozyme (0.5 mg/ml) was in either acetate buffer, pH 3.8 or in phosphate buffer, pH 6.8, containing 0.0004% methylene blue at 23°C . In lysozyme, at either pH 3.8 or 6.8, only tryptophan and methionine were oxidized. As noted previously, Weil, *et al.* did not detect any destruction of methionine when lysozyme was photooxidized in phosphate buffer, pH 8.7. However, the assays were done on the acid hydrolysates of lysozyme. Ray and Koshland have demonstrated that methionine sulf-oxide, a product of photooxidation of methionine, is reduced back to methionine during acid hydrolysis (105). In contrast to lysozyme, photooxidized RNase, at pH 3.8, showed no loss of tyrosine or histidine and at pH 6.8, all amino acids examined showed considerable loss.

McVey and Spikes recently measured the quantum yields of the photodynamic inactivation of lysozyme as sensitized with methylene blue, eosin Y and FMN (85). Effects of oxygen partial pressure and pH on the photo-oxidation reaction were examined. The quantum yield with FMN as sensitizer reached a maximum with 1-2.5% oxygen. Sensitizers eosin Y and methylene blue had greater oxygen requirements for maximal quantum yields (2.5% and 10-20%, respectively). Little enzyme was photoinactivated at low pH values with eosin Y and methylene blue. Quantum yields increased with increasing pH and reached a maximum at pH 10. In contrast, with FMN as the sensitizer the pH curve showed maximum quantum yields at pH 5-6. It is probable that FMN sensitizes by a different reaction mechanism than do methylene blue and eosin Y.

Photodynamic action on trypsin

The photodynamic inactivation of trypsin has been investigated almost exclusively by Spikes and associates (42,48,130,131). In an attempt to learn more about mechanisms of energy transfer in photodynamic systems, they have examined the effect of variables such as enzyme concentration, dye type and concentration, pH, and temperature on the rate of photodynamic inactivation.

In order to compare the effectiveness of different dyes their later papers expressed the photodynamic inactivation of trypsin in the quantitative terms of quantum yield or the reciprocal, quantum requirement. The quantum yield for a photodynamic process is the ratio: (number of enzyme molecules inactivated)/(number of light quanta absorbed).

If the reaction mixture was supplied with adequate amounts of air, the rate of inactivation was first order. A reaction mixture consisted

of 42 μM in trypsin, either 12.5 μM in methylene blue (MeB) and eosin Y (EoY) or 150 μM in riboflavin-5'-phosphate (FMN), and 0.125 M in sodium phosphate buffer, pH 8.0. The photoinactivation was carried out in air at 15° C.

Under usual conditions the quantum yields were 0.0014 with methylene blue, 0.0027 with eosin Y and 0.0023 with FMN. This compares with 0.006 reported by Shugar (119) for the riboflavin sensitized photodynamic inactivation of lysozyme. These values are much smaller than the quantum yields of the dye-sensitized photooxidation of small organic molecules such as tryptophan which is 0.038 (119).

In general, the quantum yields of trypsin photoinactivation with the three sensitizers increased rapidly with increasing dye concentration, reached a maximum, and then decreased. The quantum yields of photoinactivation increased with increasing enzyme concentration and were independent of light intensity. FMN-sensitized photodynamic inactivation had small quantum yields at low pH values and large quantum yields under basic conditions. In contrast, MeB and EoY showed no activity as sensitizers at low pH values and high activity at high pH values. Quantum yields increase slightly from 4° C to 33° C. It was suggested that photodynamic action proceeds by a different mechanism with flavins than with other dyes. This was evidenced by 1) the different response of FMN and the MeB or EoY to pH of the reaction mixture, 2) the higher ratio of oxygen consumed per trypsin molecule inactivated photodynamically (15 with FMN, 5 with MeB), 3) the need for rapid stirring during the photoinactivation of trypsin sensitized with FMN in

order to maintain a first order reaction. In contrast inactivation of trypsin as sensitized by MeB was insensitive to the stirring, and 4) the production during photooxidation of enzymatically active trypsin molecules which are sensitive to 5 M urea when FMN or riboflavin was the sensitizer but not when the inactivation was sensitized with MeB or EoY. The urea-sensitive class of trypsin molecules was detected by assaying for enzyme activity with casein or benzoyl-L-arginine ethyl ester and with a hemoglobin-5 M urea substrate. Using FMN as the sensitizer, the rate of enzyme inactivation during photodynamic treatment was found to be much greater when measured by the hemoglobin-5 M urea assay than when measured by the other two assays. The damaged fraction was also less stable upon storage at room temperature (pH 4.6) than was native trypsin. Damaged enzyme fractions sensitive to heat and/or chemical agents have also been found after U.V. and gamma irradiation (2,31).

An attempt was made to isolate the damaged class sensitive to urea by disc polyacrylamide gel electrophoresis (44). The native trypsin sample separated into a main band and three minor bands which had lower mobility. The main band contained all detectable tryptic activity. When the enzyme was photoinactivated by procedures which produced a urea-sensitive fraction of trypsin molecules, a progressive loss of all electrophoretic bands was observed as measured by stainability. No decrease in stainability was observed during photoinactivation with the two sensitizers that do not produce urea sensitive trypsin activity: MeB and EoY.

The immunological properties of trypsin apparently are not altered during FMN sensitized photoinactivation (45). Using the capillary fluid

precipitation and a modified Ouchterlony gel diffusion test, no differences were observed between inactivated and native trypsin. However, when acetylated trypsin, which is enzymatically active, was tested against trypsin antisera, no precipitation was observed using either test.

Glad and Spikes have tried to inhibit photodynamic inactivation with various chemical agents (48). Protective agents apparently act by quenching the excited state of the sensitizing dye or by competition with the substrate for photooxidation. 2-aminoethylisothioronium bromide hydrobromide (AET), which protects biological systems against ionizing radiation, also protected trypsin against photodynamic inactivation in the presence of MeB and FMN, but not EoY. Sodium iodide protected with EoY and FMN; histidine and egg albumin protected with MeB, EoY, and FMN.

Several hundred dyes have been examined as sensitizers for the photodynamic inactivation of trypsin (130). Inactive compounds include the azo, indophenol, oxazine, triarylmethane, thiazole, ketone imine, methine, nitro and nitroso dyes. Active dyes include certain acridines, flavins, azines, thiazines, anthroquinones, xanthenes and certain porphyrin and chlorophyll derivatives. Some of the substituted anthraquinones were highly specific. They either sensitized well or not at all.

Some kinetic mechanisms of photodynamic action postulate the existence of a dye-substrate intermediate. Binding, as measured by perturbation of absorption spectra of dyes in the presence of trypsin, was strong with some good sensitizing dyes and not detected at all with

other good sensitizers (133). This data does not rule out the dye-binding mechanisms for photodynamic action but tends to underline the conclusion that dyes sensitize the photodynamic inactivation of trypsin by different mechanisms. Furthermore, although the ground state dye does not bind to the substrate, it may be that the dye in the triplet state would.

Photodynamic action on chymotrypsin

The first report that chymotrypsin was inactivated by photodynamic action was in 1953 by Weil et al. (156). Crystalline chymotrypsin (5 mg/ml) in 0.2 M phosphate buffer, pH 7.4 and 37° C was sensitized with 0.005% MeB; the pH optimum of oxygen uptake during photooxidation was about 9.0. Testing for both enzyme activity for both ester and peptide bonds, they found that the rate of inactivation was the same for both assays and that the activity reached zero when 4 moles of oxygen had been taken up per mole of enzyme. Also this inactive enzyme would not react with diisopropyl fluorophosphate (DFP). At this point, amino acid analysis indicated a 53% decrease in histidine (1.02 moles) and a 40% decrease in tryptophan (3.01 moles). The tyrosine, cystine and methionine contents of the enzyme were unchanged. Control experiments lacking dye or oxygen showed no enzyme inactivation.

Similar experiments with chymotrypsinogen under identical conditions gave results closely resembling those with chymotrypsin. Photooxidized chymotrypsinogen was converted to the active enzyme by treating the zymogen with trypsin.

The absorbancy of chymotrypsin at 280 nm dropped as the enzyme was photooxidized and only a slight maximum remained by the time the enzyme

was completely inactivated. This drop in absorbancy was assigned to the photooxidation of tryptophan. As mentioned before, tyrosine was unaffected during the course of enzyme inactivation.

No precipitation of denatured chymotrypsin in 2 M NaCl solution or at its isoelectric pH was observed during the course of the reaction. Also, no increase in the relative viscosity was observed. Weil et al. offer this as proof that denaturation of the enzyme was not occurring during photooxidation.

Jandorf et al. (64) confirmed the results of Weil et al. and extended the study by comparing the photooxidation of chymotrypsin with the photooxidation of DFP-treated chymotrypsin at pH 7.0 and 25° C. They found that the rate of oxygen uptake for DFP-treated chymotrypsin, and the number of rapidly photooxidized histidine and tryptophan (1.2 and 2.8, respectively) was halved when DFP-treated chymotrypsin was photooxidized. The covalently bound diisopropyl-phosphate was not released during the process of photooxidation.

Ultracentrifugal studies at pH 4.6 and ionic strength 0.2, showed that photooxidized chymotrypsin devoid of enzyme activity or photooxidized DFP-treated chymotrypsin did not dimerize at increased protein concentration while native chymotrypsin was almost completely in the dimerized state at 5 mg/ml protein concentration and DFP-treated chymotrypsin at about 15 mg/ml protein concentration. Because of the pH dependence of the dimerizability of chymotrypsin and DFP-treated chymotrypsin, Egan et al. felt that the photooxidation of histidine rather than tryptophan was responsible for the loss of ability to dimerize. (30).

A very interesting kinetic approach to the identification of the possible role of photooxidized amino acid residues in enzymes was given in a series of papers by Ray, Koshland and co-workers (74,104,106). By assuming that each amino acid lost by photooxidation was either rapidly destroyed (presumably it was readily accessible on the enzyme surface) or slowly destroyed, they were able to accurately fit theoretical curves to the data and thus evaluate the first order kinetic constants of the photooxidative destruction of both the "fast" and the "slow" residues. These derived kinetic constants were then compared with the kinetic constant of the rate of enzyme photodynamic inactivation. If the kinetic constants were the same, this would be good evidence that the amino acid was essential for the maintenance of enzyme activity. Their kinetic technique also permits an estimate of the number of amino acids that are involved in the "fast" photooxidative destruction.

They found that the loss of activity of chymotrypsin by photodynamic action in 0.05 M phosphate buffer, pH 7 and 0.01 % MeB at 10° C gave a biphasic curve and that this curve could be correlated with the photooxidation of a "fast" histidine and a "fast" methionine. Under their conditions and in contrast to that found by Weil, et al. (150), the tryptophan residues of chymotrypsin were unreactive, thus suggesting an inaccessibility to the photooxidation reagent.

The photolysis of enzymatically inactive diazo-acetylchymotrypsin was reported by Westheimer and co-workers (117,123). p-Nitrophenyl diazoacetate stoichiometrically acylates chymotrypsin at its active center to form the intermediate, diazoacetyl chymotrypsin. Photolysis

of the diazoacetyl-enzyme yielded a mixture of regenerated, active enzyme and modified protein which retains the carbon atoms of the diazoacetyl group. This inactive modified enzyme had an o-carboxymethyl group attached to a serine residue probably arising from a reactive carbene formed as a result of the photolysis of the diazoacetyl ester. A small amount of l-carboxymethylhistidine and o-carboxymethyltyrosine were also obtained.

The effects of pH, dye concentration and the rate of stirring on the quantum yield for the photodynamic inactivation of chymotrypsin was recently reported by Hodgson and Spikes (60). They used three sensitizers; MeB, EoY and FMN. In contrast to the finding of Jandorf et al. (64) who report that no photooxidation of MeB sensitized chymotrypsin takes place at pH 4.6, they found that the rate of inactivation was appreciable at pH 3 and increased rapidly above pH 6. In comparison, Spikes and Glad (130) reported that trypsin was not photodynamically inactivated below pH 6. Quantum yields for the photodynamic inactivation increased with increasing dye concentration, reached a maximum and then declined. Unlike the findings for FMN sensitized trypsin, the inactivation of chymotrypsin was not influenced by the rate of stirring of the reaction mixture during illumination.

B. Conformation Measurements on Enzymes

The structural organization of proteins is divided into several levels. These are termed primary, secondary, and tertiary structure. Distinctions among these levels may be made in the type of interactions necessary for the maintenance of the structure. The primary structure

of a protein is defined by the amino acid residues covalently linked together in a definite sequence by peptide bonds. Some amino acid chains are also held together by specific covalent cross links called disulfide bonds. Patterns of disulfide bond linkages in lysozyme, RNase, chymotrypsin and trypsin are:

Topological patterns of enzyme structures. The heavy line represents the polypeptide chain. Source: Kato *et al.*, *Biopolymers* 4, 738 (1966).

The first major achievement in the elucidation of amino acid sequences for enzymes was the laboratory of Hirs, Stein and Moore on RNase (128). Following this spectacular success with RNase, the amino acid sequences of several additional enzymes have been established. These include lysozyme (17,66), chymotrypsinogen (148),

trypsinogen (148) and papain (9,76).

Secondary and tertiary structure

The primary structure of a linear protein chain, like that of simpler organic compounds, can have different three-dimensional conformations. In the case of flexible chains of molecular weights ranging up to 200,000 or more, the possible number of conformations has been estimated by thermodynamic procedures to be around 10^{22} accessible configurations (65) and on theoretical consideration alone, there might be as many as 10^{129} different conformations for an enzyme like lysozyme (97). Any ordering of the otherwise flexible peptide chains resulting from the formation of hydrogen bonds between the carbonyl oxygens and the amide nitrogen atoms of the polypeptide backbone is termed secondary structure. These hydrogen bond formations fall into two broad classes: helical structures and sheet structures (96). Generally, proteins that contain sheet structures are fibrous and insoluble in aqueous solvents. Thus, when studying enzymes this thesis is concerned principally with helical structures and the methods for their detection and measurement. Three techniques are used to estimate helical content in proteins: infrared spectroscopy and dichroism, optical rotatory dispersion and hydrogen-deuterium or hydrogen-tritium exchange.

Polypeptides exhibit characteristic absorption bands in their infrared spectra. The position of the amide I and amide II bands is altered during the transition of a randomly coiled molecule to an α -helix as a result of vibrational interaction among peptide groups in the chains. The measurement of infrared dichroism exploits the selective

absorption of polarized infrared of oriented solid samples when the transition moment of the concerned vibration is parallel to the electric vector of the light. Only recently have a number of globular proteins been examined in aqueous (H_2O and D_2O) solutions (139).

Optical rotatory dispersion is the most widely employed method of estimating helical regions of proteins. Optical rotatory dispersion is the variation of optical rotation at various wavelengths of monochromatic light. The rotatory power is a consequence of molecular asymmetry. Model polypeptides have provided convincing evidence that the dispersion curves are a sensitive function of secondary structure (142). Results with native proteins have been successful but uncertainties in the calculation of helical content are introduced by the lack of a theoretical basis for the Moffitt-Yang equation, side chain interactions, the possible presence of ordered structures other than α -helix or random coil and the possibility that a protein may contain segments of right-handed and left-handed α -helix. Optical rotatory properties of some enzymes are listed in Table 1.

Table 1. Optical Rotatory Properties and Hydrogen-Deuterium Exchange as an Index of Partial Helical Content in Native Enzymes.

Enzyme	Estimated Percent Right-handed Helix [‡]	Ref	Percent HEAH*	Ref	Percent Helix [‡]	Ref
Lysozyme	30	(8,140)	45	(11)	40	(10)
Chymotrypsin	22	(14,33)	43	(11)	--	--
RNase	15	(57)	35	(11)	10	(69)
Trypsin	--	--	14	(11)	--	--

[‡] Calculated from the Moffitt parameter, b_0 , of optical rotatory data

* HEAH stands for hard to exchange amide hydrogen

+ based on X-ray structure of crystalline enzymes

Hydrogen-deuterium exchange measurements estimate the helicity of polypeptides by quantitatively measuring the exchange of labile protons bonded to atoms that have unshared electron pairs, such as oxygen, nitrogen, and sulphur. Although ordinary exchange of these atoms with the protons of the solvent is very rapid the hydrogen-bonded protons of the α -helix are protected from solvent and exchange slowly. Some data on the hard-to-exchange hydrogens of several enzymes are presented in Table 1.

Exchange of protons of polypeptides may be followed in several ways. The release of D_2O can be followed by density measurements of the solvent or by infrared spectrophotometry. Tritium may be used in place of deuterium and has the advantage that it can be readily measured by liquid scintillation counting. Estimation of the percent of residues involved in α -helical structures by the hydrogen-deuterium exchange method does not always give satisfactory results. Sources of uncertainty in H-D exchange measurements include the fact that: 1) several classes of exchangeable protons are usually found, 2) possible thermal perturbation of the protein may lead to transient exposure of HEAH to the solvent, 3) structure other than α -helix may also bury certain protons in the interior and, hence, prevent their exchange and 4) helical regions near the protein surface may still exchange rather rapidly.

Tertiary structure includes the superfolding of helical segments of a polypeptide chain into a rather tight, compact structure, owing to interactions between the side chains of the various amino acid residues.

Such interactions might include hydrogen bond formation, hydrophobic interactions, charge transfer forces, salt links, and so on. Such superfolding usually results in long helical regions being broken up into shorter helical segments interspersed with segments of flexible random coils. The alteration of the specific tertiary structure of enzymes usually results in the loss of enzymatic activity. The most direct approach to the understanding of the tertiary structure of enzyme is by x-ray diffraction and electron microscopy. Presently only two enzymes have been examined by x-ray diffraction: lysozyme (10) and RNase (69). Studies on chymotrypsinogen are in progress, but the technique is very tedious and requires sophisticated equipment. Because analysis is limited to solid crystals of the enzyme, there is the unresolved question as to whether protein structures derived from x-ray diffraction measurements are closely related to those for the enzyme in aqueous solution.

The alterations of secondary and tertiary structure of proteins, including denaturation, have been the subject of intense interest to protein chemists. The value of such studies for understanding the structures of proteins and the possible role of transconformation reactions in enzyme catalysis account for some of this interest. With respect to the enzyme being a highly and specifically folded structure, we wish to get detailed information concerning the nature of such structures and to account for the inactivation of the enzyme by photodynamic action in terms of this tertiary structure. Kauzmann has defined protein denaturation as "a process in which the spatial arrangements of the polypeptide chains within the molecule is changed from that

typical of the native protein to a more disordered arrangement" (70).

A partial summary of parameters whose variations have been taken as measures of protein denaturation is presented in Table 2. The properties underlined were studied in this thesis.

Table 2. Methods for Measuring Enzyme Denaturation*

I. Shape properties of proteins

A. Hydrodynamic properties

1. Viscosity
2. Sedimentation--Diffusion
3. Specific volume
4. Molecular sieve
5. Fluorescence-polarization spectra of labeled proteins

B. Radiation scattering

1. Light scattering
2. X-ray diffraction
3. Electron microscopy

II. Short-range properties of proteins

A. Topography

1. Reactivity to chemical reagents
2. Electrophoresis
3. Ion-exchange chromatography
4. Protease digestibility
5. Solvent perturbation of U.V. spectra
6. Immunological reactivity
7. Binding of small molecules
8. Biological activity
9. H-D exchange

B. Thermodynamic properties

1. Differential thermal analysis
2. Thermodenaturation
3. Unfolding in 8 M urea
4. Solubility

C. Environmental conditions

1. Visible and ultraviolet absorption
2. Protein fluorescence and intrinsic polarization
3. Optical rotation and circular dichroism
4. Infrared absorbance and dichroism
5. High-frequency nuclear magnetic resonance

6. Extrinsic chemical reporters
7. Spectrophotometric titration

* The underlined properties were studied in this thesis.

The parameters used to study denaturation (listed in Table 2) often have limitations. Hydrodynamic properties are generally insensitive to small changes in enzyme conformation such as might occur upon interaction with substrate or the destruction of an amino acid by photo-oxidation. Another limitation is that some methods require high concentrations of protein in order to permit measurements. Finally, results of studies of denaturation are very frequently a function of the type of measurements that are made (46). Despite these limitations it should be possible to select from Table 2 parameters that have the necessary sensitivities. One need not be concerned as much with the molecular interpretation of the observed parameters when trying to describe the functional properties of the proteins as in the correlation of observables of any kind with the biological activity of the protein.

Attention is focused now upon those parameters which are employed in the research section of this thesis.

Viscosity. The ability of a protein solution to flow through a capillary tube depends upon the shape and size of the protein molecule. An increase in viscosity is a reflection of an increase in molecular volume by aggregation or asymmetry due to unfolding.

Gamma irradiation of dry RNase (37) and solutions of RNase (120) causes an increase in the viscosity of solutions of the enzyme. There is almost a direct correlation between the rate of loss of enzyme

activity and the rate of loss of structural integrity as measured by hydrogen-tritium exchange and viscosity. The denaturation of bacterial amylase by high pressure has been studied (90). The viscosity increases as the enzyme is inactivated by pressures of 9500 kg/cm^2 and decreases when reversibly denatured amylase is allowed to reactivate at atmospheric pressure. Viscosity increases are also observed upon photodynamic inactivation of lysozyme (155). In contrast, viscosity does not change when RNase or chymotrypsin is photooxidized (156, 157).

Chromatography through molecular sieves. The most useful molecular sieves for sorting out proteins according to size are certain polymeric carbohydrates or poly-acrylamides which are available in a variety of pore sizes. Commercially they are called Sephadex or Biogel. This procedure for separating different proteins is very popular mainly because of the ease and gentleness of the procedure.

Elution peaks uniquely different from those of native enzyme are observed when ultraviolet inactivated carboxypeptidase, lysozyme or chymotrypsin is filtered through a Sephadex column (99). Photooxidized RNase-S comes off a column of Sephadex in the same elution volume as native RNase-S (72). RNase inactivated by gamma irradiation in nitrogen separates into several peaks upon chromatography on Sephadex (67). These aggregated components still retain appreciable enzyme activity. Irradiation in air yields two components; one equivalent to the native enzyme, the other is an aggregate containing no enzyme activity. The elution pattern of photooxidized aspartate transaminase on a column of Sephadex G-200 is identical with that of the native enzyme (84).

Because of secondary and tertiary folding of enzymes there are a variety of states of each amino acid in proteins. Some of the residues are hydrogen-bonded or linked by hydrophobic bonds and some others are embedded in the interior of the molecules. These bound residues are different in physico-chemical and chemical properties from exposed residues. Any change in these properties would reflect a difference in the state of the residues compared to the native enzyme. Some topographical parameters sensitive to changes in the various states of certain residues are listed below.

Reactivity to chemical reagents. The reactivities of tyrosine, histidine and tryptophan residues in α -chymotrypsin, trypsin and lysozyme have been analyzed by measuring their reactivities with increasing concentrations of cyanuric fluoride diazonium-1-H-tetrazole and H_2O_2 -dioxane, respectively. As is expected, while all of the residues in alkali-denatured proteins react rapidly at low concentrations of the reagents, the native enzymes show variable reactivity, depending upon the reagent concentration (51,52). N-bromosuccinimide, a specific oxidant of tryptophan, has much slower rates of reaction with native lysozyme than with the denatured protein (103). Koshland's reagent, 2-hydroxy-5-nitrobenzyl bromide, shows increased reactivity with lysozyme as the enzyme is progressively denatured in increasing concentrations of 2-chloroethanol (7). Acetylimidazole reacts with a number of tyrosyl residues in trypsin and RNase. The stoichiometry correlates closely to the number of "free" residues measured by spectrophotometric titration. Urea denaturation of trypsin and RNase exposes the "buried" tyrosyl residues to the reagent (108,124).

Electrophoresis. A change in the surface charge distribution of a protein can be detected by an altered mobility under the influence of an external electric field. Two electrophoretic techniques are commonly employed in the study of proteins: the moving boundary type and the zone type. We will consider only the much simpler zone electrophoresis which is distinguished from moving-boundary electrophoresis principally in that the former involves the use of a support on which the material moves, while in the latter the material moves freely in solution. Typical supports included paper, cellulose acetate, starch gels, polyurethane foam and polyacrylamide gels. The gel media also simultaneously separate proteins according to size by molecular sieving. In zone electrophoresis, the protein to be examined is initially applied to the support medium, separated with the electric field and finally the protein is detected by staining or by some histological enzyme activity test.

Photooxidized trypsin sensitized with MeB or EoY has electrophoretic bands identical to those of native trypsin when subjected to (44) electrophoresis on polyacrylamide gel. While there are still no new bands when FMN is used as a sensitizer, the bands lose their ability to be stained with amido black dye as the enzyme is photodynamically inactivated. When pressure-denatured bacterial amylase is electrophoresed the mobility of the single band is slower than that of the native enzyme (90).

Ion-exchange chromatography. Denatured proteins may be separated by column chromatography on ion-exchange resins if the charge distribution is different from that of the native enzyme. Partially denatured

trypsin shows a complex elution pattern upon chromatography on Amberlite IRC-50 with phosphate buffer-8 M urea (22). Partially photodynamically inactivated RNase-S separates into three bands when chromatographed on Amberlite IRC-50 (72). One band is inactive, the other two bands are enzymatically active. Column chromatography of RNase photooxidized to 20% activity on IRC-50 elutes faster than native enzyme (147). The elution pattern consists of at least three fractions, all of which possess a small amount of enzymatic activity.

Protease digestibility. An enzyme in the native configuration is usually resistant to proteolytic digestion by trypsin or subtilisin (77). If the enzyme is in a non-native configuration it is readily digested. The all-or-none nature of this reaction makes it an excellent way to quantitatively distinguish the denatured state of a protein from the native state, independent of the degree of denaturation (94). The digestion may be followed by measuring the increase in trichloroacetic acid (TCA) soluble peptides or the increase in ninhydrin positive groups.

While only a small portion of native bacterial amylase incubated with subtilisin, trypsin and chymotrypsin in neutral pH is digested, urea-denatured amylase was rapidly digested with all proteinases (94). The extent of digestion of photooxidized myoglobin is 2.5 times that of the native protein, but only one-tenth that of urea-denatured myoglobin (111). The tryptic digestibility of photooxidized glyceraldehyde-3-phosphate dehydrogenase proceeds rapidly (38). Maximum digestion is reached in about half the time necessary for complete inactivation. The rate of tryptic hydrolysis of ultraviolet irradiated lysozyme is considerably slower than the loss of enzymatic activity (35). Ribonuclease

is digested by trypsin after gamma irradiation (125). Proteolysis of samples irradiated to 18% original activity is about half of that observed for the digestion of chemically reduced RNase.

Immunological reactivity. An antibody, synthesized in an animal following the injection of a purified protein, can identify specific groupings of amino acid residues found on the surface of the specifically folded chain. Antigen-antibody reactions are critically dependent on the conformation of the antigenic combining site in globular proteins. When the antibody is brought in contact with the native antigenic protein a precipitin reaction may occur.

Anti-RNase serum will not react with a drastically altered protein molecule having its disulfide bonds randomized during reduction and reoxidation (89) but will react with RNase-S, an active derivative of RNase where amino acid residues 1 through 20 have been removed (122). Heat and exposure to urea, however, can abolish the reactivity of RNase-S with antiRNase serum (87). Heat and alkali also reduce the immunological reactivities of pepsin and pepsinogen toward their antisera (41). Photodynamic inactivation apparently does not alter the immune response of proteins. Neither photooxidized botulinum type A toxin (158) or trypsin (45) sensitized with methylene blue show reduced combining power with their antibodies as compared to the original proteins. Furthermore, antibodies prepared to photoinactivated snake venom will protect mice against certain toxic actions of the active venom (73).

Thermodenaturation. When most enzymes are heated over the range of 20° to 100° C a conformational shift to a different structure

energetically favored under the new conditions will occur in a rather narrow temperature range. This heat perturbation can be followed by the loss of enzyme activity, an increase in intrinsic viscosity, a shift in optical rotatory dispersion parameters, spectroscopic and titration changes associated with the normalization of previously buried tyrosine residues, complete and rapid hydrogen-deuterium exchange and an increased susceptibility to proteolytic degradation. The temperature of the midpoint of this reversible transition (T_m) can be progressively shifted to lower temperatures either by decreasing the pH (15,56), or by increasing concentrations of urea (15,36,118), lithium bromide (81) or alcohols (114) and, may be shifted either up or down by increasing concentrations of various neutral salts (47,146). The gamma radiation damage to chymotrypsin decreases the net change in absorbancy of the heat transition (135) and the T_m is lowered as RNase is inactivated with monochromatic ultraviolet light of 254 nm (91). The U.V. damaged RNase also loses its ability to renature when cooled.

Unfolding in 8 M urea. When some enzymes are placed in 8 M urea, a time dependent denaturation process can be detected by reduced viscosity, ultraviolet absorbance, optical rotation, or protein fluorescence. The latter parameter is preferred because minimum protein concentrations are 10 fold lower than those needed for the other physical criteria. The kinetics of the unfolding are strongly influenced by the temperature, pH and ionic environment (19,29). Time dependent unfolding transitions in 8 M urea have been studied with amylase (141), carboxypeptidase (102) chymotrypsin (83), lysozyme (53,134), pepsinogen (29), and RNase (6,23, 93). The kinetics of unfolding reactions are usually first order.

To date, the unfolding of enzymes in 8 M urea has not been used as a technique to study other denaturing processes.

Solubility. Often the denatured state of a protein can be detected by a large decrease in its solubility. This criterion is a very convenient one, but is only available as a qualitative method because few proteins become completely insoluble on denaturation. The thermodenaturation of chymotrypsin can be followed by the production of an insoluble enzyme fraction at pH values near its isoelectric point (9). Photodynamic denaturation of ovalbumin (4) and lysozyme (155) results in a decreased solubility of the proteins at their isoelectric point. In contrast, no such precipitation is observed with photooxidized chymotrypsin (156). The rate of precipitation is much slower than the rate of loss of enzymatic activity. When RNase, trypsin or chymotrypsinogen are irradiated in the dry state with 7-kev electrons they complex with RNA to form turbid solutions (92). Dry heating also alters the structure of these proteins in such a manner that they increase the turbidity of RNA solutions.

Protein fluorescence and intrinsic polarization. Fluorescence measurements as an analytical tool in the ultraviolet range is just coming into general use. The sensitivity of fluorescence detection is much greater than colorimetry for measuring concentrations of substrates. Proteins show a characteristic intrinsic fluorescence if they absorb in the far ultraviolet region. The globular proteins can be divided into 2 classes on the basis of their fluorescence (152). One class of proteins contains tyrosine but no tryptophan, and the fluorescence spectrum is

characteristic of tyrosine, with a maximum at 303 nm. The second class contains tryptophan as well as tyrosine. In these proteins only one fluorescence peak at 350 nm is observed, that due to tryptophan. In a denaturing medium such as 8 M urea, the fluorescence quantum yields of trypsin and chymotrypsin are increased, while values for RNase are unchanged and decrease for lysozyme (138). The fluorescence efficiency of the fluorescent residues within proteins is strongly influenced by the nature of the immediate environment (134) but is not influenced by the extent of α -helix content (34). Thus fluorescence difference can be used as an indication of tertiary structural changes. The polarization of the fluorescence of the proteins, unlike the effects observed in fluorescent protein conjugates, is not sensitive to the viscosity of the medium because of the relative short lifetime of the excited state of tryptophan. Because of this, no information on the rotational diffusion of the protein (a shape sensitive parameter) may be expected. However, if the fluorescence polarization is recorded as a function of the wavelength of exciting light, changes in the number and relative orientation of the transitional moments of the absorbing chromophores and the extent of local freedom of rotation of the tryptophan residues can be followed (153).

The quantum yields of the tryptophan fluorescence of proteins are often below that of free tryptophan (138). As would be expected, unfolding of the protein by urea, reduction or other means tends to increase the quantum yield of protein fluorescence and shifts the maxima of fluorescence spectra to the red region (20,134).

Photodynamic inactivation of amino acid oxidase causes an enhanced tyrosine fluorescence at 303 nm and a reduced tryptophan fluorescence at 348 nm. RNase shows a marked depression of tyrosine fluorescence at 303 nm with gamma irradiation (86). When 84% of the enzyme activity is destroyed there is a 62 % decrease in fluorescence. The fluorescence intensity of trypsin decreases after treatment with atomic hydrogen. Presumably the decrease in fluorescence is due to the destruction of tryptophan residues.

The effect of high concentrations of urea on the polarization of protein fluorescence has been studied using several enzymes including pepsin, chymotrypsin, lactic dehydrogenase, lysozyme and yeast enolase (16,15). In 8 M urea the two polarization excitation bands at 270 and 305 nm persist but are much lower than in water solutions. The decrease in the 305 nm maximum is more pronounced. When urea is removed by dialysis, lactic dehydrogenase and chymotrypsinogen do not recover the polarization spectrum characteristics of the native protein.

Altered but active enzymes. Not all enzymes having altered primary, secondary and/or tertiary structure need lose their biological structure. A classic example is the demonstration that approximately 120 of the 180 amino acid residues of papain can be removed by means of aminopeptidase without loss of enzymatic activity (58). Thus an enzyme may still have an intact catalytic site but be abnormal by other criteria. The abnormal enzyme may have altered enzyme kinetics, unique physicochemical properties or enzymatic activity unusually sensitive to perturbants. Table 3 presents an incomplete

summary of measurements used to detect altered but active enzyme fractions in a partially inactivated reaction mixture. The underlined methods are used in the experimental portion of this thesis.

Table 3. Methods for detection of altered but active enzyme

Method	Reference
<u>Thermosensitivity</u>	(109)
<u>Sensitivity to digestion</u>	(82)
<u>Urea sensitivity</u>	(42)
<u>Salt sensitivity</u>	(149)
Sensitivity to specific reagents	(110)
Reduction-reoxidation sensitivity	(54)
<u>Active site titration</u>	(74)
Increased specific activity	(99)
Altered binding constants	(161)
Altered esterase--peptidase ratios	(143)
<u>Chromatography</u>	(72)

* Methods being used in the experimental portion of this thesis.

11. STATEMENT OF THE PROBLEM

It is generally agreed that only a small number of the amino acid side chains participate directly in the catalytic region of the enzyme. A much larger portion of the hundred or so amino acids found in the linear amino acid chain participate in the formation of secondary and tertiary bonding. The protein chain is folded in a specific manner forming an enzyme having a characteristic catalytic function.

Photodynamic action of enzymes almost invariably destroys the catalytic activity and, in the process, only a few types of amino acids are photooxidized. Some of these amino acids are critically involved in the accompanying destruction of enzyme activity. These observations bring up the question: What are the changes induced by photodynamic action which produce a loss in the characteristic function of an enzyme?

The loss of catalytic activity can occur by two mechanisms: the chemical alteration of amino acid side chains in the active site, or by disruption of the essential enzyme configuration. Several investigators have accepted the first mechanism to interpret their experimental results without making an effort to critically eliminate the second mechanism (3,5,121,136). To date, no photodynamically inactivated enzyme has been examined primarily for changes in conformation. In the case of the few biophysical measurements reported, the techniques used to attempt to detect shape change often lacked the precision necessary to detect subtle modifications in enzyme conformation.

This thesis is devoted largely to an examination of the effects of photodynamic inactivation on the configuration of four crystalline enzymes: ribonuclease (RNase), lysozyme, trypsin and chymotrypsin. Two approaches were used to investigate mechanisms of photodynamic action not involving the active site. The direct approach was to measure conformation sensitive parameters such as protein fluorescence and immunological reactivity. The indirect approach was to detect active enzyme molecules that have altered biophysical properties. Although the observed effects of any single conformation sensitive measurement usually cannot be unequivocally interpreted, its correlation with other structural properties of the enzyme should form a powerful tool to understanding the mechanism of photodynamic inactivation.

111. MATERIALS AND METHODS

A. Reagents

Trypsin stock solution: 40 mg of 2X recrystallized, salt-free trypsin (Worthington Biochemical Corporation, Lot TRSF 6403) was dissolved in 3 ml of 0.01 N HCl and then made up to 10 ml with 0.01 M NaH_2PO_4 . This 164 μM (4 mg/ml) solution was kept at 4° C for two or three weeks without losing enzymatic activity.

Other enzymes: The following enzymes were used directly from the bottle: -Chymotrypsin, 3X crystallized, (Worthington Biochemical Corp., Lot CDI 6146-7); bovine pancreas ribonuclease, 5X crystallized (Mann Research Laboratories, Inc., Lot N 1027); and egg white lysozyme, 3X crystallized (Mann Research Laboratories, Inc., Lot N 1855).

Benzoylarginine ethyl ester (BAEE) substrate for trypsin: 10.0 ml of 0.5 M sodium phosphate buffer, pH 8 (see below) was added to 10 mg of BAEE (Mann Research Laboratories, Inc.). The solution was made up to 100 ml with distilled water and stored at 4° C. This solution could be used for about 2 weeks. If the solution was also to be 5 M in urea, 30 grams of urea recrystallized from hot 95% ethanol was added prior to adding the distilled water.

N-Acetyl-L-tyrosine ethyl ester (ATEE) substrate for chymotrypsin: 32 mg of ATEE (Mann Research Laboratories, Inc.) was added to 100 ml of 0.05 M sodium phosphate buffer, pH 7.0. This material was slow to dissolve. It was stirred at room temperature for 2-3 hours and stored at 4° C.

Cytidine 2'',3''-cyclic phosphate (C23P) substrate for ribonuclease:

20 mg of C23P (Schwartz Bioresearch, Inc.) was dissolved in 100 ml of 0.1 M sodium acetate buffer, pH 5.0. The acetate buffer was made adding about 212 mls of 0.1 M sodium acetate (3.0 g $\text{NaAc} \cdot 3\text{H}_2\text{O}$ /220 ml H_2O) to a mixture of 86.5 ml H_2O and 0.5 ml of glacial acetic acid. It was stored at 4° C and used within 2 weeks.

Micrococcus lysodeikticus substrate for lysozyme: 15 mg of

lyophilized M. Lysodeikticus (Mann Research Laboratories, Inc.) was suspended in 100 ml of 0.1 M sodium phosphate buffer, pH 7.0. It was stored at 4° C and the bacteria were resuspended by shaking just before use. 0.1 M phosphate buffer, pH 7.0 was made by adding 15.5 ml of 0.5 M Na_2HPO_4 solution and 9.0 ml of 0.5 M NaH_2PO_4 solution to 95 ml of distilled water.

0.5 M phosphate buffer, pH 8.0: 0.30 g $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ and 6.7 g Na_2HPO_4 were dissolved in 95 ml of distilled water.

Dye stock solutions: Methylene blue (California Biochemical Corp. No. 4605) and Eosin Y (Allied Chemical Certificate NE37) were prepared at 5×10^{-5} M and flavin mononucleotide (FMN) (Nutritional Biochemical Corp.) was prepared at 6×10^{-5} M.

B. Enzyme Assays

Kinetic assays for the four enzymes were performed on the Cary 14 recording spectrophotometer or, occasionally, on the Beckman DB spectrophotometer equipped with a Sargent SLR recorder. Except for lysozyme, where distilled water was used, the reference consisted of the substrate solution without added enzyme. The following tabulation gives the

necessary parameters for the assays as carried out in a 4 ml 1 x 1 cm quartz cuvette:

Enzyme and final concentration	Substrate (ml)	Enzyme (nl)	Wavelength setting (nm)	Direction of absorbance change	Chart speed (in/min)	Assay reference
Chymotrypsin (4 mg/ml)	3.0	10	237	↓	2	(115)
Trypsin (1 mg/ml)	3.0	10	253	↑	1	(115)
Lysozyme (5 mg/ml)	3.0	10	450	↓	1	(120)
RNase (5 mg/ml)	2.0	100	280	↑	1	(25)

An assay would consist of adding the enzyme to the cuvette, stirring rapidly and, after closing the cuvette compartment, activating the spectrophotometer and recorder. Slopes are calculated from the recorded plot of absorbancy versus time. Initial slopes must be estimated for non-linear recordings of chymotrypsin and RNase. The slopes are a direct measure of the enzymatic activity of the enzyme solution since constructed standard curves were linear for all four enzymes.

C. Reaction Mixtures

Typical 10 ml reaction mixtures consisted of enzyme, water, buffer and dye as tabulated below. The final enzyme concentration is also listed below.

	-Chymotrypsin	Trypsin	Lysozyme	RNase
Enzyme	40 mg	2.5 ml*	5.0 ml*	50 mg
H ₂ O	5.0 ml	2.5 ml	--	5.0 ml
Phos. buffer pH 8	2.5 ml	2.5 ml	2.5 ml	2.5 ml
Stock dye	2.5 ml	2.5 ml	2.5 ml	2.5 ml
Final enz. conc. (mg/ml)	4.0	1.0	5.0	5.0

*From stock solution of 168 nM trypsin or 1% lysozyme.

D. Irradiation Procedures

Reaction mixtures were prepared in clear 10 dram plastic vials (Lerner Plastics, Inc.) and placed in a special, glass bottom, water bath maintained at a constant temperature of 15° C. The solution was illuminated with a 150-watt projector floodlight underneath the water bath. The light path from the floodlight to the bottom of the vial was about 5 inches. The reaction vials were gently shaken during illumination at about 1 cycle per second. Controls were enzyme solutions either exposed to the light in the absence of dye or kept in the dark in the presence of dye. These controls established that no extraneous inactivation of the enzyme solutions occurred during the normal time course of photoinactivation which ran up to 2 hours.

When FMN was used as sensitizer another apparatus with higher light intensity was used. The reaction solution was placed in a water jacketed, 1.0 ml capacity plastic reaction cell mounted on an optical

bench. The reaction mixture was illuminated with a water cooled G.E. A-H6 high pressure mercury lamp. A glass plate was placed between the light source and the reaction mixture to filter out ultraviolet radiation. The plastic reaction cell was stirred with a motor driven vibrating plastic-coated wire. Only when the solution was stirred would the time course of inactivation proceed with first-order kinetics. Again the temperature was maintained at 15° C. The time-course of enzyme inactivation was determined by removing samples of the reaction mixture after successive periods of illumination and assaying for enzymatic activity.

E. Ion Exchange Chromatography

Procedures reported by Cole and Mendiola (22) were used for ion-exchange chromatography of photodynamically treated trypsin in buffers containing urea. Ion exchange chromatography of ribonuclease was by the method of Hirs et al. (59).

1. Preparation of 0.2 M phosphate buffer-urea: 1056 g of reagent grade urea was dissolved in 2.20 liters of distilled water. To deionize the urea, it was passed through a 200 ml column of Amberlite MB-1 resin. To two liters of the 8 M urea 35.8 grams of $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ was added and 3.70 g of Na_2HPO_4 added to the remaining 200 ml of 8 M urea solution. About 160 mls of the latter solution was added to the 2 liters to get pH 6.10. The solution was stored at 4° C. Eluent buffer for chromatography of ribonuclease was prepared by adding 18 g $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ and 10 g Na_2HPO_4 to one liter of distilled water. This 0.2 M buffer had a final pH of 6.2 and was stored at 4° C.

2. Preparation of column: Amberlite cation exchange CG-50 resin (200-400 mesh) was changed to the sodium form by adding about 100 ml (wet volume) of resin to 1 N NaOH with stirring. The resin was rinsed with distilled water and soaked in 4% HCl for 15 minutes, washed, and resoaked in 4% HCl for 30 minutes. This cycle was repeated three more times and finally the resin was washed very thoroughly with distilled water. Either 100 ml buffer or buffer-urea solution was added to the resin and enough 50% NaOH was added to maintain a pH of 6.0. When the rate of pH change slowed down, the buffer was replaced with fresh buffer. A 0.9 x 30 cm column of resin was poured and several column volumes of buffer or buffer-urea run through it.

3. Chromatography of the enzyme: A solution of photooxidized trypsin or ribonuclease containing 10-15 mg protein in 0.01 M phosphate buffer, pH 8.0 was put on top of the column. Elution of the enzyme with buffer or buffer-urea proceeded overnight with a flow rate of 3-5 ml per hour. Collecting one ml of effluent per tube, the protein peaks came off the column between tubes 10 and 50. The chromatography was carried out at 4° C in the coldroom.

F. Cellulose Acetate Electrophoresis

A Colab universal electrophoresis apparatus was used. Samples of photooxidized RNase were applied to the middle of 2 1/2 x 12 cm cellulose acetate strips. The strips were electrophoresed at 200 volts, 1 m.a. per strip at room temperature for 2 hours in sodium barbital buffer pH 8. The strip was warmed over a hot plate to fix the protein and

then stained with 0.02% Nigrosin dye over night. Excess dye was removed with a 5% acetic acid wash.

G. Gel Chromatography

The enzymes were filtered through a 0.9 x 30 cm column of Sephadex G-75 or Biogel P-60. The gel was equilibrated and eluted with 0.125 M sodium phosphate buffer, pH 7.0, in 8 M urea.

H. Immunology Tests

1. Preparation of antisera: Antisera were prepared in rabbits employing complete Freund's adjuvant with added Mycobacterium butylicum. To 0.5 ml adjuvant, 0.5 ml normal saline containing 5 mg RNase was slowly added until a stable emulsion was obtained. This preparation was injected into a toe pad in each of the four extremities (0.2 ml) and the final 0.2 ml injected subcutaneously. In addition, 0.5 ml of adjuvant containing 1 mg of RNase was given subcutaneously twice a week for three weeks. On the fourth through the sixth week after immunization serum was combined, preserved with 1:10,000 Merthiolate and stored in the freezer.
2. Double diffusion in an agar plate: (0.8% in 0.9% saline) was by the method of Ouchterlony (95). All antigens were added to their respective wells at concentration of 1.0 mg/ml and undiluted antiserum was added to the center antibody well.
3. Quantitative precipitin curves and the amount of protein in the precipitate were determined by the method of Kalbat and Mayer (68), employing 0.25 N acetic acid to dissolve precipitates and measure the

absorbance at a wavelength of 280 nm. In performing the test, 0.25 ml of undiluted antisera was mixed with 0.75 ml of saline. The amount of antigen needed to give maximum precipitation (equivalence point) was determined from the absorbance of a series of dilutions of RNase. Ten lambda of 5 mg/ml RNase per tube was close to the equivalence point for the system.

I. Protein Digestibility

The all-or-none assay reported by Okunuki et al. (94) was used. Fifty ul of subtilisin (1 mg/ml water) was added to 1.0 ml of reaction mixture (5 mg/ml lysozyme) and incubated at room temperature for 40 minutes. The digestion was stopped by adding 2.0 ml of cold 10% TCA and stored at 4° C for 1 hour. The precipitate was centrifuged to the bottom of the tube in a clinical centrifuge and 1 ml of the supernatant decanted into a cuvette and its absorbancy determined in a Beckman DB Spectrophotometer at 278 nm. The "blank" consisted of a zero time sample of lysozyme incubated for 40 minutes before TCA was added. Then 50 ul of subtilisin was added.

J. Fluorescence Polarization

An Aminco spectrophotofluorometer equipped with a pair of Glan Thompson prism polarizers was used to measure fluorescence polarization of the protein. Fluorescence intensity readings were made at the settings EE, BE, EB and BB using a 1 x 1 cm cell. The first letter corresponds to the position of the polarizers on the excitation side

and the last letter, the setting of the emission side. The excitation wavelength was increased in 5 nm steps scanning from 215 to 315 nm. The following slit arrangements were used on the excitation side (see instruction manual of the Aminco spectrophotofluorometer): arrangement no. 3 was used until the settings were over 100% transmittance with the meter multiplier setting at 0.001. Then slit arrangement #2 was installed and the scanning continued to 315 nm. The emission side had slit arrangement number 5 and included a Corning filter 0-54 to filter out scattered ultraviolet light. For maximum sensitivity the EE or EB setting, whichever was greater, was set at 100% T by adjusting the photomultiplier scale before the other settings were recorded. Stray light from the solvent-dye solution alone was subtracted from the fluorescence intensity of the reaction mixture. While MeB and EoY in the solutions did not interfere with the measurements, FMN fluoresced too strongly to be used as a sensitizer. The following formula was used to calculate the polarization (101):

$$P = \frac{I_{EE} - I_{EB} (I_{BE} / I_{BB})}{I_{EE} + I_{EB} (I_{BE} / I_{BB})}$$

where I_{EE} , I_{EB} , I_{BE} , and I_{BB} were the fluorescence intensities at polarizer settings EE, EB, BE and BB respectively. As a test of technique, the polarization spectrum of the fluorescence of Rhodamine B in 90% glycerol was measured as illustrated in Fig. 1. The spectrum was in good agreement with measurements made by others (27,150).

Figure 1. Polarization spectrum of the fluorescence of 2×10^{-5} M Rhodamine B in 90% glycerol.

K. 8 M Urea Fluorescence Transition

An Aminco spectrophotofluorometer was used to follow the change of protein fluorescence after the solution was made 8 M in urea. The output of the phototube amplifier was connected to a Brown recorder having a scan speed of 2 in/min. The reaction was initiated by rapidly adding 1 ml of 8.4 M urea in 0.125 M sodium phosphate buffer to a spectrophotofluorometer (SPF) cuvette containing 100 μ l of enzyme solution. Then the shutter was opened and the chart drive turned on. The fluorescence transition was recorded until the fluorescence intensity reached a maximum or, with very slow transitions, after 70-80% of the fluorescence increase had taken place. Excitation was at 290 nm and emission recorded at 254 nm. Slit arrangement #2 (see Aminco instruction manual) was used on the excitation side and slit arrangement #5 on the emission side of the SPF. The wavelength settings on the spectrophotofluorometer were calibrated from the emission lines of a pencil mercury lamp placed in the cuvette holder. Temperature was controlled to $\pm 0.1^\circ \text{C}$ with a water jacket around the cuvette holder. While MeB and EoY in the solutions did not interfere with the measurements, FMN fluoresced too strongly to be used as a sensitizer.

L. Protein Concentration Determinations

Protein eluted from chromatography experiments was followed by the colorimetric method of Lowery et al. (78) or, more directly, by the ultraviolet absorption maxima of the enzyme.

M. Tryptophan Assay

The method of Spies and Chambers (129) can measure the tryptophan content of intact proteins. To a 0.5 ml aliquot of enzyme solution (4-10 mg/ml), 5.0 ml of DMABA in sulfuric acid was added and mixed. After incubating overnight in the dark (12 hr) at room temperature, 0.1 ml of 0.04% NaNO_2 was added and mixed. After 0.5 hours at room temperature, the absorbancy at 590 nm was measured. A series of controls were run to determine the limits and base-line of this assay. Blanks containing protein and all reagents except p-dimethy-aminobenzaldehyde (DMADA) showed a small amount of color development and was preferred over the other logical choice of a blank, the complete tryptophan reagent without protein. This latter blank was always colorless. It was found that light inhibits the development of color in the assay. If the color development took place under ordinary room lighting the final color was reduced by about 20% compared to an identical experiment developed in darkness. Finally and most important, when native RNase was assayed for tryptophan content at the same concentration as the trypsin solutions, no color was found. This was expected because RNase has no tryptophan residues. However, after prolonged photooxidation, the RNase sample gave about 10% of the color value of native trypsin. Chromogenic materials detectable by the Spies and Chambers assay apparently are produced during photodynamic inactivation of proteins.

Tryptophan reagent was a mixture of 59 ml 23 N H_2SO_4 and 6 ml of a solution of DMABA (2.78 ml conc. H_2SO_4 added to 97 ml H_2O and 1.5 g DMABA).

N. Oxidation of Tryptophan Residues With H_2O_2 -Dioxane

Following a technique worked out by Hachimori et al. (51), 1.0 ml of the enzyme, 1.4 ml distilled water, 0.3 ml of fresh 1.0 M $NaHCO_3$ buffer, pH 8.0 and 0.3 ml of dioxane were placed in a test tube. Then up to 0.18 ml of 0.3% H_2O_2 solution (zero to 6.0×10^{-3} M H_2O_2 , final concentration) was added to the series of tubes and the final volume adjusted to 3.2 ml with 0.12 to 0.30 ml of dioxane. Experimentally, the H_2O_2 solution was the last thing added because this initiated the oxidation reaction. The effect of incubation time on the reactivity of tryptophan residues in 22.4 μ M trypsin was studied. At 4° C, an incubation period of 6 hours was necessary to complete the reaction of 6 μ M H_2O_2 or less with the tryptophan residues. This incubation period was considerably longer than the 2.5 hour incubation used by Hachimori et al. At lower enzyme concentrations the minimal reaction time decreased somewhat. Nevertheless all reaction mixtures were incubated 6 hours to assure complete oxidation of all reactive groups. In some experiments photo-oxidized enzyme was studied. At 1.0 M sodium bicarbonate buffer, pH 8.0, must be substituted for the usual 0.125 M phosphate buffer in photooxidation reaction mixtures because phosphate ion interferes with the H_2O_2 -dioxane reaction.

O. Active Site Titration

The active site of α -chymotrypsin was titrated with N-trans cinnamoylimidazole following a modification of procedure B of Schonbaum et al. (113). To 2.50 ml of 0.2 M acetate buffer, pH 5, was added

500 μ l of 5 mg/ml chymotrypsin solution. The phosphate buffer concentration of the reaction mixture for photoinactivation was decreased to 0.06 M compared to the usual 0.125 M final concentration. Then 100 μ l of 5 mg/10 ml dry acetonitrile substrate solution was quickly added and the absorbancy was followed on the Cary 14 spectrophotometer at 335 nm and a chart speed of 2 in/min. The reference cell consisted of native enzyme, acetate buffer and 100 μ l of acetonitrile. Standard curves for the titration of various dilutions of native chymotrypsin were linear. Therefore the following formula was used to express the data in percent active site (π) of the "zero time" sample:

$$\pi = \frac{\Delta A_t}{\Delta A_B - \Delta A_0} \times 100$$

where ΔA_t is the difference in the initial and (extrapolated) final values of the absorbancy at the time of addition of the substrate, ΔA_0 is the same absorbance difference of a native, "zero time" enzyme sample and ΔA_B is the same absorbance difference of the blank.

P. Thermosensitivity

Thermosensitive fractions of photodynamically treated lysozyme and RNase were detected by incubating test solutions of these enzymes for sixty minutes at 70° C and 60° C, respectively, before assaying for enzymatic activity. The percent of initial enzymatic activity after (A) and before (B) the enzyme was incubated at elevated temperatures at given times of illumination was determined. The following relationship was used to calculate the fraction of thermosensitive

enzyme activity (F_{sens}), present at various times after initiation of photodynamic inactivation:

$$F_{\text{sens}} = 1 - \frac{A}{B}$$

Q. Thermodenaturation

The "melting curves" of the enzymes were measured over the temperature range of 10-50° C. A 200 ml sample of enzyme and 1.7 ml of 0.03 N HCl was put into a 1 x 1 cm quartz cuvette; the cuvette securely capped, and then cooled to 5° C in ice-water. Recordings of the difference spectra at 292 nm were begun immediately after the cuvette was placed in the Cary 14 spectrophotometer. The reference cell contained an identical enzyme-HCl solution kept at room temperature. The sample was allowed to warm up from 10-25° C by absorbing heat from the surroundings. A hair dryer kept the air circulating in the cuvette chamber to prevent the cuvette surface from clouding up from water vapor condensation. From 25-50° C the temperature was increased at a rate of about one degree per minute by pumping water from a one liter water bath sitting on a hot plate into the brass water jacket surrounding the cuvette holder. The 0-0.1 absorbancy scale was used and the chart speed was 1/6 inches per minute.

IV. RESULTS

A. Ribonuclease

The effects of photodynamic action on the configuration of ribonuclease (RNase) were examined. Two approaches were used to investigate mechanisms of photodynamic action not involving the active site. The direct approach was to measure conformation sensitive parameters such as protein fluorescence, molecular sieve chromatography, ion-exchange chromatography, electrophoresis, photoinactivation in urea, immunological reactivity and protein digestibility. The indirect approach was to detect active enzyme molecules that have altered biological properties such as salt sensitivity, urea sensitivity, and thermosensitivity. Results are broken into the above categories and presented in that order.

Ribonuclease (RNase) was photoinactivated in a first-order manner when sensitized at 16° C with 12.5 μ M MeB or EoY or with 150 μ M FMN. The reaction mixtures were buffered with 0.125 M sodium phosphate, pH 8.0 and the final enzyme concentration was 5 mg/ml. In discussing the following conformation sensitive measurements, the above reaction conditions and concentrations for photoinactivation should be assumed unless stated otherwise.

Fluorescence. As shown in Figure 2, the tyrosine fluorescence of RNase dropped rapidly to about one-half of the original value during the course of MeB-sensitized photoinactivation. After the initial rapid decrease, the fluorescence continued to decline at a slower,

Figure 2. The time course of photoinactivation of RNase and decrease in protein fluorescence. The reaction mixture was 5 mg/ml in RNase, 0.125 M in phosphate buffer pH 8, 12.5 μ M in MeB. The fluorescence emission maximum was 306 nm with excitation 275 nm.

constant rate. Furthermore, the rate of fluorescence decrease appeared to be similar to the rate of enzyme photoinactivation. Identical kinetics of fluorescence change were observed when EoY was used as the sensitizer.

Molecular sieve chromatography. The elution order of molecular sieve chromatography is inversely proportional to the molecular weight. Figure 3 shows the elution pattern from Sephadex G-75 in a 0.9 x 30 cm column, of native RNase and RNase photoinactivated to 50% and 15% of the initial activity using MeB as the sensitizer. There was a progressive decrease in protein having an elution volume characteristic of native RNase and an increase in a more rapidly eluting fraction. The new heterogeneous peak consisted of higher molecular weight material.

Similar elution patterns were obtained when photoinactivated RNase was passed through a column of Biogel P-60. The main difference between these two gels is that Sephadex is a dextran while Biogel is a polyacrylamide.

Ion-exchange chromatography. Ion-exchange chromatography separates proteins according to their surface ionic charge distribution. As seen in Figure 4, when MeB-sensitized RNase illuminated to 55% remaining activity was chromatographed on Dowex CG-50, less protein eluted in the area characteristic of native enzyme and more protein eluted earlier in an area characteristic of protein less basic than native RNase.

Electrophoresis. As seen in Figure 5, native RNase migrated as a single band toward the cathode when electrophoresed on cellulose acetate in barbitone-acetate buffer pH 8, while electrophoresis of a

Figure 3. Chromatography of photoinactivated RNase on a Sephadex G-75 column (0.9 x 30 cm). The protein concentration was determined spectrophotometrically at 287 nm using 0.125 M sodium phosphate, pH 7.0 as the elution buffer. Using MeB as a sensitizer, photodynamically treated RNase was inactivated to 50% and 15% of the original activity.

Figure 4. Chromatography of MeB-sensitized RNase on Dowex CG-50. Elution was in 0.2 M phosphate buffer, pH 6.2. Protein concentration was measured at 286 nm. Photoinactivated RNase had 55% of the original activity.

sample of completely photoinactivated RNase stained evenly over a region extending from the origin to the location where native RNase would have moved. Apparently, a good portion of photoinactivated RNase consisted of altered proteins having a range of isoelectric points lower than native RNase but higher than 8.

Figure 5. Cellulose acetate electrophoresis of native and photoinactivated RNase in barbatone-acetate buffer, pH 8. RNase was photoinactivated to less than 1% of the original activity using MeB as the sensitizer. Arrow points to the line of application.

Photoinactivation in urea. The folding of an enzyme could shield reactive amino acid residues from the oxidizing entities produced during photodynamic treatment. If any of the shielded residues are essential for the maintenance of enzymatic activity then any treatment increasing their rate of destruction should also increase the rate of enzyme inactivation.

RNase is reversibly unfolded in 8 M urea. The rate of photoinactivation of RNase was much faster when 8 M urea was present in the reaction mixture than in its absence. (see Figure 6). Compared to the rate of photoinactivation in buffer, the rate was 2.0 times greater for the MeB-sensitized photoinactivation and 2.4 times greater for

Figure 6. The time-course of photoinactivation of RNase in the presence of 8 M urea, 0.125 M phosphate buffer and 12.5 μ M EoY. The control had no urea in the reaction mixture.

the EoY-sensitized photoinactivation.

Immunological reactivity. The immunological activity of RNase was examined using two different techniques. The double diffusion agar technique of Ouchterlony (95) qualitatively detects differences in both the molecular weight and reactivity of the antigen. Diffusion through agar gel, like molecular sieve chromatography, separates macromolecules according to size. The quantitative test is used to determine changes in the amount of antigen-antibody precipitate.

Using the double diffusion agar technique of Ouchterlony, no decrease in the density or location of precipitation lines was seen when photoinactivated RNase, as sensitized with EoY or MeB, was tested against anti-RNase serum (see Figure 7).

Figure 7. Double diffusion in agar of photoinactivated RNase. Ten μ l of antigens were added to the outer wells at concentrations of 5.0 mg/ml and undiluted antiserum was assayed simultaneously to the center well. Percentages are amount of initial activity remaining after photodynamic treatment.

Quantitative precipitin curves and the amount of the protein in the precipitate were determined by the method of Kalbat and Mayer (68).

As shown in Figure 8, the equivalence point was 200 mg RNase per ml

Figure 8. Quantitative precipitin curves of anti-RNase serum. The antigen-antibody complex was dissolved in 0.25 N acetic acid and the absorbancy determined at 278 nm (68).

of antiserum. The effects of MeB-sensitized photoinactivation of RNase on the amount of precipitation with anti-RNase serum is summarized in Table 4. Neither MeB nor FMN sensitized photoinactivation resulted in a significant change in the precipitation response of RNase with anti-RNase serum.

Table 4. The Precipitation of Photoinactivated RNase by Anti-RNase Serum.

	100	65	41	17	3
Percent enzyme activity*	100	65	41	17	3
Precipitate (A_{278})	0.38	0.37	0.37	0.36	0.36

* MeB sensitized photoinactivation

There is the possibility that the photoinactivated RNase could be unfolded more easily in urea and, thus might fail to react with anti-RNase at urea concentrations where native RNase still formed a precipitate. Merigan and Potts have demonstrated the reversible inhibition of RNase-S to react with antiserum at concentrations of urea that inhibit very little the reaction of the antiserum with RNase (87). They demonstrated an inhibition of the precipitation reaction of the RNase-antiRNase system when 2-4 M urea was present in the reaction system. The present work has confirmed the findings (see Figure 9). To test for the above urea sensitive fraction, previously photoinactivated samples of RNase were incubated with anti-RNase in 2 M urea. The extent of urea inhibition of the precipitation reaction with anti-RNase using photoinactivated RNase sensitized

Figure 9. The effect of urea on the immunological reactivity of native RNase. The antibody-antigen precipitate was dissolved in 0.25 N acetic acid and the absorbancy determined at 278 nm (68).

with MeB or FMN was no different from the extent of inhibition using native RNase as the antigen.

Protein digestibility. Most native proteins are quite resistant to proteolytic digestion. Attempts were made to measure the production of trichloroacetic acid (TCA)-soluble digestion products of photoinactivated RNase after incubation with subtilisin. However, TCA did not completely precipitate RNase after 1 hour at 4° C and thus, results were unreliable. Powerful protein precipitants such as phosphomolybdic acid or tungstic acid were tried as substitutes for TCA but gave such high absorbance backgrounds in ultraviolet that the absorbance of hydrolysed protein was obscured.

Salt sensitivity. Several tests were employed to detect the production of active, but altered, RNase as a result of photodynamic treatment. The approach was to follow the kinetics of photoinactivation with the usual assay and with an assay solution containing a perturbant such as high concentrations of salt. In most cases the perturbant caused a decrease in the activity of native RNase. However, it was also found that progressively greater portions of active RNase were destroyed by the perturbant as the enzyme was photoinactivated. This is considered evidence that an altered but enzymatically active enzyme fraction is produced during photodynamic treatment.

Although the activity of RNase was inhibited by two-thirds when assayed with substrate containing 1.2 N NaCl, no increase in salt sensitivity was observed when RNase was photodynamically treated using MeB or EoY as sensitizers.

Urea sensitivity. When native RNase was assayed in substrate solution containing 5 M urea, the activity was about one-half of the activity as measured in substrate alone. Similar to the results of the test for salt sensitivity, there was no difference in the rate of photoinactivation of RNase as measured by the hydrolysis of C2,3P in the presence and absence of 5 M urea when MeB or EoY was used as the sensitizer. However, when the enzyme was photoinactivated with FMN as sensitizer, the rate of inactivation as determined by the substrate-urea assay was significantly faster than the inactivation rate measured with substrate solution lacking 5 M urea. (See Figure 10.) The percent of urea-sensitive activity produced during the course of photodynamic inactivation is shown in Figure 11.

Thermosensitivity. When native RNase was incubated at 60° C for 60 minutes, the activity of the enzyme decreased to 77% of the initial activity. A sample of photoinactivated RNase having 30% of the activity of an unirradiated control was much more thermosensitive. (See Table 5.)

Table 5. Percent Relative Activity of RNase After Incubation at 60° C. The activity of the MeB sensitized photoinactivated sample was 30% of the native enzyme.

Enzyme	Incubation Time		
	0 min	20 min	60 min
Native	100	95	77
Photoinactivated	100	40	25

Figure 10. Time course of photoinactivation of RNase as assayed with substrate or substrate-5 M urea. The reaction conditions were 0.125 M phosphate buffer, 150 μ M FMN and 5 mg/ml enzyme.

Figure 11. The production of urea sensitive RNase activity during the course of FMN-sensitized photodynamic inactivation. See Figure 10 for reaction conditions.

Figure 12 shows the rate of photoinactivation of RNase as sensitized with MeB when the activity of the enzyme sample was determined directly after being incubated at 60° C for 60 minutes. The controls were identical samples of photoinactivated RNase assayed for enzyme activity without any previous heat treatment. The difference in rates indicate that photodynamic inactivation produced an active form of the enzyme having increased heat sensitivity. The increases in thermosensitive enzyme activity during photoinactivation of RNase using MeB, EoY and FMN as sensitizers are shown in Figure 13.

B. Lysozyme

The effects of photodynamic action on the configuration of lysozyme were examined. Two approaches were used to investigate mechanisms of photodynamic action not involving the active site. The direct approach was to measure conformation sensitive parameters such as protein fluorescence, photoinactivation in urea, fluorescence polarization and protein digestibility. The indirect approach was to detect active enzyme molecules that have altered biological properties such as salt sensitivity, urea sensitivity, digestible activity and thermosensitivity. Results are broken into the above categories and presented in that order.

Photoinactivation of lysozyme followed first-order kinetics when sensitized at 16° C with 12.5 μ M MeB or EoY or with 150 μ M FMN. The reaction mixture was 0.125 M in sodium phosphate buffer, pH 8.0, and

Figure 12. The time course of RNase as assayed after (●) incubation at 60° C for 60 minutes. Reaction conditions using MeB as the sensitizer were the same as in Figure 2. The controls (○) were identical samples assayed without any previous heat treatment.

Figure 13. The production of thermosensitive RNase activity during the course of dye-sensitized photoinactivation. Total activity is determined by assaying enzyme activity before heat treatment. Sensitizing dyes used were MeB (●), EoY (▲), and FMN (■).

the final enzyme concentration was 4 mg/ml. The above reaction conditions were used for all experiments unless otherwise stated.

Fluorescence. The fluorescence of tryptophan residues in lysozyme was monitored at 342 nm by exciting samples of the enzyme with 290 nm light in an Aminco-Bowman spectrophotofluorometer. As shown in Figure 14, the decrease in fluorescence intensity is due to the loss of total fluorescence and not to a shift in the fluorescence peak. The fluorescence intensity of lysozyme dropped rapidly during the initial times of irradiation and began to level off as irradiation continued, plateauing at about 15% of the fluorescence intensity of native lysozyme (see Figure 15). Kinetic analysis of the first-order plot of the fluorescence intensity during photoinactivation yields two rate constants along with other kinetic constants (see Table 6). The rate of the rapid fluorescent decrease observed in the initial phase of the photoinactivation of lysozyme was the same as the rate of enzyme photoinactivation.

The rapid decrease in tryptophan fluorescence could be caused either by conformational changes or the photooxidation of tryptophan residues as lysozyme is photodynamically inactivated. The explanation, that loss of the tryptophan residues in lysozyme by photooxidation accounts for the decrease in fluorescence intensity, can be tested. It requires that the rate and extent of rapidly photooxidized tryptophan residues be equal to the rate of "fast" fluorescence decrease. The colorimetric assay of Spies and Chambers (129) using p-dimethylamino benzaldehyde (DMABA) reagent, was selected to directly measure the loss of tryptophan residues in lysozyme during dye-sensitized

photooxidation. Kinetic analysis of the tryptophan content of photooxidized lysozyme yielded the kinetic rate constants listed in Table 6. The number of tryptophan residues rapidly photooxidized using MeB and EoY as sensitizers, were 1.0 and 1.5, respectively. About 85% of the total protein fluorescence was quenched upon the photodynamic destruction of 1 to 1.5 tryptophan residues in lysozyme. Thus, it appears that the fluorescence decrease in lysozyme during photodynamic inactivation is not directly due to the loss of a corresponding proportion of tryptophan residues.

Table 6. Some Rate Constants For the Photooxidation of Lysozyme.

Property assayed	$k \text{ (min}^{-1}\text{)}$	
	MeB	EoY
Enzyme activity	0.12	0.027
"Fast" fluorescence	0.12	0.027
"Fast" tryptophan	0.042	0.030
"Slow" fluorescence	0.00031	0.006
"Slow" tryptophan	0.00031	0.00051

The rate of loss of enzymatic activity with EoY as the sensitizer, was similar to the rate of "fast" tryptophan destruction. In contrast, when MeB was the sensitizer, the rate of inactivation was three times faster than the rate of photooxidation of "fast" tryptophan residues. With both dyes, the rate of the "slow" fluorescence decrease was equal or close to the rate of loss of "slow" tryptophan.

Figure 14. The decrease in tryptophan fluorescence of lysozyme during photodynamic treatment using MeB as the sensitizer. Fluorescence was at 306 nm with 275 nm exciting light. Remaining activity after partial photoinactivation was 100% for curve 1, 80% for curve 2, 48% for curve 3, 26% for curve 4 and 8% for curve 5.

Figure 15. The time-course of photoinactivation, protein fluorescence decrease and photooxidation of tryptophan residues of lysozyme using MeB as the sensitizer.

Photoinactivation in urea. One might expect the rate of photoinactivation to be faster in the presence of 8 M urea. If an enzyme is unfolding in 8 M urea, buried residues that participate in the maintenance of the active configuration may be exposed. Lysozyme was photoinactivated in 0.125 M phosphate buffer in the presence and absence of 8 M urea. There was no difference in the rate of MeB or EoY sensitized photoinactivation of lysozyme in the presence of 8 M urea buffer compared to the rate of photoinactivation in buffer alone (see Figure 16).

Fluorescence polarization. The polarization spectra of tryptophan containing proteins are sensitive to conformational changes (152). Figure 17 gives the results of exciting protein fluorescence with various wavelengths of polarized ultraviolet light and recording the polarization of the fluorescence at 342 nm. The range of polarization spectra of lysozyme photoinactivated to 30% activity was limited because of the low fluorescence values of lysozyme after photodynamic treatment (Figure 15). Measurements had to be made near the limits of resolution of the Aminco spectrophotofluorometer. In general, the polarization values of photoinactivated lysozyme were larger than the polarization values of native lysozyme in the spectral range of 270 to 305 nm.

Protein digestibility. Most native enzymes are resistant to protease digestion. However, if an enzyme is inactivated by heat and acid, the enzyme is readily hydrolyzed by proteases into fragments which are not precipitated in trichloroacetic acid (TCA) solutions. Digestibility is followed by measuring the concentration of TCA-soluble peptides and amino acids. Figure 18 shows that lysozyme

Figure 16. The time-course of photoinactivation of lysozyme in the presence of 8 M urea, 0.125 M phosphate buffer, pH 8 and 12.5 μ M MeB. The control (0) had no urea in the reaction mixture.

Figure 17. Polarization spectra of the fluorescence of lysozyme in 0.125 M sodium phosphate, pH 8.0. Native lysozyme, O; MeB-sensitized, ●, photoinactivated lysozyme which had 30% of activity of native sample.

Figure 18. The increase in subtilisin digestibility of lysozyme during the course of photoinactivation. The absorbance (A) of TCA soluble digestion products were measured at 278 nm. The solid line is a theoretical curve. (See Discussion for details.) The dashed line is percent enzyme activity remaining after MeB-sensitized photoinactivation.

becomes more susceptible to protease digestion during the course of MeB-sensitized photoinactivation. Using a Guggenheim plot (127); the final absorbancy of the digestion mixture at infinite irradiation time was estimated. A biphasic first-order curve of the digestive susceptibility of lysozyme was then constructed (Figure 19). The slower first-order reaction accounted for up to 70% of the digestive products after prolonged photoinactivation of lysozyme. Only three data points were available for graphical kinetic analysis of the "fast" digestion reaction. Although three data points are too few to permit accurate calculations of the rate constant, the rate constant was consistent with the first-order photoinactivation rate constant of 0.125 min^{-1} . Figure 18 shows the fit of a theoretical curve constructed on the assumption that the rate of the fast phase of digestion was the same as the rate of photodynamic inactivation. The formula is derived in the Discussion section. A similar treatment of data for EoY-sensitized photoinactivated lysozyme tested for increased digestibility gave identical results and conclusions. The slow phase of the digestion reaction observed with EoY was much slower than the slow phase reaction observed with MeB.

Thermosensitivity. Several tests were used to detect altered but enzymatically active lysozyme molecules. The perturbants used were high salt and urea concentrations, and high temperature. Possible digestive susceptibility of active enzyme to proteases was examined also.

Figure 20 shows that the remaining enzymatic activity of partially photoinactivated lysozyme was more sensitive to incubation at 70°C

Figure 19. A first-order plot of the digestibility and activity of lysozyme during photodynamic treatment. The data is the same as in Figure 18. The enzyme was photoinactivated using MeB as the sensitizer.

Figure 20. How the time of exposure to 70° C effects the activity of native and partially photoinactivated lysozyme. Using MeB as the sensitizer, the enzyme was inactivated to various percentages of the original activity. The photooxidation and high temperature media was 0.125 M phosphate buffer, pH 8.

than was the native enzyme. The thermosensitive activity was destroyed by incubating partially photoinactivated lysozyme at 70° C for 60 minutes. The damaging effect of elevated temperature on enzyme activity was complete in 60 minutes. When samples of photoinactivated lysozyme as sensitized with MeB were exposed to 70° C for 60 minutes, a progressively larger fraction of the remaining active enzyme was thermosensitive. This is seen in Figure 21 by the more rapid rate of photoinactivation compared to the control, which did not receive the 70° C challenge. Similar results were observed with EoY and FMN as the sensitizers. Figure 22 illustrates the production of thermosensitive lysozyme activity during the course of dye-sensitized photodynamic action.

Initially, there were problems in obtaining reproducible data on thermosensitivity. First-order plots of the time course of photoinactivation measured after incubation at 70° for 60 minutes were rather variable. The time lapse between the photooxidation of the enzyme and the initiation of the high temperature incubation was found to be critical for reproducible results. A time-dependent decrease in the fraction of thermosensitive enzyme activity was observed when the partially photoinactivated enzyme was stored at 4° C (see Figure 23).

Salt sensitivity. An attempt was made to assay the activity of lysozyme in the presence of high salt concentrations. An assay solution containing 1.2 M NaCl completely eliminated any activity of the native enzyme. Even after the salt concentration was reduced to 0.15 M NaCl, the native enzyme was inhibited 85%. The rate of

Figure 21. The time-course of lysozyme photoinactivation measured before (O) and after (●) incubation of the native enzyme at 70° C for 60 minutes. Reaction conditions using 12.5 μ M EoY as sensitizer, were the same as in Figure 16.

Figure 22. The production of thermosensitive lysozyme activity during the course of dye-sensitized photoinactivation.

Figure 23. The decrease in thermosensitivity of photoinactivated lysozyme when stored at 4° C prior to the incubation at 70° C. The lysozyme sample was photoinactivated to 66% activity using MeB as sensitizer.

photodynamic inactivation as sensitized by MeB, EoY and RMN did not show any difference when the activity was assayed in the presence and absence of 0.15 M NaCl.

Urea sensitivity. As with high concentrations of salt, lysozyme activity was completely inhibited when assayed in the presence of 5 M urea. When the concentration in the substrate solution was decreased to 0.83 M urea, native lysozyme was still 80% inhibited. There was no difference in the rate of MeB, EoY or FMN sensitized photoinactivation when the enzyme was assayed in the presence and absence of 0.83 M urea.

Digestible activity. When native lysozyme was incubated for 90 minutes at 30° C with 0.1 mg/ml subtilisin or trypsin, twenty-five percent of the activity was destroyed by protease digestion. The rate of MeB or EoY sensitized photoinactivation did not change when the remaining lysozyme activity was assayed after exposure to the proteolytic action of subtilisin or trypsin for 90 minutes as compared to measuring the remaining activity directly after photodynamic treatment. Thus, photoinactivation did not increase the fraction of protease digestible lysozyme activity when sensitized with MeB or EoY.

C. Chymotrypsin

The effects of photodynamic action on the configuration of chymotrypsin were examined. Two approaches were used to investigate mechanisms of photodynamic action not involving the active site. The direct approach was to measure conformation sensitive parameters such

as protein fluorescence, thermal denaturation, molecular sieve chromatography, viscosity, photoinactivation in urea, fluorescence transition in urea, enzyme solubility, and polarization fluorescence. The indirect approach was to detect active enzyme molecules that have altered biological properties such as salt sensitivity, urea sensitivity, and low turnover activity. Results are broken into the above categories and presented in that order.

Chymotrypsin was photoinactivated in a first-order manner when sensitized at 16° C with 12.5 μ M MeB or EoY or with 150 μ M FMN. The reaction mixtures were buffered with 0.125 M sodium phosphate, pH 8.0 and the final enzyme concentration was 2 mg/ml. In discussing the following conformation sensitive measurements, the above reaction conditions and concentrations for photoinactivation should be assumed unless otherwise stated.

Fluorescence. When solutions of chymotrypsin were excited with 290 nm light, the tryptophan residues fluoresced with maximum intensity at 341 nm. During MeB or EoY sensitized photoinactivation, the tryptophan fluorescence intensity of chymotrypsin decreased (see Figure 24). When the enzyme activity had dropped to less than 1%, the fluorescence intensity decreased to 70% of its original value. In order to determine if tryptophan fluorescence changes in photoinactivated chymotrypsin were due to the photooxidation of the amino acid residues responsible for the protein fluorescence, the tryptophan residues remaining after MeB and EoY sensitized photoinactivation were measured by the method of Spies and Chambers (129) using DMABA reagent. As seen in Figure 24,

Figure 24. The time-course of photoinactivation, tryptophan fluorescence decrease and tryptophan destruction of chymotrypsin. The reaction mixture consisted of 12.5 μM MeB and 0.125 M sodium phosphate buffer, pH 8.0. Irradiation was done at 16° C. Kinetic analysis of tryptophan destruction was according to Ray and Koshland (106).

only a few of the seven tryptophan residues were destroyed during the course of MeB-sensitized photoinactivation and the kinetics were essentially the same as the kinetics of protein fluorescence quenching.

Kinetic analysis of the rate of destruction of enzyme activity and tryptophan residues of lysozyme yielded the rate constants listed in Table 7.

Table 7. Some Rate Constants for the Photoinactivation of Chymotrypsin.

Assayed	k (min ⁻¹)	
	MeB	EoY
Enzyme activity	0.12	0.051
"Fast" tryptophan	0.045	0.043
"Slow" tryptophan	0.00012	0.0057

The rate of "fast" tryptophan destruction was one-third the rate of photoinactivation with MeB. The rate for the photooxidation of "fast" tryptophan residues was probably not significantly different from the rate of the MeB-sensitized photoinactivation of trypsin. The number of tryptophan residues that were rapidly photooxidized using MeB or EoY as sensitizers, were estimated to be 2.0 and 1.2 respectively.

Thermal denaturation. Figure 25 shows the results of the spectrophotometric titration of samples of photoinactivated chymotrypsin as the temperature was increased from 10° to 50° C. The enzyme underwent an unfolding transition on heating that was followed spectrophotometrically at 292 nm using the expanded absorbancy scale (0-0.1) of the Cary 14 spectrophotometer. The midpoint temperature of the thermal

Figure 25. Thermal transitions of EoY-sensitized photoinactivated chymotrypsin followed by difference absorption at 280 mμ. Curve A, native enzyme; curve B and C, photoinactivated enzyme having 46% and 8% activity, respectively, of native enzyme.

transition (T_m) of native chymotrypsin was 35.5° C. As chymotrypsin was progressively photoinactivated with EoY as a sensitizer, the transition zone became broader and the T_m decreased. The decrease in the T_m during the course of photoinactivation is seen in Figure 26. Similar results were found with MeB as the sensitizer.

Molecular sieve chromatography. This type of chromatography separates proteins according to molecular size with the larger proteins eluting more rapidly. As seen in Figure 27, when MeB-sensitized photoinactivated chymotrypsin was filtered through a 0.9 x 30 cm column of Sephadex G-75, it eluted slightly faster than a sample of native enzyme. This small difference was consistently reproduced in three separate experiments.

Viscometry. No change in viscosity of chymotrypsin was observed when the enzyme was photoinactivated using 12.5 μ M MeB as a sensitizer. (See Table 8).

Table 8 . Viscosity of Photoinactivated Chymotrypsin (1%, pH 8.0).

Percent Activity	Specific Viscosity
100	0.023
64	0.023
19	0.024

Measurements were made with an Ostwald type viscometer, placed in a water-bath maintained at 15° C.

Figure 26. The midpoint temperature of the thermal transition (T_m) of chymotrypsin during the course of EoY-sensitized photodynamic inactivation. O, T_m ; ●, per cent enzymatic activity.

Figure 27. Chromatography of photoinactivated chymotrypsin on a Sephadex G-75 column (0.9 x 30 cm). The eluting buffer was 0.125 M sodium phosphate, pH 7.0 in 8.0 M urea. Protein concentration was measured at 282 nm. The photoinactivated sample was sensitized with MeB and had 4% of the activity of a native sample.

Photoinactivation in urea. Solvent perturbation of the ultraviolet spectra of chymotrypsin in buffer, chymotrypsin in 8 M urea, and a model mixture of amino acids, indicate that chymotrypsin is completely unfolded in 8 M urea (57). Exposure of chymotrypsin to 8 M urea during photooxidation might expose photooxidizable amino acid residues normally buried in the native enzyme. If any of the exposed amino acids are essential for the maintenance of enzyme activity, their destruction would prevent the reversible reactivation of chymotrypsin after removal of the concentrated urea. The results of photooxidizing chymotrypsin in 8 M urea, 0.125 M phosphate buffer and 12.5 μ M EoY is shown in Figure 28. The rate of photoinactivation of chymotrypsin was 3.2 times faster in the presence of 8 M urea than in its absence. In contrast, no difference in photoinactivation rates were observed in the presence and absence of 6 M urea. After removing the 6 M or 8 M urea by the process of rapid dilution, native chymotrypsin was reactivated to 50 or 25%, respectively, of the original activity.

Fluorescence transition in urea. A first-order increase in the tryptophan fluorescence intensity and a shift in the emission maximum from 241 nm to 256 nm occurs in a period of several minutes when chymotrypsin is rapidly added to 8 M urea (63). The fluorescence change monitors the denaturation process of chymotrypsin in 8 M urea. Figure 29 shows the results of MeB-sensitized photoinactivation of chymotrypsin on the fluorescence transition.

As chymotrypsin was photoinactivated using MeB or EoY as sensitizers, the rates of fluorescence transition in 8 M urea increased and,

Figure 28. The time-course of photoinactivation of chymotrypsin in the presence of 8 M urea, 0.125 M phosphate buffer and 12.5 μ M EoY. The control had no urea in the reaction mixture.

Figure 29. The fluorescence transition of photoinactivated chymotrypsin in 8 M urea. The curves show the percent activity of photodynamically-treated chymotrypsin, as sensitized by MeB. This figure also illustrates the noise in the recordings.

as previously noted, the fluorescence intensity decreased to 70% of that of the native enzyme. As seen in Figure 30, the transition rate constants in 8 M urea increased 10 fold following photodynamic treatment.

Enzyme solubility. Denaturation often decreases the solubility of an enzyme. Photodynamically treated chymotrypsin was examined for a possible decrease in solubility by incubation in a precipitating buffer consisting of 0.1 M glycine-HCl, pH 3, and 1.2 M NaCl at 4° C for 3 hours (9). No loss of enzyme due to precipitation was detected during the course of MeB-sensitized photodynamic inactivation.

Polarization fluorescence. Upon denaturation, the polarization of the tryptophan fluorescence of an enzyme often changes. The polarization spectrum of chymotrypsin photoinactivated to 18% activity is shown in Figure 31. Compared to native chymotrypsin polarization, the values for the inactivated enzyme were lower when excited at 250-270 nm and higher when excited at 290-320 nm.

Low turnover activity. Several tests were made for altered but enzymatically active fractions on photodynamically treated chymotrypsin. One of the most interesting tests was that used by Ray and Koshland (106). While studying the MeB-sensitized photoinactivation of chymotrypsin, they demonstrated the production of a fraction of chymotrypsin having a very low turnover number. The reagent, cinnamoylimidazole, combines stoichiometrically with the active site of all active chymotrypsin molecules and reacts with the low turnover enzyme in the same way as with native enzyme. In contrast, a kinetic enzyme assay would,

Figure 30. The increase in rate constants of the 8 M urea fluorescence transition as chymotrypsin is photoinactivated with MeB (O) or EoY (●) as the sensitizers. The first-order transition rates are relative to native chymotrypsin.

Figure 31. The polarization spectra of the fluorescence of chymotrypsin in 0.125 M sodium phosphate buffer, pH 8.0. O, Native chymotrypsin; ●, MeB-sensitized photoinactivated chymotrypsin having 18% activity of the native enzyme.

by its very nature, overlook an enzyme fraction with a very low turnover.

Figure 32 illustrates the difference in the rate of the FMN-sensitized photoinactivation of chymotrypsin as measured by a kinetic assay and by an active site titration. The increased rate of photoinactivation when the activity is measured by active site titration shows that the fraction of low turnover enzyme activity increased during photodynamic treatment. Similar increases in the fraction of low turnover enzyme during photoinactivation were observed when MeB or EoY were used as the sensitizer. A plot of the increase in low turnover chymotrypsin during the course of photodynamic inactivation using MeB, EoY or FMN as the sensitizer is given in Figure 33.

Urea sensitivity. Photoinactivated chymotrypsin was tested for the presence of an active enzyme form having an unusual sensitivity to high concentrations of urea. As seen in Figure 34, there was no significant difference in the rate of FMN-sensitized photoinactivation of chymotrypsin when assayed by the hydrolysis of ATEE in the presence and absence of 5 M urea. Similar results were found using MeB or EoY as the sensitizer.

Salt sensitivity. Similarly, photoinactivated chymotrypsin was tested for the presence of an active enzyme form with unusual sensitivity to 1.2 M NaCl. The presence of this concentration of NaCl in the substrate solution inhibited native chymotrypsin about 20%. The rate of MeB and FMN-sensitized photoinactivation was decreased when the activity was assayed with ATEE in 1.2 M NaCl compared to being assayed with ATEE alone (see Figure 35).

Figure 32. The time-course of photoinactivation of chymotrypsin as assayed by active site titration and by kinetic assay. The sensitizer was 150 μ M FMN in 0.125 M phosphate buffer, pH 8.0. Cinnamolyimidazole was the titrant and ATEE was the substrate for the kinetic assay.

Figure 33. The production of low-turnover chymotrypsin activity during the course of dye-sensitized photoinactivation.

Figure 34. The time-course of FMN-sensitized photoinactivation of chymotrypsin as assayed by the hydrolysis of ATEE in the presence (●) and absence (○) of 5 M urea.

Figure 35. The time-course of FMN-sensitized photoinactivation of chymotrypsin as assayed by the hydrolysis of ATEE in the presence of (●) and absence (○) of 1.2 M NaCl.

D. Trypsin

The effects of photodynamic action on the configuration of trypsin were examined. Two approaches were used to investigate mechanisms of photodynamic action involving the active site. The direct approach was to measure conformation sensitive parameters such as tryptophan fluorescence, oxidation with H_2O_2 -dioxane, molecular sieve chromatography, photoinactivation in urea, polarization fluorescence, solubility, and ion-exchange chromatography. The indirect approach was to detect active enzyme molecules that have altered biological properties such as low turnover activity, urea sensitivity, and salt sensitivity. Results are broken into the above categories and presented in that order.

Trypsin was photoinactivated in a first-order manner when sensitized at 16°C with $12.5\text{ }\mu\text{M}$ MeB or EoY or with $150\text{ }\mu\text{M}$ FMN. The reaction mixtures were buffered with 0.125 M sodium phosphate, pH 7.0 and the final enzyme concentration was 1 mg/ml . In discussing the following conformation sensitive measurements, the above reaction conditions and concentrations for photoinactivation should be assumed unless stated otherwise.

Fluorescence. The fluorescence intensity of tryptophan residues in trypsin decreased during the course of photoinactivation. By the time all enzyme activity was destroyed, the fluorescence had dropped to one-half that of the original intensity when sensitized with EoY, (see Figure 36) and one-third when sensitized with MeB. Fluorescence continued to decrease with further illumination even after the enzyme was completely photoinactivated, but at a slower rate.

Figure 36. The time-course of photoinactivation, protein fluorescence decrease and tryptophan destruction of trypsin. The reaction mixture consisted of 12.5 μM EoY and 0.125 M sodium phosphate buffer, pH 8.0. Irradiation was done at 16° C. Kinetic analysis of tryptophan destruction was according to Ray and Koshland (106).

In order to determine if tryptophan fluorescence changes in photoinactivated trypsin were due to photooxidation of the amino acid residues responsible for the protein fluorescence, the tryptophan content in trypsin was followed using the DMABA reagent of Spies and Chambers (129). By the time all enzymatic activity had been destroyed by photodynamic treatment, the tryptophan content had decreased to one-half that of the native enzyme with EoY as the sensitizer, and to one-third with MeB as the sensitizer. A first-order plot of the rate of loss of tryptophan residues, gave a biphasic curve. Kinetic analysis of the data yielded rate constants for the loss of enzymatic activity, "fast" tryptophan and "slow" tryptophan as listed in Table 9.

Table 9. Rate Constants for the Dye-Sensitized Photoinactivation of Trypsin.

Property assayed	k (min ⁻¹)	
	MeB	EoY
Enzyme activity	0.043	0.031
"Fast" tryptophan*	0.053	0.032
"Slow" tryptophan*	0.0030	<0.001

* probable error of $\pm 10\%$

The number of tryptophan residues per trypsin molecule rapidly photooxidized was estimated graphically to be 2.0 with both MeB and EoY as sensitizers. The rate for the photooxidation of "fast" tryptophan residues was probably, not significantly different from the rate of the MeB-sensitized photoinactivation of trypsin. The rate of "fast"

tryptophan loss and enzyme activity loss were the same when EoY was the photosensitizer.

The presence of 6 M urea during photoinactivation as sensitized with EoY increased the rate of photooxidation of tryptophan residues. Kinetic analysis of tryptophan destruction during EoY-sensitized photoinactivation in 6 M urea indicated that the number of tryptophan residues rapidly photooxidized, had increased to 3.0 ± 0.2 . Also, the rate of photooxidation of "fast" tryptophan in urea was about the same as the rate of photodynamic inactivation of the enzyme activity.

Some of the products of photooxidized free tryptophan are known to react with DMABA when the reagent is used as an indicator for paper chromatography (50). It was not clear whether any of the photooxidation products of trypsin could react with DMABA reagent, thereby giving a false estimate of the tryptophan content of the photoinactivated samples of the enzyme. To test for this, a sample of trypsin was auto-hydrolyzed by incubating the enzyme at 36.5°C for 2.5 hours. The final activity of this preparation was 1.8%. Extensive photooxidation of the hydrolysate did not eliminate all materials reactive with DMABA. As seen in Figure 37, a 10% background of color-producing substances persisted despite the likelihood that all tryptophan residues were completely destroyed. That photooxidation can produce materials which react with DMABA reagent was convincingly shown by photooxidizing RNase, an enzyme that does not have tryptophan, for 4 hours. Addition of DMABA reagent gave a small amount of color equivalent to 10% of the value obtained when native RNase was "salted" with 4 moles of tryptophan per mole of enzyme.

Figure 37. The time-course of photooxidation of hydrolyzed trypsin measured by the method of Spikes and Chambers (129). Reaction mixture was 0.125 M sodium phosphate, 12.5 μ M EoY and 2 mg/ml autolysed trypsin. Trypsin was hydrolysed to 1.8% of the original activity by incubating the reaction mixture at 36.5° C for 2.5 hours.

Oxidation of tryptophan with H_2O_2 -dioxane. Kinetic analysis of the tryptophan content of trypsin during the course of photoinactivation indicated that two out of the four tryptophan residues were rapidly destroyed. By adapting a technique used to specifically oxidize tryptophan residues in enzymes, these conclusions were reconfirmed. Hachimori et al. (51) have reported that the extent of oxidation of tryptophan residues in trypsin varies with the concentration of hydrogen peroxide in a solution of 0.5 M bicarbonate buffer, pH 8.1, containing 10% dioxane. As seen in Figure 38, treatment with increasing concentrations of H_2O_2 -dioxane lowers the absorption band of trypsin. The extent of the absorbance decrease (ΔA) of trypsin photoinactivated with EoY as the sensitizer was one-half that of native trypsin. Assuming that the change in absorbance of native trypsin is due to the oxidation of all of its four tryptophan residues, the change in absorbance of photoinactivated trypsin resulted from the photooxidation of two tryptophan residues. Thus, EoY-sensitized photoinactivation of trypsin destroyed two ($4-2 = 2$) tryptophan residues.

Hachimori et al., uses the change in the molar extinction coefficient of H_2O_2 -dioxane treated acetyltryptophan to estimate the moles of oxidized tryptophan residues in protein (51). They calculate that only three out of the four residues of native trypsin are oxidized. Since the extent of oxidation of photoinactivated trypsin was one-half that of native trypsin, the number of tryptophan residues remaining in photoinactivated trypsin would be one and one-half and not two as calculated above. Actually, it is risky to assume that the

Figure 38. The oxidation of tryptophan residues of native and completely photoinactivated trypsin with H_2O_2 -dioxane. The protein concentration was 7.0×10^{-6} M. Trypsin was sensitized with $12.5 \mu M$ EoY in 1.0 M bicarbonate buffer, pH 8.0 until it had less than one percent of the original activity.

extinction coefficient of acetyltryptophan is identical to the extinction coefficient of tryptophan residues in trypsin. Also, the extent of photooxidation of trypsin varies considerably with the length of exposure to H_2O_2 -dioxane. Figure 39 demonstrates that the extent of oxidation of tryptophan increased with increasing incubation periods of the H_2O_2 -protein mixture. Further, others have questioned the assumption that changes in absorbancy of trypsin are only due to the oxidation of tryptophan (98), because treatment with H_2O_2 -dioxane will oxidize residues in RNase, an enzyme lacking tryptophan. Therefore, in addition to measuring absorbancy changes, tryptophan residues were directly measured during the course of H_2O_2 -dioxane oxidation using DMABA reagent. As the data in Table 10 show, all four of the tryptophan residues in trypsin are oxidized in native trypsin and two residues in completely photoinactivated trypsin. As was found to occur with photooxidized enzymes, the 10% residual activity at 6 mM H_2O_2 concentration may be due to the reaction of H_2O_2 oxidation products with DMABA reagent.

Table 10. The Tryptophan Content of Native and Completely Photo-inactivated Trypsin in Different Concentrations of H_2O_2 -dioxane. The bicarbonate buffer and H_2O_2 -dioxane were removed by lyophilization prior to determining tryptophan content by the method of Spies and Chambers (). Trypsin was photoinactivated to 1% activity using EoY as the sensitizer.

H_2O_2 Concentration (μM)	Number of Residues	
	Native	Photoinactivated
0	4*	2.0
3	1.5	0.8
6	0.4	0.3

*The tryptophan residues in native trypsin were taken as 4 (71).

Figure 39. The extent of oxidation of tryptophan residues of native trypsin in increasing concentrations of H_2O_2 -dioxane. The different curves show the effect of varying the incubation time of $22.4 \mu M$ trypsin with H_2O_2 reagent at $4^\circ C$.

When native trypsin was oxidized with H_2O_2 -dioxane, 20% to 80% of the activity was lost, depending upon the experiment. It is not clear why the percent inactivation varied from experiment to experiment. Apparently, much of the remaining activity is a damaged fraction because the hydrolysis of BAEE was reduced 50% if 5 M urea was present in the assay solution. A urea sensitive fraction was also found when trypsin or RNase was oxidized by FMN-sensitized photodynamic treatment. These latter findings are detailed in other parts of the results section.

Molecular sieve chromatography. In an effort to detect changes in molecular size, photoinactivated trypsin was filtered through a column of Sephadex G-75. Trypsin photoinactivated to 10% activity with MeB had a broad elution peak which came off the column slightly ahead of the native sample of trypsin. (See Figure 40).

Photoinactivation in 8 M urea. As seen in Figure 41, the rate of photodynamic inactivation of trypsin was increased when 8 M urea was present in the reaction mixture. Compared to controls, which had 0.125 M phosphate buffer, pH 8.0 and 12.5 μ M dye but no urea, MeB-sensitized photoinactivation in the presence of 8 M urea was 14 times faster and the EoY-sensitized reaction, 10 times faster. Enzyme samples were taken from the reaction mixture containing 8 M urea and assayed by dilution of a small aliquot (10 μ l) in 3.0 mls volume of substrate solution. Native trypsin activity was inhibited 50-60% when assayed by this procedure.

Figure 40. The time-course of photoinactivation of trypsin in the presence of 8 M urea, 0.125 M phosphate buffer and 12.5 μ M MeB. The control had no urea in the reaction mixture.

Figure 41. Chromatography of photoinactivated trypsin on a Sephadex G-75 column (0.9 x 30 cm). The eluting buffer was 0.125 M sodium phosphate, pH 7.0 in 8.0 M urea. Protein concentration was measured at 280 nm. The photoinactivated sample was sensitized with MeB and had 10% of the activity of a native sample.

If the reaction mixture containing 8 M urea was not preincubated in the dark for 10 minutes at 15° C before the photoinactivation reaction was initiated, the rates of inactivation in 8 M urea were considerably slower than samples which were preincubated prior to photoinactivation but still 3.6 times faster than in the absence of urea. Extended dark incubation of the 8 M urea-reaction mixture did not inactivate the enzyme.

Polarization fluorescence. The fluorescence polarization spectra of native trypsin and trypsin having 30% activity is shown in Figure 42. Polarization values of the inactivated enzyme were higher than those of native trypsin over the entire spectral range examined.

Solubility. Mullaney and Preiss (92) reported that trypsin subjected to gamma irradiation in the dry state forms a turbid solution when added to RNA. In a single experiment, photoinactivated trypsin sensitized with MeB did not increase the turbidity of an RNA solution. It is not known whether this negative result was due to the fact that inactivation took place in solution rather than in the dry state, or whether it was due to the probability that the dye-sensitized photoinactivations of trypsin gave reaction products different from gamma irradiation.

Ion-exchange chromatography. Crystallin trypsin was chromatographed on a 0.9 x 22 cm column of Dowex CG-50 resin and eluted with a buffer system containing 8 M urea (see Figure 43). The 8 M urea was present in the chromatography system in order to prevent autolysis of trypsin during chromatography. As has been previously shown (22),

3

Figure 42. Polarization spectra of the fluorescence of trypsin in 0.125 M sodium phosphate buffer, pH 8.0. Native trypsin, O; MeB-sensitized, photoinactivated trypsin having 18% activity of the native enzyme ●.

Figure 43. Ion-exchange chromatography of native and photoinactivated trypsin on a 0.9 x 22 cm Dowex GC-50 column containing 8 M urea, following the procedures reported by Cole and Mendiola (21). Symbols: protein concentration of native trypsin, ○ and of 42% photoinactivated trypsin, ●; enzyme activity of native trypsin, □ and of 42% photoinactivated trypsin, ■.

the elution pattern of native trypsin was heterogeneous and the activity was associated with a minor, slower eluting peak. The elution pattern of photoinactivated trypsin using MeB as the sensitizer was generally like that of native trypsin, but less of the slower moving peak was present and the earlier eluting peaks now contained much more protein. The pattern of activity of the 42% photoinactivated trypsin was heterogeneous; at least two enzymatically active species were present.

Low turnover activity. In an attempt to detect an active fraction of enzyme having a lower turnover as was done with chymotrypsin, an attempt was made to titrate the active sites of trypsin with p-nitrophenol N²-benzyloxycarbonyl-L-lysinate. Unfortunately, the minimum concentration of trypsin required for the successful titration of the active site is around 10 mg/ml. In sodium phosphate buffer, pH 8, trypsin, at this high concentration tended to precipitate out of solution. Thus, titration data was unreliable for trypsin reaction mixtures maintained at pH 8.

Urea sensitivity. A difference in the rate of the FMN-sensitized photoinactivation of trypsin when assayed with BAEE or with hemoglobin containing 5 M urea has been noted previously (42). This difference in the rate of photoinactivation was interpreted, but not proven, to result from the presence of 5 M urea in one of the assay systems. The 5 M urea inactivated an active fraction of the enzyme that had an unusual sensitivity to this denaturant. Urea-sensitive active fractions were not produced when the sensitizer for the photoinactivation

of trypsin was MeB or EoY. These findings have been confirmed using a more defined assay system, that is, casein solutions with and without 5 M urea or BAEE solutions with and without 5 M urea. As shown in Figure 44, the rate of FMN sensitized photoinactivation was more rapid when the casein solution contained 5 M urea. Identical results were obtained using BAEE in place of casein as the substrate. No significant difference was seen in the rate of photoinactivation using substrate with and without 5 M urea when MeB or EoY were the photosensitizers. The increase in the portion of urea-sensitive activity as trypsin was photoinactivated is shown in Figure 45. When the native enzyme was assayed in the presence of 5 M urea, the activity was decreased 40%.

Salt sensitivity. Using the same approach as used for the detection of urea-sensitive activity, the possible production of salt sensitive enzyme activity during the course of photoinactivation was tested. Although the activity was inhibited 25% when assayed in 1.2 M NaCl, no difference was noticed when photoinactivated trypsin, as sensitized by MeB, EoY, or FMN, was assayed in the presence or absence of 1.2 M NaCl.

Figure 44. Time-course of photoinactivation of trypsin as assayed with casein substrate (O) or casein substrate-5M urea (●). The reaction conditions were 0.125 M phosphate buffer, pH 8, 150 uM FMN and 1 mg/ml enzyme.

Figure 45. The production of urea sensitive trypsin activity during the course of FMN-sensitized photodynamic inactivation. See Figure 44 for reaction conditions. The different symbols designate separate experiments.

V. DISCUSSION

A. Ribonuclease

A number of different conformation sensitive parameters were examined during the photodynamic inactivation of RNase as sensitized by MeB, EoY and, at times, FMN. Molecular sieve chromatography, electrophoresis on cellulose acetate, ion-exchange chromatography and immunophoresis lead to the conclusion that a large conformational change occurs during dye-sensitized photodynamic inactivation.

The rapid filtration through Sephadex G-75 or Biogel-60 indicate that at least part of photoinactivated RNase has become a heterogenous spectrum of aggregates. The ionic charge on the surface of the protein is also altered. While native RNase migrates as a single band towards the cathode when electrophoresed on cellulose acetate at pH 8, the photoinactivated enzyme is spread over a region starting at the origin and ending at the band characteristic of native RNase. This broad range of lowered electrophoretic mobilities indicate that photooxidation produces a disperse series of less basic proteins. Also, photoinactivated RNase tends to elute more quickly upon ion exchange chromatography on Dowex CG-50. This too is characteristic of a less basic protein than native RNase. These results would be expected if basic amino acids such as histidine were photooxidized during the process of photodynamic action. Weil reports that this is exactly what happens when RNase is photoinactivated using MeB as a sensitizer (157). Similar alterations in the ionic charge of photooxidized

insulin occur. Weitzel et al. report that the isoelectric point is shifted toward lower pH values (162).

Biophysical properties more sensitive to small conformational changes include fluorescence, protein digestibility, and antigen-antibody reactivity. For a long time the last technique, antigen-antibody reactivity, has been considered to be extremely sensitive to changes in the spatial arrangements of the antigen surface. We are unable to demonstrate any decrease in RNase-antiRNase reactivity as the enzyme is photoinactivated in the presence of MeB. Nor are changes in the reactivity observed when photoinactivated RNase is exposed to 2-4 M urea. It was hoped that these moderate concentrations of urea would cause any damaged enzyme to unfold and consequently lose its ability to react with the anti-RNase serum. Inasmuch as other criteria have shown that RNase was structurally and ionically altered during photodynamic treatment, the possibility exists that an antigen-antibody reaction is not as sensitive to conformation changes as was anticipated. There is one other case where photoinactivated enzyme was examined for possible altered antibody-antigen reactivity (45). Here also, MeB-sensitized inactivation did not cause any decrease in reactivity of trypsin to anti-trypsin serum.

The tyrosine fluorescence of RNase drops rapidly during the initial stages of photoinactivation as sensitized by MeB or EoY. The initial rate of fluorescence decrease is very similar to the rate of enzyme inactivation. Not all of the fluorescing tyrosine residues, however, are destroyed during photoinactivation because 50% of the

fluorescence still persists even after prolonged photoinactivation. It is likely that the rapid loss of tyrosine fluorescence is due to a rapid alteration in enzyme conformation (i.e., quenching) rather than the rapid photooxidation of several of the tyrosine residues. When Weil and Seibles (157) photooxidized RNase in 0.2 M phosphate buffer, pH 8.0 and 37° C, using MeB as the sensitizer, tyrosine residues were not destroyed, even after extensive photooxidation.

When RNase is photoinactivated using MeB or EoY as sensitizers in the presence of 8 M urea, the rate of inactivation is about twice as fast as the rate of photoinactivation under similar conditions in the absence of 8 M urea. Thus it appears that there are several photooxidizable amino acid residues which are buried in the interior of the RNase molecule. Furthermore, photooxidation of some of these buried residues prevents the refolding of RNase into the active configuration after removal of urea.

Weil et al. found that the rate of MeB-sensitized photodynamic inactivation of insulin was also increased two-fold when 8 M urea was present in the reaction mixture (160). They also showed that the increased reactivity in 8 M urea was not due to any solvent effect. The rates and extent of photooxidation of a model amino acid mixture were the same whether water or 8 M urea was the solvent (160). The rate of MeB-sensitized photooxidation of myoglobin is increased several fold when 7.5 M urea is in the reaction mixture (111).

One of the objectives of this thesis is to determine whether the photodynamic inactivation of enzymes can occur by a change in

conformation or by other mechanisms not involving the direct destruction of amino acids in the active site. In addition to conformation measurements, another approach is to determine whether photodynamic inactivation produces active but altered enzyme molecules.

Photodynamic treatment might alter an enzyme in such a way that it still retains its activity, but shows unusual sensitivity to some denaturing conditions (e.g., high urea or salt concentrations; elevated temperatures, etc.). Such altered molecules could be detected by the difference in the total activity of a solution of partially photoinactivated enzyme when assayed with and without the denaturing conditions.

During the course of RNase photoinactivation using MeB or EoY as sensitizers, we are unable to detect production of an enzyme fraction which is unusually sensitive to 1.2 N NaCl. However, an enzyme species unusually sensitive to 5 M urea is produced when FMN is used as the photosensitizer. The fraction of the urea-sensitive residual activity produced during the FMN-sensitized photoinactivation of RNase increases rapidly with time of illumination. By the time the total activity has dropped to 25%, the urea sensitive fraction makes up approximately 50% of the activity remaining. No urea sensitive fraction is detected when MeB or EoY were the sensitizers.

Similar results are found by Ghiron and Spikes (42) who report a lower rate of FMN-sensitized photoinactivation of trypsin when assayed with BAEE or casein compared to the rate of photodynamic inactivation when assayed with hemoglobin in 5 M urea. Furthermore, they also are unable to detect a urea sensitive fraction when trypsin

is photoinactivated with MeB or EoY.

The production of a urea sensitive fraction during FMN-sensitized photoinactivation of RNase suggests that the specific, catalytically active configuration in these enzymes is dependent on the participation of certain photooxidizable amino acid residues. These amino acid residues need not be involved in the catalytic center of the enzyme.

Richards (107) has shown that the peptide fragments of subtilisin-digested RNase functions as a determinant of proper folding in the rest of the molecule. Both native RNase and the active subtilisin derivative are stable in aqueous solution. In urea the macromolecular fraction, RNase-S, is rapidly denatured and cannot be reactivated by the addition of the peptide, even following removal of the dissociating agent. Native RNase itself, is completely reactivated when 8 M urea is removed.

We have shown that photoinactivation as sensitized by FMN produces an altered enzyme fraction that is catalytically active in aqueous solutions. Unlike native RNase, it is unable to return to a catalytically active conformation after unfolding in 5 M urea. Thus FMN-sensitized photoinactivation may bring about the destruction of amino acid residues that are exclusively involved in the refolding process after the enzyme has been unfolded by exposure to 5 M urea.

Finally all three dyes (MeB, EoY, or FMN) sensitize the production of an active but thermo-sensitive fraction as RNase is photoinactivated. Thermosensitive activity is also produced when pepsin or myosin (ATPase activity) is photodynamically treated with MeB or eosin (32,80) and after trypsin is ultraviolet irradiated.

Based on the findings of Mueller (91) who studied the ultraviolet inactivation of RNase, an explanation for thermosensitive RNase can be offered. It is well known that RNase undergoes a rather sharp unfolding transition as the temperature is raised to 50-60° C. This thermotransition, termed melting, is completely reversible with native RNase. However, when the enzyme is inactivated by ultraviolet irradiation the reversibility of melting decreases. Presumably the same thing happens to photoinactivated RNase. Apparently, limited photooxidation of amino acid residues during photodynamic treatment does not alter the enzymatic activity of the enzyme but destroys certain amino acid residues essential for proper refolding to an active enzyme configuration after heat-denaturation.

The different effects of heat and urea on altered enzymes produced during MeB or EoY sensitized photodynamic action might have been anticipated. Biltonen (9) finds that thermodenaturation is different from urea-denaturation. At least in the case of chymotrypsin, optical rotatory dispersion measurements indicate that thermally unfolded enzyme can be unfolded even further in high concentrations of urea. The fact that MeB or EoY can sensitize the photoproduction of an active enzyme sensitive to 60° C but not to 5 M urea, while FMN can sensitize the photoproduction of both thermosensitive and urea-sensitive RNase emphasized the conclusion that the pathways of photoinactivation are neither simple nor consistent with the three sensitizing dyes used.

B. Lysozyme

Three conformation-sensitive measurements were made on lysozyme during photodynamic inactivation with MeB, EoY and FMN; tryptophan fluorescence, fluorescence polarization and subtilisin digestibility. All three parameters indicate that a conformation change occurs during the process of dye-sensitized photoinactivation.

The fluorescence intensity of tryptophan residues in lysozyme decreases rapidly during photodynamic inactivation. After prolonged illumination, about 15% of the fluorescence intensity still remains. A kinetic analysis of the decrease in fluorescence during the course of MeB or EoY sensitized photooxidation shows that the rate of fluorescence decrease is equal to the rate of photoinactivation. Since a simple colorimetric assay for tryptophan residues in an intact protein is available (129), the time course of tryptophan photooxidation during photodynamic treatment could be conveniently followed. Of the seven tryptophan residues present in lysozyme, only one or two of the residues are photooxidized during photoinactivation. Kinetic analysis of a first-order plot of the photooxidation of tryptophan residues yields rate constants for two classes of tryptophan molecules. One class of tryptophan residues reacts rapidly during the process of photoinactivation. These residues are termed "fast" tryptophans. The number of "fast" tryptophan residues observed with MeB and EoY as sensitizers was 1.0 and 1.5

Table 11 compares the present results on the photodynamic destruction of tryptophan in lysozyme with the work of others. In some cases

quantitative data is lacking. As can be seen, the reaction conditions vary, making critical comparisons impossible. As mentioned in the Introduction, the pH and temperature can cause large differences in the types and numbers of amino acid residues which are photooxidized.

Table II. Tryptophan Residues Photooxidized After Complete MeB-sensitized Photoinactivation of Lysozyme.

Authors	pH and temperature of the reaction mixture		Tryptophan destroyed
DasGupta and Boroff (26)	3.8	23° C	+
DasGupta and Boroff (26)	6.8	23° C	+
Shugar (119)	7.1	25° C	+
Weil <u>et al.</u> (155)	8.7	37° C	3
Our data	8.0	16° C	1

+ means that tryptophan residues were destroyed.

There are two possible ways to explain how the destruction of so few tryptophan residues can account for such a large decrease of tryptophan fluorescence. Steiner et al. (134) and Teale (138) report that the fluorescence intensity of tryptophan decreases as the dielectric constants of the immediate environment increases. Either the rapidly photooxidizable tryptophan residues are in very hydrophobic regions where their fluorescence intensity accounts for 85% of the total tryptophan fluorescence of the protein, or photooxidation of these residues causes a large conformational change that brings a good portion of the six remaining tryptophan residues out of a hydrophobic environment.

The first possibility can be tested. In a very hydrophobic environment, the fluorescence intensity of tryptophan might be as much

as 3.5 times higher than in aqueous solvent (138). If it is assumed that all tryptophan residues but the 1 to 1.5 "fast" tryptophans in lysozyme are in aqueous environments (which is probably too generous), the theoretical percent decrease in fluorescence as a result of the destruction of the 1 to 1.5 "fast" residues would at the very most be 54%.

Since in our experiments 85% of the initial fluorescence was lost after the photodynamic destruction of the fast tryptophan residues, this interpretation can not account for the observed results. For this reason we must conclude that the majority of the fluorescence change of lysozyme during photodynamic treatment results from conformational changes of the enzyme

In the case of the EoY-sensitized photoinactivation of lysozyme, the rate of change in conformation as measured by fluorescence parallels the rate of inactivation. This suggests, but does not prove, that the photodynamic inactivation of lysozyme in this case is caused by a change in enzyme configuration. Since the rate of photoinactivation of lysozyme with MeB is about three times faster than the rate of change in conformation as measured by fluorescence, a similar conclusion is not possible with this sensitizer.

It is tempting to suggest that residues 62 and 63 are the site of rapid tryptophan photooxidation during the photodynamic inactivation of lysozyme. The argument follows.

Blake et al. (10) have elucidated the complete three dimensional structure of lysozyme by x-ray crystallography. The molecule has a

hydrophobic core consisting mainly of the 6 tryptophan side chains, four of which are found in α -helical conformations. Of these four, residue 123 and 108 are on the surface of the enzyme. The two other tryptophan residues, 62 and 63, also protrude beyond the molecular surface where they could readily react with the active intermediates of the photodynamic process.

Of the four surface tryptophan residues, residue 108 can be modified with ozone without any loss of activity (100). Therefore, its photooxidation cannot account for the inactivation of lysozyme.

Residues 62 and 63, being adjacent to each other, represent a microregion where the concentration of tryptophan is higher than elsewhere in the enzyme molecule. The probability of an active intermediate of photodynamic action randomly reacting with surface residue 62 or 63 is considerably higher than with one of the other two tryptophan residues. Also residue 62 is situated on the enzyme surface in such a way that it is very reactive and selectively oxidized by N-bromosuccinimide and oxidation of this residue causes lysozyme to lose its activity almost completely (55).

Most native enzymes are quite resistant to digestion by proteases. However, if the enzyme is denatured by heat or acid, it becomes readily digestible. Enzyme digestibility has been called an "all-or-none" type assay because any conformation other than the native conformation is digestible by protease. The photoinactivation of lysozyme also increases its digestibility by subtilisin. As detailed in the Results section, the protease digestion of photoinactivated

lysozyme appears to be made up to two different proteolytic reactions. While it is possible to determine the first-order kinetic rate constant of the slower digestion, it is not possible to determine the rate constant of the faster digestion process. It does appear however, that the faster rate might be similar to the rate of inactivation of the enzyme. To test this, a theoretical protease susceptibility curve for the MeB-sensitized photoinactivated lysozyme was derived which incorporates both the first-order kinetic constant for the "slow" digestion reaction and the first-order rate constant for photoinactivation.

Derivation of an equation for the time course of lysozyme digestion using two first-order reactions with rate constants k_f and k_s is as follows: The fractions of enzyme resistant to slow or fast digestion remaining at time, t , will be given by the expressions in Equations 1, where x_f or x_s is the concentration of protein resistant to the fast or slow digestion and the subscript zero refers to the concentration of native protein resistant to digestion.

$$\frac{(x_f)}{(x_f)_0} = e^{-k_f t} ; \quad \frac{(x_s)}{(x_s)_0} = e^{-k_s t} \quad (1)$$

The time dependence for the fraction of digestion resistant lysozyme lost via photoinactivation, $\frac{[X]}{[X]_0}$, is given by Equation 2:

$$\frac{[X]}{[X]_0} = \frac{(x_f)}{(x_f)_0} \times \frac{(x_s)}{(x_s)_0} = e^{-(k_f + k_s) t} \quad (2)$$

While the fraction of lysozyme that is digested at any given time is given by Equation 3, where $[Y]$ is the concentration of lysozyme digested.

$$\frac{[Y]}{[Y]_0} = 1 - e^{-(k_f + k_s) t} \quad (3)$$

In order to express the fraction of digestible lysozyme, $\frac{[Y]}{[Y]_0}$ in absorbance units, A , we set $Y = A$ and $Y_0 = A_\infty - A_0$. Rearranging, Equation 4 is derived:

$$A + A_0 = A_\infty - (A_\infty - A_0) (e^{-(k_f + k_s) t}) \quad (4)$$

Where A_0 is the absorbance of digestion products of native enzyme and A is the absorbance of digestion products of enzyme that is completely photooxidized.

The curve in Figure 18 comes from Equation 4, taking constants A_0 , A_∞ , and k_s from the experimental data and assuming that the rate constant for the fast digestion reaction was equal to the rate of inactivation of the enzyme.

The good fit of the theoretical curve to the data supports the assumption that two different reactions are occurring in photodynamically treated lysozyme. Both reactions (presumably changes in conformation), make the enzyme susceptible to the proteolytic action of subtilisin. The slower reaction accounts for 70% of the net digestibility of lysozyme after prolonged photooxidation. The rate of the faster reaction can be directly correlated with the photoinactivation of lysozyme. Therefore, at least part of the digestibility of lysozyme and, hence, change in conformation can be directly

correlated with the process of photoinactivation.

Over the wavelength range 270-305 nm, the polarization spectra of the fluorescence of photoinactivated lysozyme sensitized with MeB is slightly higher than the native enzyme. These differences in polarization are similar to those seen when native lysozyme is denatured in 8 M urea (152).

The rate of photooxidation of lysozyme using MeB or EoY as a sensitizer in 8 M urea was the same as the rate of photooxidation in buffer alone. These results can be explained in two ways: either there are no photooxidizable amino acid residues essential for activity normally buried within the lysozyme molecule that become exposed to the surface of the enzyme in the presence of 8 M urea or lysozyme is not unfolded in 8 M urea to any appreciable extent. The latter explanation is supported by the findings of Williams et al. who show by solvent perturbation of the ultraviolet absorption spectra that lysozyme is folded almost as much in 8 M urea as it is in buffer alone (163). Further, Hamaguchi and Kurono report that the optical rotatory properties of native lysozyme do not change in 8 M urea (53) and Takahashi et al. find little if any increase in the oxidation of tryptophan residues by N-bromosuccinimide after addition of 5 M urea to reaction mixture (137).

Several tests were used in attempts to detect altered but enzymatically active lysozyme molecules. During the photodynamic inactivation of lysozyme as sensitized by MeB, EoY and FMN, no damaged fractions showing unusual sensitivity to urea or NaCl were

observed. These negative results must be qualified, however, because the concentration of NaCl used had to be decreased 8 fold from the usual 1.2 M to 0.15 M and the usual concentration of urea used had to be decreased 6 fold from 5.0 M to 0.83 M. In the presence of the higher concentrations of salt or urea, no enzymatic activity of a native sample of lysozyme can be detected. This apparent inhibition may result from an alteration of the M. lysodeikticus cells used for assay, making them unsuitable as a substrate for lysozyme. Even 0.83 M urea prevents the lysis of the substrate when the bacterial suspension was exposed to the urea several hours before the enzyme was added. As previously discussed, the tertiary structure of lysozyme is not altered much by concentrations of urea as high as 8 M.

While a portion of native lysozyme activity is lost when incubated with subtilisin or trypsin, no further increase in the fraction of activity destroyed by digestion is seen when lysozyme is photoinactivated using MeB or EoY as the sensitizer.

A thermosensitive fraction of lysozyme is produced during the photoinactivation process. All three dyes, MeB, EoY and FMN sensitize the photoproduction of the thermosensitive fraction. A possible explanation for thermosensitive activity follows the same lines of reasoning as mentioned in the discussion section for RNase: the reversibility of the thermal transition of lysozyme is progressively lost during photoinactivation. One can calculate that when only 25% of the total activity of lysozyme remains, 75% of that activity

consists of a thermosensitive enzyme. The portion of thermosensitive activity found in the total activity seems to reach a limit of 75% as the percent total activity continues to drop to zero.

While testing for thermosensitive, but active lysozyme, a most unexpected observation was made. When partially photoinactivated lysozyme is incubated at 4° C, up to 50% of the thermosensitive fraction is converted back to a non-thermosensitive enzyme. The "repair" process has a half-life of about one hour.

A similar time-dependent repair process occurs after fully reduced RNase is reoxidized in air (1). Although the basic structural properties of the enzyme are reestablished on air oxidation as followed by the disappearance of -SH groups and an increase in specific optical rotation, certain finer adjustments are necessary to establish the final specific tertiary structure. This lag may be as much as several hours.

Presumably then, photodynamic treatment suddenly perturbs some of the enzyme molecules into an altered configuration which retain enzymatic activity, but which are unusually sensitive to heat. Given enough time and the right conditions, up to 50% of this damaged enzyme reverts to a more thermodynamically stable configuration having characteristic biophysical properties of the native enzyme.

C. Chymotrypsin

Six conformation sensitive measurements were made upon lysozyme during photodynamic treatment with MeB, EoY and FMN as sensitizers.

During the course of photoinactivation as sensitized by MeB or EoY, the tryptophan fluorescence of chymotrypsin drops to about 70% of its original value. This decrease in the tryptophan fluorescence intensity is due to the photooxidation of one or two tryptophan residues. The rapidly photooxidized tryptophan residues in chymotrypsin are probably the same tryptophan residues responsible for the loss of fluorescence intensity. This conclusion is based on the observation that the kinetics of fluorescence decrease is identical to the kinetics of tryptophan destruction. Hence, in the case of photoinactivated chymotrypsin as sensitized by MeB or EoY, the change in fluorescence intensity probably is not a monitor of conformational transitions.

The tryptophan content of chymotrypsin decreases 20-30% by the time MeB or EoY-sensitized photoinactivation is complete. Table 12 compares the present results on the photodynamic destruction of tryptophan in chymotrypsin with the work of others. As can be seen, the reaction conditions vary, making critical comparisons impossible. As mentioned in the Introduction, the pH and temperature can cause large differences in the types and numbers of amino acid residues which are photooxidized.

Table 12. Tryptophan Residues Photooxidized After Complete MeB-sensitized Photoinactivation of Chymotrypsin.

Authors	pH and temperature of reaction mixture		Tryptophan destroyed
Weil, <u>et al.</u> (156)	7.4	37° C	3
Jandorf, <u>et al.</u> (64)	7.0	25° C	1
Ray, <u>et al.</u> (104)	7.0	10° C	0
Present data	8.0	16° C	2

Kinetic analysis of the time-course of tryptophan photooxidation indicates that the reaction proceeds by two first-order processes, one considerably faster than the other. With MeB as the sensitizer, the rate of enzyme inactivation is three times faster than the rate of "fast" tryptophan photooxidation. The rate of enzyme inactivation is slightly faster than the rate of "fast" tryptophan photooxidation when EoY is the sensitizer. Because the first-order rate constants of tryptophan destruction have errors of $\pm 10\%$, the difference in these two rates may not be significant.

Thermal transition curves of samples of photoinactivated chymotrypsin were recorded spectrophotometrically. Analysis of a family of curves as the enzyme was progressively inactivated using EoY or MeB as sensitizers, show that the transition midpoint (T_m) decreases and the overall transition curves become broader. The decrease in the T_m is characteristic of an altered enzyme structure. Similar decreases in the T_m have been observed when chymotrypsin is exposed to urea or alcohol (15,36,114,118) although the decreases are usually considerably larger.

The two-state definition of conformational transitions of proteins assumes that only a single active thermodynamic state and a single "denatured" thermodynamic state are in simple equilibrium. If a large number of states are intermediate between the original and final state and not part of a cooperative transition, the thermal transition would occur over a wider temperature range. The broadening of the transition zone of photodynamically inactivated chymotrypsin is indicative of

multiple state behavior (79). It appears that native chymotrypsin is structurally altered during the course of photodynamic inactivation forming a heterogeneous population of protein molecules.

Evidence for a change in the molecular structure of chymotrypsin is also indicated by molecular sieve chromatography, and by measurements of polarization fluorescence and unfolding transitions in 8 M urea. Photoinactivated chymotrypsin elutes slightly faster than native enzyme when filtered through a column of Sephadex G-75. This reproducible difference is not large enough to suggest some sort of enzyme aggregation but probably results from an increase in the molecular volume of the inactivated chymotrypsin. When chymotrypsin is photoinactivated using MeB as a sensitizer, the polarization spectra of the tryptophan fluorescence decreased in the excitation wavelength region of 250-270 nm and increased in the excitation wavelength region of 290-320 nm. Weber reported similar fluorescence polarization changes for native chymotrypsin in the excitation wavelength region of 250-280 nm when chymotrypsin was placed in 8 M urea (152).

The denaturation of chymotrypsin in 8 M urea was followed by the time-dependent shift in the fluorescence spectrum. As chymotrypsin is photoinactivated using MeB or EoY as sensitizers, the first-order rate constant of the fluorescence transition in 8 M urea increases 10 fold. A change in the first-order rate of denaturation in 8 M urea is indicative of structural changes in the enzyme. The enzyme has become more permeable, allowing the urea to denature the protein more quickly.

Photoinactivation of chymotrypsin in buffered 8 M urea is over three times faster than photoinactivation in buffer alone. Williams, et al., have shown that chymotrypsin becomes extensively unfolded in 8 M urea (163). Therefore, it is not surprising that some of the residues of chymotrypsin normally buried in the interior are photooxidized in the presence of 8 M urea. It is not possible to decide whether the increased rate of photodynamic inactivation in 8 M urea is due to the destruction of residues necessary for the primary refolding process after urea has been removed or to the secondary process of establishing subtle configurational relationships necessary for enzymatic activity.

A number of different tests were employed to detect altered but active enzyme fractions during the photoinactivation of chymotrypsin. A comparison of the rate of photoinactivation as measured by active site titration and by a kinetic assay show that MeB, EoY, and FMN all sensitize the production of an altered enzyme having a very low enzyme turnover. By the time that chymotrypsin had been 75% photoinactivated, approximately 50% of the remaining activity was in the form of low turnover enzyme. Ray and Koshland had previously reported that the MeB-sensitized photoinactivation of chymotrypsin produces a low turnover enzyme fraction (105). They suggested that this low turnover enzyme might be a chymotrypsin derivative having a single oxidized methionine residue.

During the course of the photodynamic inactivation of chymotrypsin no activity having unusual sensitivity to the denaturing effects of 5 M urea or having decreased solubility in 1.2 M NaCl or 0.1 M

glycine-HCl, pH 3, was detected.

An unexpected effect was observed with the possible production of an active enzyme showing increased activity in 1.2 N NaCl. One possible explanation is that this reactivation of photoinactivated chymotrypsin in the presence of high concentrations of NaCl is due to the presence of contaminating Ca^{++} ions found in the salt solution. It is well known that Ca^{++} ions protect the enzyme from the denaturing effects of urea and heat (83).

D. Trypsin

During the time-course of the MeB, EoY and FMN sensitized photodynamic inactivation of trypsin, five conformational sensitive measurements were made; tryptophan fluorescence, gel filtration, thermodenaturation, fluorescence polarization, and fluorescence transition in urea. Possible charge changes were detected by ion-exchange chromatography. All of these biophysical measurements confirm that trypsin is altered by photodynamic action, but do not necessarily indicate that large changes in conformation are involved.

The fluorescence intensity of tryptophan residues in trypsin decrease rapidly on photodynamic treatment. With prolonged illumination about one-third of the original fluorescence intensity remains when MeB is used as the sensitizer and about one-half of the original fluorescence intensity remains when EoY is used. There are two explanations for this drop in fluorescence: The change in fluorescence would be due either to a conformational change or simply to the destruction of tryptophan residues.

As a test of the latter explanation, the loss of tryptophan residues was measured during the course of photoinactivation of trypsin. The kinetics of the fluorescence decrease are identical to the kinetics of tryptophan destruction. Thus, the decrease in fluorescence probably is not a monitor of conformation change during the photoinactivation of trypsin. Kinetic analysis of the tryptophan destruction during the course of photoinactivation indicate that two out of the four residues are rapidly destroyed using either MeB or EoY as sensitizers. The rate of photooxidation of "fast" tryptophan is equal to the rate of enzyme inactivation with EoY or MeB as sensitizers. The fact that these two rates are similar suggests that a tryptophan residue may be essential for the maintenance of enzyme activity.

When photoinactivated trypsin sensitized with MeB is filtered through Sephadex G-75 the protein elutes slightly faster than the native enzyme. It is unlikely that this small difference could be caused by protein aggregation. More likely, it indicates that only small changes in the conformation of the enzyme occur during the process of photoinactivation. Chromatography on ion-exchange resin also suggests the presence of an altered enzyme after photoinactivation. As the enzyme is photoinactivated using MeB as the sensitizer, it elutes more rapidly from the ion-exchange column. This would be expected if the altered enzyme is less basic than native trypsin. The slight differences observed in the polarization of fluorescence values over the range of 250-230 nm between native and photodynamically treated enzyme may indicate that a conformation change has occurred in

photoinactivated trypsin. There are no simple interpretations for changes in fluorescence polarization (151). The rate at which the fluorescence of trypsin changes in 8 M urea can indicate the permeability of the enzyme to the unfolding action of urea. During the course of the MeB or EoY sensitized photoinactivation of trypsin, the rate of fluorescence transition in 8 M urea, which takes several minutes with native trypsin, increases very little. This indicates that the "looseness" of trypsin does not change much during photodynamic inactivation.

Apparently, several photooxidizable amino acid residues are buried within the interior of native trypsin. When the enzyme is photoinactivated in the presence of 8 M urea, the rate of inactivation increases remarkably. Using MeB as the sensitizer, the rate of photooxidation in 8 M urea is 14 times faster than that found in aqueous solvent alone. With EoY as sensitizer, the rate of photooxidation in urea is ten times faster than that found in buffer alone. As might have been expected from the studies of fluorescence transition in 8 M urea where the unfolding takes several minutes to go to completion, trypsin-urea mixtures must be preincubated in the dark for a few minutes before illumination in order to get maximal rates of photodynamic inactivation.

An attempt was made to destroy the tryptophan residues in trypsin by exposing the enzyme to various concentrations of H_2O_2 -dioxane. The extent of oxidation of tryptophan residues at various concentrations of H_2O_2 -dioxane was reproducible. The extent of

oxidation of tryptophan residues in trypsin was measured by ultraviolet spectrophotometry and by the colorimetric method of Spies and Chambers (129). When a sample of trypsin photoinactivated with EoY is exposed to the H_2O_2 -dioxane reagent, two tryptophan residues are destroyed. All four tryptophan residues in native trypsin are oxidized by treatment with H_2O_2 . This confirms the earlier conclusions that photodynamic action destroys 2 out of the 4 tryptophan residues found in trypsin.

Two tests were used to detect active but altered enzyme molecules in photodynamically treated trypsin. None of the remaining activity in partially photoinactivated trypsin as sensitized with MeB, EoY, or FMN shows increased sensitivity to 1.2 N NaCl. Nor does the remaining activity show any increased sensitivity to 5 M urea with MeB or EoY as the sensitizer. However, with FMN as the sensitizer, urea sensitive activity is produced during the course of photoinactivation.

The production of a damaged fraction of photodynamically treated trypsin having unusual sensitivity to urea was previously reported by Ghiron and Spikes (42). The present results confirm their finding that FMN but not MeB or EoY can sensitize the production of this fraction. More defined assay systems containing substrates with and without 8 M urea were used. With these assay systems the validity of their assumption that the destruction of the damaged fraction is due to urea is established.

E. Comparisons

A number of interesting comparisons are possible with the data on the photodynamic inactivation of RNase, lysozyme, chymotrypsin and trypsin. It was possible to make several types of measurements on all four enzymes, including fluorescence, photoinactivation in the presence of urea, and molecular sieve chromatography. Other conformation-sensitive measurements, as well as tests for active but altered enzymes could not be applied to all of the enzymes because of dye interference, autolysis, lack of tryptophan in the enzyme, etc. However, the following measurements were made on two or three enzymes; ion-exchange chromatography, tryptophan content, and fluorescence transitions in urea.

Fluorescence measurements were made on all four enzymes during the course of photoinactivation as sensitized by MeB and EoY. The greatest decreases in protein fluorescence are observed with lysozyme and RNase. The rate of decrease of fluorescence with both of these enzymes is the same as the rate of inactivation. The fluorescence intensity of trypsin decreases to one-third to one-half of the original value even after extensive photodynamic treatment. The smallest effect was seen with chymotrypsin where the decrease in fluorescence intensity was only 30%. When EoY is used as the sensitizer, the initial rates of decrease in fluorescence of chymotrypsin, trypsin, and lysozyme, during photodynamic treatment are equal to the rates of loss of enzyme activity. The same relationship was found between these two rates when trypsin was sensitized with MeB. In

contrast, when lysozyme and chymotrypsin were photoinactivated with MeB as the sensitizer, the rate of decrease of fluorescence is only one-third that of the rate of decrease of enzyme activity.

Tryptophan analysis of photodynamically-treated trypsin and chymotrypsin show that the extent and rate of fluorescence decreases can be completely accounted for by the photooxidation of certain rapidly destroyed tryptophan residues. While the rate of decrease in tryptophan fluorescence in lysozyme is the same as the rate of loss of "fast" tryptophan residues during photooxidation, the extent of the fluorescence decrease is considerably greater than can be accounted for by the photooxidation of tryptophan residues. This difference can be explained by assuming that large conformational changes occurred during the course of photoinactivation of lysozyme.

When all four enzymes are sensitized with EoY, the rate of "fast" tryptophan photooxidation is not only very similar to the rate of fluorescence decrease but also similar to the rate of enzyme inactivation. The same relationships held with photoinactivated trypsin as sensitized with MeB. In contrast, the rate of "fast" tryptophan photooxidation is only one-third as fast as the rate of photoinactivation for chymotrypsin and lysozyme as sensitized with MeB.

Despite the similarity of the rates of photooxidation of "fast" tryptophan in all three tryptophan-containing enzymes, the rate of enzyme inactivation varies over a range of an order of magnitude. The rate of photoinactivation of chymotrypsin and lysozyme is very similar when MeB was the sensitizer and three times faster than the rate of the

photodynamic inactivation of trypsin. Comparisons of rates of photoinactivation among the various enzymes are approximate, however, because conditions of photoinactivation such as light intensity, were not intentionally kept constant. Table 13 summarizes the above comparisons with the three tryptophan-containing enzymes.

Table 13. Some Kinetic Values of Tryptophan Fluorescence, Activity and Tryptophan Content of Photodynamically Treated Trypsin, Chymotrypsin and Lysozyme.

Measurement	Trypsin		Chymotrypsin		Lysozyme	
	MeB	EoY	MeB	EoY	MeB	EoY
Rate constant of enzyme activity (Min^{-1})	.053	.032	.045	.043	.042	.030
Rate constant of "fast" tryptophan (Min^{-1})	.043	.031	.12	.051	.12	.027
Residues of "fast" tryptophan	2.0(4)		2.0(7) 1.4(7)		1.0(6) 1.5(6)	
Percent loss of fluorescence	60%		30%		85%	

Total tryptophan content of the enzyme in parenthesis.

Molecular sieve chromatography of trypsin, chymotrypsin and RNase before and after photodynamic treatment with MeB demonstrated that these enzymes have eluted faster after being photoinactivated. The elution pattern of photoinactivated RNase indicates that RNase undergoes aggregation during photodynamic inactivation. Chymotrypsin and trypsin are altered more subtly.

Ion-exchange chromatography of photoinactivated trypsin and RNase revealed that both proteins have become less basic than the native

enzyme. Electrophoretic studies with RNase also show that photodynamically treated protein is less basic than the native protein.

The rates of the sensitized photooxidation in the presence of 8 M urea of trypsin, chymotrypsin and RNase are considerably faster than in buffer alone. The increase in rate ranges from 2 to 14 times faster. In contrast, lysozyme is not photoinactivated in 8 M urea any faster than in buffer. As chymotrypsin is photoinactivated using MeB or EoY as sensitizers, the fluorescence transition rate in 8 M urea increases up to 10 fold. In contrast, the fluorescence transition in urea of photoinactivated trypsin as sensitized by MeB or EoY increases very little.

The following tests were used to detect active but altered enzymes: urea sensitivity, salt sensitivity, and thermosensitivity. The last two tests could not be applied to photoinactivated trypsin or chymotrypsin because these enzymes undergo autolysis under the conditions used for the particular tests. When trypsin and RNase are photoinactivated using FMN as sensitizer, a urea sensitive fraction is produced but no urea sensitivity is detected for these enzymes when MeB or EoY is used as the sensitizer. Increased sensitivities to 1.2 N NaCl after photodynamic treatment are not detected with the four enzymes using any of the three sensitizers.

An active fraction of enzyme having unusual sensitivity to heat is detected with photoinactivated lysozyme and RNase when any of the three sensitizers are used. Photoinactivated chymotrypsin, which gives negative responses to tests for urea sensitivity and salt

sensitivity, does have an active fraction showing much lower enzyme turnover. Therefore, photodynamic treatment of all four enzymes can produce damaged enzymes still having activity under certain conditions.

From the above discussion on thermosensitive as well as urea sensitive fractions, it is clear that at least two pathways for the photoinactivation of the enzyme exist:

There are two ways one can obtain the experimentally observed first-order rates of inactivation for both the loss of total enzyme activity and the loss of "native" activity. The reaction rate of pathway 1 must be either much, much less than the reaction rate of pathway 2, or the reaction rate of pathway 1 must be equal to the reaction rate of pathway 3.

The data suggest that pathway 1 is a minor route, and that much of the photoinactivated enzyme first goes through an intermediate stage where it exists as a structurally altered but enzymatically active molecule. In order to accumulate active but altered enzymes during photodynamic treatment, the reaction rate of pathway 3 must be smaller than the reaction rate of pathway 2. Thus, even if the reaction rate of pathway 1 is equal to the reaction rate of pathway 3, the rate of these two reaction pathways must be lower than the reaction rate of pathway 2.

VI. SUMMARY

Conformational changes in ribonuclease, trypsin, chymotrypsin, and lysozyme during the course of photodynamic inactivation were studied. Methylene blue, eosin Y, and in some experiments, flavin mononucleotide were used as sensitizing dyes. Conformation-sensitive parameters studied include fluorescence, the behavior on molecular sieve and ion-exchange chromatography, electrophoresis mobility on cellulose acetate, immunological reactivity, sensitivity to thermal denaturation, fluorescence polarization, viscosity, solubility, fluorescence transitions in urea and protein digestibility.

A secondary objective of this thesis was to determine whether altered forms of enzyme molecules still possessing catalytic activity were produced as a result of photodynamic treatment. The altered characteristics examined were urea sensitivity, salt sensitivity, thermosensitivity, digestibility, and low enzyme turnover. For comparative purposes, the enzymes were also photoinactivated in 8 M urea.

All four enzymes showed some structural alteration. With ribonuclease and lysozyme, the rate of alterations correlated directly with the rate of loss of enzyme activity. This is evidence that at least some of the photoinactivation of these enzymes occur by changes in protein conformation. Chymotrypsin and trypsin showed slight conformational changes but the rate of these changes did not correlate directly with the loss of enzyme activity. When eosin Y

was used as the sensitizer for all four enzymes, one or two tryptophan residues were rapidly destroyed at a rate similar to the rate of enzyme photoinactivation, indicating that a tryptophan residue may be involved in the maintenance of enzyme activity. Charge interactions of the respective proteins were disrupted; ion-exchange chromatography and electrophoresis patterns of the photoinactivated enzymes were characteristic of less basic proteins than the native enzymes. The rates of photooxidation of trypsin, chymotrypsin, and ribonuclease in 8 M urea were, in general, several times faster than the rates in buffer alone. The native enzyme structure protects some amino acid residues from destruction by photodynamic action.

During the photodynamic inactivation of all four enzymes, active but altered enzyme molecules were produced. Urea-sensitive enzyme fractions were found with trypsin and ribonuclease using flavin mononucleotide but not methylene blue or eosin Y as the sensitizer. Thermosensitive enzyme fractions were produced during the photodynamic inactivation of ribonuclease and lysozyme with all three sensitizers. Because of their autolytic properties, trypsin and chymotrypsin could not be tested for thermosensitivity. A low turnover enzyme was detected during the photodynamic inactivation of chymotrypsin as sensitized by all three dyes. Partially photoinactivated enzyme with unusual sensitivity to 1.2 N sodium chloride, was not found with any of the four enzymes.

Thus, we have demonstrated that photodynamic treatment can produce altered enzymes which still retain intact active sites. Furthermore,

some enzymes apparently lose catalytic ability as a result of the destruction of secondary and tertiary structure.

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