Photodynamic Action of Porphyrins on Amino Acids and Proteins. I. Selective Photooxidation of Methionine in Aqueous Solution*

Giulio Jori, Guido Galiazzo, and Ernesto Scoffone

ABSTRACT: The efficiency of porphyrins in sensitizing the photooxidation of amino acids was found to be dependent upon the presence and upon the nature of a complexed metal ion and upon the nature of the side chains which protrude from the tetrapyrrolic core of the molecule. In particular, chlorophyll a, which contains the diamagnetic ion Mg^{2+}, causes a very fast photooxidation of tryptophan, methionine, histidine, and tyrosine in aqueous solution, pH 6.1; on the contrary, under the same conditions hemin, which contains the paramagnetic ion Fe^{3+}, is devoid of photodynamic activity.

Moreover, hematoporphyrin, which contains no metal ion, acts selectively on the amino acids tryptophan and methionine both in aqueous solution, in the pH range 2.5–6.5, and in 5–100% acetic acid solution. Since tryptophan is no longer photoreactive when the α-amino group is protected, the hematoporphyrin-sensitized photooxidation can be utilized for the specific modification of the methionyl residues in a polypeptide molecule. The feasibility of the method in the case of proteins was tested with hen's egg-white lysozyme. Irradiation of the enzyme in the presence of hematoporphyrin and in neutral aqueous solution results in a reduction of the enzymic activity to about 54%; amino acid analysis showed that this partially active product contains one methionine sulfoxide residue per protein molecule: this uniquely photoreactive residue was identified as methionine-12. The other methionyl residue which is present in lysozyme, *i.e.*, methionine-105, is not susceptible of photooxidation, unless the conformation of the protein molecule is modified by running the irradiation in concentrated acetic acid solution. The different photoreactivity of the two methionyl residues has been interpretated on the basis that methionine-105 is less accessible to the solvent than methionine-12 in the native lysozyme.

The dye-sensitized photochemical modifications of proteins provide answers on several different levels of inquiry into the protein structure. First of all, dye-sensitized photooxidations allow one to modify certain amino acid residues with a high degree of selectivity and, consequently, to elucidate their importance for the biological action (see, for a recent review, Spikes and Straight, 1967). Furthermore, this method can be utilized as a tool for the three-dimensional mapping of the amino acid residues in polypeptides and proteins, by estimating the type and the number of the photoreactive amino acids which are exposed at the surface or buried in the interior of the molecule (Ray and Koshland, 1962). New routes of research are being developed as the photooxidation products of the susceptible amino acids are characterized; for example, the conversion of tryptophan into kynurenine, which occurs on proflavine-sensitized photooxidation in formic acid or in acetic acid solution (Benassi *et al.*, 1967; Galiazzo *et al.*, 1968), opens the way for the specific cleavage of a polypeptide chain similar to that for tryptophyl residues (Veronese *et al.*, 1969).

Previous work from this laboratory (Jori *et al.*, 1968a) investigated the photochemical oxidation of methionine to methionine sulfoxide as a function of pH, of the solvent, and of the sensitizer; it was pointed out that the competitive photooxidation (Sluyterman, 1962; Weil, 1965) of the histidyl, tyrosyl, and tryptophyl residues can be prevented by performing the irradiations in aqueous acetic acid solution and in the presence of rose bengal or methylene blue as sensitizer. The selectivity for methionine of this photooxidative procedure was successfully tested with RNase A[1] (Jori *et al.*, 1968a) and with lysozyme (Jori *et al.*, 1968b).

It is well known, however, that several proteins are unstable at low pH values (Smillie and Neurath, 1959); in any case, strongly protic solvents, such as formic or acetic acid, can induce one or more types of conformational changes in protein molecules (Singer, 1962), although, as it was found with lysozyme and RNase A, these alterations may be reversed when the protein returns to an aqueous medium. Therefore, in order to enlarge the scope of the aforesaid photochemical techniques and to extend their potential usefulness, it would be desirable to find experimental conditions of irradiation which simultaneously fulfill the requisites of selectivity for a given amino acid and of inability to modify the native conformation of protein molecules. For this reason, we spread our investigations over new classes of sensitizers, whose electronic excited states would, at least in theory, have different properties from those of the dyes commonly employed and, hence, eventually promote original reaction mechanisms.

* From the Institute of Organic Chemistry, University of Padova, and Centro Nazionale di Chimica delle Macromolecole del C. N. R., Padua, Italy. *Received January 3, 1969.*

[1] RNase A, the principal chromatographic component of bovine pancreatic ribonuclease. The amino acids, peptides, and peptide derivatives mentioned are of the L configuration. The abbreviations are those recommended by the IUPAC–IUB Commission on Biochemical Nomenclature, *Biochemistry*, *5*, 1445 (1966).

In the present paper, we describe our findings about the photodynamic action of some porphyrin derivatives on amino acids and proteins. Porphyrins were chosen because the photochemistry of tetrapyrrolic molecules has been investigated in great detail (Robinson, 1964; Goedheer, 1966; Azizova *et al.*, 1966); in particular, it has been demonstrated that the chelation of metal ions to the porphyrin molecules greatly affects the yield of the intersystem crossing from the first excited singlet state to the first triplet state, which is the reactive one in photoreactions involving porphyrins (Fujimori and Livingston, 1957); the lifetime of the triplet state is also appreciably affected (Livingston and Pugh, 1959). As far as we know, however, only very few and qualitative experiments concerning the use of porphyrins as sensitizers for the photodamage of proteins are reported in the literature (Spikes and Glad, 1964; Della Pietra and Dose, 1965).

Our results indicate that the selective photochemical modification of methionine can be readily achieved in neutral aqueous solution if hematoporphyrin is used as the sensitizer. Moreover, a concerted study of the photooxidation of the two methionyl residues in lysozyme by the present method and by the above described procedure in aqueous acetic acid (Jori *et al.*, 1968a,b) allowed us to differentiate one buried from one exposed residue.

Materials

The amino acids were the products of Fluka AG (Basel, Switzerland). The protected tryptophan and methionine peptides Z-Trp-Gly-OEt, Z-Ala-Trp-OMe, Z-Trp-Met-OMe, and Z-Met-Asp-OH were synthetized in this Institute (Veronese *et al.*, 1967). Hen's egg-white lysozyme, salt free and twice crystallized (lot LYSF 8 D B), was purchased from Worthington Biochemical Corp. (Freehold, N. J.); its purity was checked by chromatographic and spectrophotometric analysis, as previously described (Jori *et al.*, 1968b).

Water-soluble magnesium–chlorophyll a, hematoporphyrin hydrochloride, protoporphyrin IX dimethyl ester, and hemin (ferriprotoporphyrin IX chloride) were obtained from Fluka, and were used without further purification. Thin-layer chromatography of the single dyes in different solvents always showed one spot, except in the case of chlorophyll a where traces of a second component (perhaps chlorophyll b) appeared to be present. The porphyrins were stored in the dark at 0°.

Cyanogen bromide, analytical grade, was obtained from Fluka. The 2-chloro-3,5-dinitropyridine was kindly furnished by Professor A. Signor of this Institute. Acetic acid (99–100%), analytical grade, was a product of Merck AG (Darmstadt, Germany). Sephadex G-25, medium grain size, was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden); Amberlite CG-50 (200–400 mesh) was purchased from British Drug Houses, England. All other chemicals were purchased commercially and were reagent grade products, unless otherwise stated.

Methods

Procedure of Photooxidation. The photoreactions were run in a water bath with a controlled temperature at ±0.1°, and the reaction vessels (Pyrex test tubes, 12 × 1.2 cm) were illuminated, at a distance of 40 cm, by four 150-W incandescent light bulbs placed on either side of the transparent walls of the Plexiglas container. A typical photooxidation system was prepared by mixing 2 ml of a 2×10^{-3} M substrate[2] solution with 2 ml of an equimolar sensitizer solution. During the irradiation, a stream of purified oxygen was slowly fluxed through the reaction mixture. Controls without oxygen, light, or dye were also run.

The following reaction media were tested: distilled water (pH 6.1),[3] aqueous-buffered solutions in the pH range 2.5–6.5 (0.1 M sodium phosphate buffer), and 5–100% acetic acid. For the experiments in aqueous solutions involving hematoporphyrin or protoporphyrin dimethyl ester, the porphyrins appeared to be dispersed through the medium. However, the reaction mixture was visually clear, and there was no tendency for the porphyrins to precipitate out of the solution, unless it was allowed to stand for several hours.

Amino Acid Analyses. In the case of amino acids and of oligopeptides, the progress of the reaction was followed by chromatographic and spectrophotometric analysis, according to the procedure previously described (Jori *et al.*, 1968a).

In the case of lysozyme, after irradiation, the solvent was removed by lyophilization, the residue was taken up with 5% acetic acid, and the solution was freed of the sensitizer and of the salts by gel filtration through a column (60 × 1.2 cm) of Sephadex G-25, using 5% acetic acid as the eluent. In the eluates, the lysozyme concentration was evaluated on the basis of the absorbance at 278 nm, since the extinction at this wavelength was found to remain essentially constant at all stages of photooxidation. The enzyme was recovered by lyophilization.

Samples for amino acid analysis were hydrolyzed in 6 M HCl within evacuated sealed tubes at 110° for 22 hr. The acid was removed by rotatory evaporation under reduced pressure. The amino acid composition of the resulting hydrolysate was determined on a Technicon automatic analyzer. The results obtained were standardized against valine, against leucine, and against phenylalanine, since these three amino acids are known to be stable toward acid hydrolysis and are also apparently unaffected by photooxidation. The content of methionine and of methionine sulfoxide was evaluated by alkaline hydrolysis in 3.75 N NaOH (Jori *et al.*, 1968b). The content of tryptophan was quantitated on the intact protein both by the spectrophotometric method of Goodwin and Morton (1946) and by reaction with NPS-Cl, as recently proposed by Scoffone *et al.* (1968).

Kinetic Measurements. The time course of methionine photooxidation was determined by removing known aliquots of the reaction mixture at intervals during the illumination and by chromatographing them on paper, using water as eluent. All the operations were carried out in a dimly lit room. At the end, the methionine spots were cut out of the sheets in strips of identical size and extracted from the paper with water; the resulting solution was treated with ninhydrin according to the method of Troll and Cannan (1953), and its optical density was measured at 570 nm. The amounts of methionine were calculated by interpolation with a calibration curve obtained with known amounts of control. An equal area of un-

[2] In the case of lysozyme, the molarity of the solution was calculated on the basis of a molecular weight of 14,386 (Jollès, 1960).

[3] In all the experiments reported in the present paper, the pH of the solutions of amino acids and of lysozyme in deionized water did not change during the irradiations.

TABLE I: Per Cent Recovery of Amino Acids after Irradiation in the Presence of Various Porphyrins.[a]

Sensitizer	Amino Acid				
	Cystine	Methionine	Histidine	Tyrosine	Tryptophan
Chlorophyll a	98.0	0.0	12.4	50.2	18.6
Haemin	100.0	95.6	97.8	99.0	97.0
Hematoporphyrin	100.0	0.0	98.6	100.0	61.5
Protoporphyrin IX dimethyl ester	100.0	98.5	99.0	98.5	98.7

[a] The irradiation was carried out in deionized water solution (pH 6.1) at 37°; the reaction mixture was 10^{-3} M in amino acid and in sensitizer. The recovery was evaluated after 10-min exposure to light. There was no change in the pH of the solutions during this period of irradiation.

stained sheet was treated with the same volume of water and used as a blank for the spectral reading.

Chromatographic Analyses. The samples of native and of dye-freed photooxidized lysozyme were analyzed chromatographically on a column (60 × 0.9 cm) of Amberlite CG-50, according to the method of Tallan and Stein (1953). The experimental procedure was the same as previously described (Jori *et al.*, 1968b).

Enzymic Activity. The assay of the enzymic activity of the dye-freed photooxidized lysozyme was based on the method of Smolelis and Hartsell (1949), using *Micrococcus lysodeikticus* as the substrate. *Micrococcus* (1.5 mg) was suspended in 3 ml of a 0.1 M sodium phosphate buffer solution (pH 6.2) and added with 20 μl of a 3.5×10^{-4} M enzyme solution. The hydrolysis of the substrate was followed at room temperature by measurements of the decrease in absorbance at 540 nm. The activity was calculated from the slopes of initial velocities. A solution of native lysozyme at the same protein concentration was always assayed along with the photooxidized enzyme.

Reaction with Cyanogen Bromide. The reaction of lysozyme with cyanogen bromide was carried out according to the method of Gross and Witkop (1962), as adapted for lysozyme by Bonavida *et al.* (1967), and further modified by us. To a solution of 14.4 mg (1 mmole) of lysozyme in 10 ml of 80% formic acid which had been thoroughly deaerated with nitrogen, a 50-fold molar excess of cyanogen bromide was added. The solution was shaken for 1 hr at 50° and for an additional 24 hr at room temperature within a tightly closed glass-stoppered container. The excess of cyanogen bromide and the volatile side products of the reaction, such as methyl thiocyanate and hydrogen chloride, were removed by lyophilization and by subsequent gel filtration on a column (40 × 0.9 cm) of Sephadex G-25, which had been previously equilibrated with 5% acetic acid. Protein (11.8 mg) was recovered by lyophilization.

Amino End-Group Analysis. The amino-terminal groups of the CNBr cleavage products were determined by reaction of lysozyme with 2-chloro-3,5-dinitropyridine, following the method of Signor *et al.* (1964). The 2,4-dinitropyridyl protein was hydrolyzed in 6 N HCl within evacuated sealed vials for 30 min at 100°; under these conditions, the quantitative detachment of the 2,4-dinitropyridylamino acids is achieved. The hydrolysate was adjusted to a pH around 2 and the 2,4-dinitropyridyl derivatives were extracted with ethyl acetate. The mixture of the 2,4-dinitropyridylamino acids was separated by descending paper chromatography on Whatman No. 1, using a 1.5 M sodium phosphate buffer solution (pH 6.15) as the eluent. The yellow spots of the 2,4-dinitropyridylamino acids, identified by comparison of the R_F values with those of controls, were cut out of the paper and eluted with a 1% sodium bicarbonate solution, and the concentration of the resulting solution was determined by measurements of the optical density at 340 nm on the basis of the reported extinction coefficients for the single 2,4-dinitropyridylamino acids (Signor *et al.*, 1964).

Instrumentation. An Hitachi Perkin-Elmer Model 139 spectrophotometer was used for absorbance measurements at single wavelengths; continuous spectra were recorded with an Optica CF 4 DR spectrophotometer. Two matched quartz cuvets of 1-cm path length were employed. Difference spectra were obtained by the procedure previously described (Jori *et al.*, 1968b). The measurements of optical rotation were carried out by means of a Perkin-Elmer Model 141 polarimeter, with a mercury arc as the light source.

Results

Susceptibility of Amino Acids to the Photodynamic Action of Porphyrins. The relative efficiency of some selected porphyrins in sensitizing the photodegradation of amino acids in deionized water solution (pH 6.1) is shown in Table I. Out of the naturally occurring amino acids, only those which are potentially susceptible to the photodynamic action of dyes (Weil *et al.*, 1951) were tested. The values quoted in the table indicate the per cent recovery of the single amino acids after 10-min illumination. These data cannot be compared directly, since no corrections were applied for the molar extinction coefficients of the different porphyrins and for the spectral distribution of the light energy emitted by the tungsten lamps employed. However, a rather clear-cut relationship between the structure of the dye and the ability to sensitize the photooxidation of amino acids in these particular experimental conditions appears to exist.

Chlorophyll a, which contains the diamagnetic ion Mg^{2+}, was the most efficient sensitizer, since it catalyzed the photooxidation of methionine, histidine, tyrosine, and tryptophan at a high rate. This was expecially true in the case of methio-

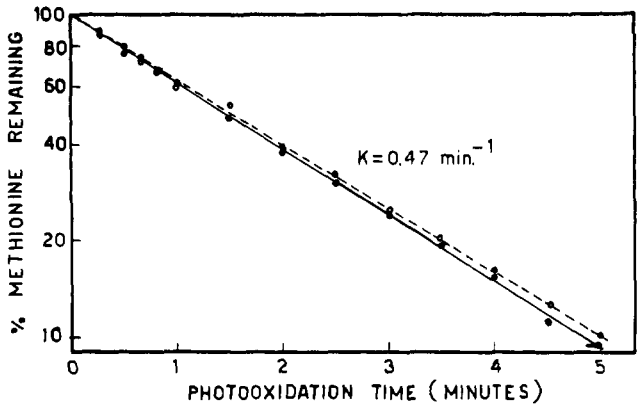

FIGURE 1: Time course of the hematoporphyrin-sensitized photooxidation of L-methionine (●—●) and of *N*-carbobenzyloxy-L-methionyl-L-aspartic acid (○--○) in deionized water solution (pH 6.1) at 37°. The reaction mixture was 10^{-3} M in both the substrate and in the sensitizer. The pH of the solution did not change during the irradiation.

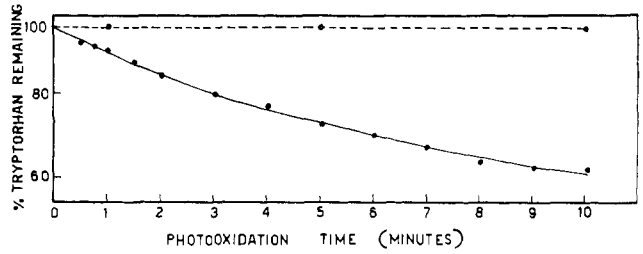

FIGURE 2: Time course of the hematoporphyrin-sensitized photooxidation of L-tryptophan (●—●) and of *N*-carbobenzyloxy-L-tryptophylglycine ethyl ester (○--○) in deionized water solution (pH 6.1) at 37°. The reaction mixture was 10^{-3} M both in the substrate and in the sensitizer. The pH of the solution did not change during the irradiation. The results obtained with other tryptophan-containing oligopeptides (see Materials) were identical with those obtained in the case of Z-Trp-Gly-OEt.

nine, which was oxidized to the extent of 50% in less than 2 min. Substitution of the paramagnetic ion Fe^{3+} for magnesium in the porphyrin molecule caused a dramatic loss of photodynamic activity; actually, when hemin was used as the sensitizer, all the tested amino acids were recovered in amounts higher than 90%, even if the exposure to light was prolonged for several hours.

Some important differences are also found if one compares the results obtained in the presence of hematoporphyrin and of protoporphyrin IX dimethyl ester. These dyes contain no metal ions, and differ from each other in the side chains which protude from the tetrapyrrolic core of the molecule; in protoporphyrin, the carboxyl functions of the two propionic substituents are methylated and two vinyl groups are in the place of the two hydroxyethyl residues (Vernon, 1961). It appears from Table I that protoporphyrin was devoid of photocatalytic efficiency toward amino acids, at least in the aforesaid experimental conditions. By contrast, in the presence of hematoporphyrin, the photooxidation of both tryptophan and methionine did occur; once again, the latter substrate was the most rapidly oxidized.

In all the reaction systems studied, cystine was quantitatively recovered from the irradiated solutions. This lack of photoreactivity is in agreement with previous findings (Sluyterman, 1962; Benassi *et al.*, 1967; Jori *et al.*, 1968a) and further supports the conclusion that the disulfide bridges in polypeptides and proteins are not affected during dye-sensitized photooxidations.

Since the pH and the polarity of the solvent are known to be important parameters in dye-sensitized photooxidations, a few experiments with hematoporphyrin and chlorophyll a were also run in aqueous buffered solutions and in acetic acid solutions. The results obtained in 0.1 M phosphate buffer (pH 6.5) were coincident with those achieved in deionized water. However, the rate of the chlorophyll-sensitized photooxidation of histidine and tyrosine was markedly depressed by lowering the pH of the solution, and these amino acids were no longer photoreactive at pH values less than 5, or if acetic acid in concentrations higher than 5% was added to the system. On the other hand, methionine and tryptophan were susceptible of photooxidation also in pH 2.5 buffered solutions or in strongly acidic media, such as 99–100% acetic acid. In particular, the reaction rate was enhanced when the irradiation was carried out in the presence of acetic acid.

In all the cases above described, no loss of the single amino acids occurred in the light and dark control experiments, or if the solutions were thoroughly degassed prior to irradiation by the freezing–thawing method. The destruction of amino acids in our systems, therefore, satisfies all of the requirements for a photodynamic process (McLaren and Shugar, 1964).

Hematoporphyrin-Sensitized Photooxidation of Tryptophan and Methionine. Clearly, hematoporphyrin appeared to afford the most selective reaction conditions for the photooxidation of tryptophan and methionine in neutral aqueous solution. A more detailed investigation about the photodynamic action of this sensitizer was therefore undertaken with the aim to further differentiate the two amino acids.

At first, the photooxidation rates of tryptophan and methionine in deionized water solution at 37° were compared with those of some model peptides. As one can see from Figure 1, the incorporation of methionine into a peptide molecule did not appreciably affect the reaction kinetics: both for the free amino acid and for Z-Met-Asp-OH, the time course of photooxidation was first order, within experimental error, at least down to 90% of conversion; in both cases the monomolecular rate constant was 0.47 min^{-1}. Moreover, methionine was quantitatively converted to one product, *i.e.*, methionine sulfoxide.

On the contrary, when bound in a peptide molecule, tryptophan showed a drastic drop in reactivity (see Figure 2). No significant loss of the tryptophyl residue was detected after irradiation of several tryptophan-containing oligopeptides (see Materials) in the presence of hematoporphyrin, both in aqueous and in acetic acid solution. A similar discrepancy between the susceptibility to photooxidation of tryptophan, whether free or bound in a peptide, had been previously observed on irradiation of this amino acid in acetic acid solution, sensitized by rose bengal or by methylene blue (Jori *et al.*, 1968a). This dual behavior was shown to be due to the involvment of the α-amino group of tryptophan in a photocyclodehydrogenation reaction, leading to β-carboline derivatives (Jori *et al.*, 1969); no reaction took place when the α-amino group was involved in a peptide linkage. A direct implication of the α-amino group in the photooxidative degradation of

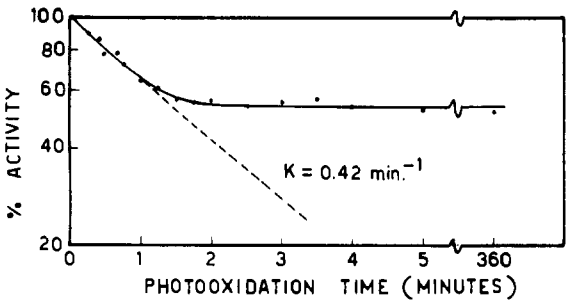

FIGURE 3: Time course of the hematoporphyrin-sensitized photoinactivation of lysozyme in deionized water solution (pH 6.1) at 37°. The reaction mixture was 10^{-3} M both in lysozyme and in the dye. In all assays, a suspension of dead cells of *M. lysodeikticus* was used as the substrate. The activities are expressed as the percentage of the activity observed before the irradiation of the solution began.

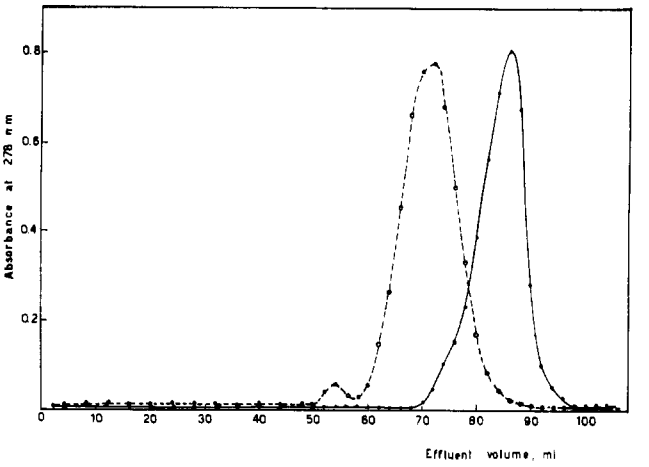

FIGURE 4: Chromatography of native (●—●) and of 15-min photooxidized (○--○) lysozyme on an Amberlite CG-50 column (60 × 0.9 cm). The eluting buffer was 0.2 M sodium phosphate (pH 6.47). The flow rate was 10 ml/hr.

tryptophan is probable also in this case. Actually, thin-layer chromatography of the irradiated tryptophan solutions showed that the disappearance of the amino acid was not paralleled by the formation of any ninhydrin-positive product; this suggests that the α-amino group was destroyed or masked in some way during photooxidation.

A further check of the unreactivity of tryptophan, when protected at the amino group, under these reaction conditions, was carried out by irradiation of the dipeptide Z-Trp-Met-OMe, which contains both the susceptible amino acids. Paper chromatographic analysis showed that the corresponding Z-Trp-Met(O)OMe was obtained as the only product in quantitative yields.

It is apparent from these data that hematoporphyrin acts on the methionyl residues with absolute specificity in a great variety of reaction media, ranging from pH 2.5 to 6.5 and from 5 to 100% acetic acid. We wish to emphasize the possibility of converting methionine selectively to methionine sulfoxide in neutral aqueous solutions. This result cannot be achieved with the sensitizers commonly used owing to the competitive photooxidation of other amino acid residues.

Besides the selectivity for methionine, a noteworthy feature of the described method is the high rate of the photooxygenation of the thioether function of methionine. In order to shed further light on the pathway of this photoreaction, we investigated whether an interaction of some sort between the amino acid and the sensitizer took place. Actually, some proposed mechanisms for photodynamic action postulate the occurrence of a binding reaction between dye and substrate; such reaction might alter the absorption spectrum of the dye (Ghiron and Spikes, 1965). In the present case, however, no changes in the visible absorption spectrum of haematoporphyrin have been observed on the addition of 10^{-3} M methionine. The measurements were performed over the pH range 2.5–6.5, as well as in acetic acid solution. In particular, there was no variation in the extinction at the wavelengths corresponding to the absorption peaks of the dye.

The effect of temperature on the kinetics of methionine photooxidation was also examined. The rate was found to decrease slightly with decreasing temperature, indicating that the hematoporphyrin-sensitized photooxidation of methionine is not a pure photochemical process. However, over the temperature range studied (2–37°), the observed variations were small and may also be accounted for by the participation of diffusion-controlled processes.

Photooxidation of the Methionyl Residues in Lysozyme. The actual feasibility of the procedure above described for the selective modification of the methionyl residues in proteins was tested with lysozyme. This enzyme was chosen since the selective photooxidation of the two methionyl residues had been previously performed by irradiation in 84% acetic acid solution, and in the presence of methylene blue (Jori *et al.*, 1968b); therefore, suitable data for comparison with the present findings were available.

The results of a typical experiment, in which the samples of lysozyme were irradiated for various times in deionized water (pH 6.1) freed of dye and assayed for enzymic activity, are shown in Figure 3. Quite similar plots were obtained in aqueous-buffered solutions (pH 6.5). It can be seen that, under the conditions employed, the enzymic activity of lysozyme decreased rapidly and then leveled off at values around 54% after about 2-min exposure to light. Prolonging the irradiation for several hours caused no further lowering of the catalytic efficiency of the enzyme. It is of interest that these curves followed first-order kinetics for approximately 1 min; the rate constant for inactivation, as calculated from the slope of the linear portion of the curve, was 0.42 min^{-1}. This value was very close to that of the first-order rate constant experimentally determined for the photooxidation of free methionine. The coincidence of the two constants was a clear indication, although not a proof, that the modification of one or both the methionyl residues was critically connected with the lowering of the enzymic activity of lysozyme.

The limited inactivation of lysozyme on photooxidation could result from the formation of a partially active enzyme or from a mixture of fully active and of inactive species. The latter possibility, however, was ruled out by chromatographic analysis of the photooxidized product on the carboxylic acid resin Amberlite CG-50. It appears from Figure 4 that the 15-min photooxidized lysozyme was eluted from the column as one peak, with a slightly higher R_F than the native enzyme. The fractions corresponding to the tubes from 60 to 68 were

TABLE II: Amino Acid Content of Untreated and of 10-min Photooxidized Lysozyme.[a]

Amino Acid	Theory	Untreated	Photooxidized in Deionized Water (pH 6.1)	Photooxidized in 84% Acetic Acid
Methionine sulfoxide[b]	0	0.0	0.9	1.9
Methionine[b]	2	1.8	1.2	0.0
Half-cystine	8	7.8	7.7	7.8
Tryptophan[c]	6	6.0	5.8	5.8
Tyrosine	3	2.8	2.8	2.6
Histidine	1	0.8	0.8	1.0

[a] The amino acids were determined chromatographically with a Technicon automatic analyzer after 22-hr hydrolysis in 6 N HCl at 110°. The table includes only those amino acids which are known to be affected by photooxidation. No appreciable change was found in the other amino acids analyzed. The values in the table denote number of residues per molecule. [b] Evaluated by automatic chromatographic analysis after 14-hr hydrolysis in 3.75 N NaOH at 100° (Jori *et al.*, 1968b). [c] Evaluated on the intact protein both by the method of Goodwin and Morton (1946) and by reaction with 2-nitrophenylsulfenyl chloride, as proposed by Scoffone *et al.* (1968). The two methods agreed very well.

pooled and lyophilized and the recovered product was found to have a 52% residual activity. Identical elution patterns and recoveries of enzymic activity have been obtained with samples irradiated for longer times up to 6 hr. Clearly, only negligible amounts, if any, of native lysozyme were present in the photooxidized sample.

The amino acid composition of the 54% active lysozyme, after 10-min irradiation in deionized water (pH 6.1), is shown in Table II and compared with that of the native enzyme. All the amino acids which are present in lysozyme (Canfield, 1963) were examined but the table includes only those which are theoretically susceptible to photooxidation. It can be seen that the photooxidized enzyme differed from the untreated enzyme in one point: one methionine sulfoxide residue appeared in the place of one methionine residue. This result was repeated several times with samples which had been irradiated for different periods. The standard deviation from the value reported in the table was ±0.2. One must conclude, therefore, that, under the experimental conditions used, only one out of the two methionyl residues in lysozyme is available for photooxidation; the conversion into sulfoxide of the thioether function of this uniquely reactive residue is responsible for the reduced catalytic efficiency of the enzyme.

The monosulfoxide derivative of lysozyme showed no appreciable changes in the ultraviolet absorption spectrum with respect to the native lysozyme. Actually, the difference absorption spectrum, obtained by reading a 5×10^{-4} M aqueous solution of photooxidized lysozyme against an equimolar solution of the unirradiated protein, was devoid of noteworthy features, except for two small troughs at 276 nm and at 294 nm. In particular, no positive absorption maximum appeared around 300 nm, that is, in a spectral region where only the tryptophyl residues significantly contribute to absorption. On the other hand, the specific rotation $-[\alpha]_{366}^{30}$ (c 0.2%, water) increased from 230.5° for the native enzyme to 241° for the photooxidized sample.

Now, when lysozyme was subjected to methylene blue sensitized photooxidation in 84% acetic acid solution, both the methionyl residues were converted into the sulfoxide and the enzymic activity dropped to about 6% (Jori *et al.*, 1968b). Since all the other conditions were the same, the discrepancy between the previous and the present results could derive either from the different sensitizer or from the change in the polarity and acidity of the solvent. However, if lysozyme was irradiated in 84% acetic acid solution and in the presence of hematoporphyrin, a disulfoxide derivative with 4% enzymic activity was obtained (see Table II). This product was indistinguishable as to chromatographic behavior on Amberlite CG-50, difference absorption spectra, and optical rotation values at λ 366 nm from the one obtained in the methylene blue sensitized photooxidation. These data point out that no difference exists between the two products in the amino acid composition as well as in the spatial configuration.

Consequently, the conclusion must be drawn that the different susceptibility to photooxidation of the two methionyl residues is due to the changes in the conformation of the lysozyme molecule which are induced by the presence of concentrated acetic acid. A similar dependence of the reactivity of the single tryptophyl residue in lysozyme upon the nature of the solvent was previously observed by Previero *et al.* (1967).

Identification of the Photoreactive Methionyl Residue. In order to identify the location of the photoreactive methionine residue in the polypeptide chain of lysozyme, the 54% active photooxidized protein was allowed to react with cyanogen bromide and subsequently analyzed for the amino end groups. Since methionine sulfoxide is insensitive to the attack from cyanogen bromide (Gross and Witkop, 1962), the lysozyme molecule should be cleaved similar to the unreacted methionyl residue. According to the reported sequence of lysozyme (Canfield, 1963), the amino acids lysine and asparagine are, respectively, located at the carboxyl end of the Met-12 and Met-105 residues. Unfortunately, a lysyl residue is also placed at the NH_2-terminal end of the polypeptide chain of lysozyme; in order to draw definite conclusions, therefore, the yields in the 2,4-dinitropyridyl derivatives must be as quantitative as possible.

Preliminary experiments (G. Galiazzo and G. Jori, unpublished results) have shown that lysozyme reacts very sluggishly

with cyanogen bromide under the conditions proposed for RNase A (Gross and Witkop, 1962). On the other hand, an appreciable extent of cleavage was achieved by running the reaction in strongly acid media, such as concentrated formic acid, as proposed by Bonavida *et al.* (1967). Furthermore, the destruction of methionine could be markedly enhanced by increasing the temperature and the reaction time, and by saturating the reaction flask with nitrogen prior to the addition of the cyanogen bromide (see Methods).

Under these conditions, the native enzyme yielded 1.7 moles of bis(2,4-dinitropyridyl)lysine and 0.65 mole of 2,4-dinitropyridylaspartic acid per mole of protein. By contrast, the photooxidized lysozyme appeared to contain 0.8 mole of bis(2,4-dinitropyridyl)lysine and 0.8 mole of 2,4-dinitropyridylaspartic acid, respectively. Although these values are somewhat lower than the theoretical ones, there is clear evidence that only Met-105 is susceptible of reacting with cyanogen bromide in irradiated lysozyme. Therefore, Met-12 is the site of attack during the hematoporphyrin-sensitized photooxidation of lysozyme.

Discussion

It is apparent from our results that the photodynamic effectiveness of the porphyrin dyes is strikingly dependent on the molecular structure. In this connection, two factors seem to be of major importance: the presence and the nature of a complexed metal ion, and the type of the side chains attached at the corners of the pyrrole rings.

Mg^{2+}–chlorophyll a is a very efficient sensitizer, if compared with the other porphyrins examined. This is to be expected if the triplet state is the reactive one in porphyrin-sensitized photochemical processes, it has been suggested by several authors (Fujimori and Livingston, 1957; Pekkarinen and Linschitz, 1960). Actually, it has been shown that porphyrins containing diamagnetic metal ions have a stronger phosphorescence than the corresponding free compounds (Allison and Becker, 1963), as well as a much higher steady-state triplet population and a longer triplet lifetime (Azizova *et al.*, 1966), due to the lowering of the rate of nonradiative decay. Accordingly, one could assume in the present case that the introduction of the Mg^{2+} ion into the chlorophyll molecule leads to a high population of the triplet state of the dye, which in turn increases the efficiency of the photodynamic process. This hypothesis is further supported by the ineffectiveness of haemin as sensitizer, since it is well known that paramagnetic ions are very good quenchers of the triplet state (see, for example, Calvert and Pitts, 1966). It is clear, however, that more detailed investigations are necessary before any definite conclusion can be drawn on the role performed by the triplet state in the porphyrin-sensitized photooxidations of amino acids.

A comparison of the results obtained with hematoporphyrin and with protoporphyrin IX dimethyl ester suggests that the nature of the lateral substituents also markedly affects the pathway and the rate of the amino acid photooxidations. This factor may partially contribute to enhance the photodynamic activity of chlorophyll a, since the lateral substituents of chlorophyll a are different from those of the other porphyrins examined. Work is in progress in order to further elucidate the relative importance of the two factors discussed above in determing the efficiency of a given porphyrin as sensitizer.

In any case, such a remarkable dependence of the photodynamic efficiency of porphyrins upon the molecular structure introduces a wide range of new prospects into the field of the photochemical modifications of amino acids in biological systems.

A typical example of the original possibilities opened by the use of this class of sensitizers is given by the selective photooxidation of the methionyl residues in neutral aqueous solution, that is, in a medium which does not alter the native conformation of polypeptides and proteins, as well as in media which can perturb the three-dimensional geometry of proteins (*e.g.*, concentrated acetic acid).

This peculiarity enabled us to differentiate the two methionyl residues which are present in lysozyme as to their "exposure" to solvent in the native protein. Actually, the fact that only methionine-12 is readily converted to the sulfoxide in neutral aqueous solution, that is, when lysozyme is in its native conformation, strongly suggests that this residue is located at or near the surface of the molecule.

The slight increase in the levorotation of the monosulfoxide lysozyme, if compared with the native enzyme, suggests that a partial denaturation of the lysozyme molecule takes place on photooxidation. However, the comparison of the present data with those obtained for the disulfoxide lysozyme (Jori *et al.*, 1968b) points out that the degree of unfolding of the polypeptide chain is rather limited; in particular, the unfolding process does not involve the segments of the chain which include the tryptophyl residues, as one can deduce from the absence of any absorption around 300 nm in the difference spectrum of the photooxidized lysozyme *vs.* the native lysozyme.

The second methionyl residue of lysozyme, *i.e.*, methionine-105, is not altered by photooxidation, unless the enzyme is dissolved in 84% acetic acid. Evidently, in the native protein, this residue is buried in a hydrophobic cluster and is therefore shielded from contact with the photoexcited dye molecule.

These conclusions are consistent with previous studies, since physicochemical measurements have established that lysozyme has a tightly folded inflexible conformation (Williams *et al.*, 1965), which is resistant to the action of physical and chemical denaturing agents (Hamaguchi and Rokkaku, 1960; Tanford *et al.*, 1966). Moreover, from the X-ray structure of lysozyme (Phillips, 1967), it is apparent that the situation of the two methionyl residues is appreciably different. Methionine-12 is included in the N-terminal segment of the polypeptide chain which gets out of the core of the molecule; on the other side, methionine-105 is almost "sandwiched" between two tryptophyl side chains, that is, in a very effective hydrophobic environment.

In conclusion, it appears that further potential useful applications of the described method are the auditing of tertiary structures which have been determined by X-ray analysis or other procedures, or obtaining original information about the spatial location of the methionyl residues in proteins or enzymes whose tertiary structures are still unresolved.

Acknowledgment

We gratefully acknowledge the skillful technical assistance of Mr. Orfeo Buso during this work.

References

Allison, J. B., and Becker, R. S. (1963), *J. Chem. Phys. 67*, 2662.

Azizova, O. A., Gribova, Z. P., Kayushin, L. P., and Pulatova, M. K. (1966), *Photochem. Photobiol. 5*, 763.
Benassi, C. A., Scoffone, E., Galiazzo, G., and Jori, G. (1967), *Photochem. Photobiol. 6*, 857.
Bonavida, B., Miller, A., and Sercarz, E. (1967), *Federation Proc. 26*, 339.
Calvert, J. G., and Pitts, J. N., Jr. (1966), Photochemistry, New York, N. Y., Wiley, p 302.
Canfield, R. E. (1963), *J. Biol. Chem. 238*, 2698.
Della Pietra, D., and Dose, K. (1965), *Biophysik 2*, 347.
Fujimori, H., and Livingston, R. (1957), *Nature 180*, 1036.
Galiazzo, G., Jori, G., and Scoffone, E. (1968), *Biochem. Biophys. Res. Commun. 31*, 158.
Ghiron, C. A., and Spikes, J. D. (1965), *Photochem. Photobiol. 4*, 901.
Goedheer, J. C. (1966), *in* The Chlorophylls, Vernon, L. P., and Seely, G. R., Ed., New York, N. Y., Academic.
Goodwin, T. W., and Morton, R. A. (1946), *Biochem. J. 40*, 628.
Gross, E., and Witkop, B. (1962), *J. Biol. Chem. 237*, 1856.
Hamaguchi, K., and Rokkaku, K. (1960), *J. Biochem. (Tokyo) 48*, 358.
Jollès, P. (1960), *Enzymes 4*, 435.
Jori, G., Galiazzo, G., and Gennari, G. (1969), *Photochem. Photobiol. 9*, 179.
Jori, G., Galiazzo, G., Marzotto, A., and Scoffone, E. (1968a), *Biochim. Biophys. Acta 154*, 1.
Jori, G., Galiazzo, G., Marzotto, A., and Scoffone, E. (1968b), *J. Biol. Chem. 243*, 4272.
Livingston, R., and Pugh, A. C. (1959), *Discussions Faraday Soc. 27*, 144.
McLaren, A. D., and Shugar, D. (1964), *in* Photochemistry of Proteins and Nucleic Acids, Alexander, P., and Bacq, Z. M., Ed., London, Pergamon, p 26.
Pekkarinen, L., and Linschitz, H. (1960), *J. Am. Chem. Soc. 82*, 2407.
Phillips, D. C. (1967), *Proc. Natl. Acad. Sci. U. S. 57*, 485.
Previero, A., Coletti-Previero, M. A., and Jollès, P. (1967), *J. Mol. Biol. 24*, 261.
Ray, J. W., Jr., and Koshland, D. E., Jr. (1962), *J. Biol. Chem. 237*, 2493.
Robinson, G. W. (1964), *Ann. Rev. Phys. Chem. 15*, 311.
Scoffone, E., Fontana, A., and Rocchi, R. (1968), *Biochemistry 7*, 971.
Signor, A., Biondi, L., Terbojevich, M., and Pajetta, P. (1964), *Gazz. Chim. Ital, 94*, 619.
Singer, S. J. (1962), *Advan. Protein Chem. 17*, 1.
Sluyterman, L. A. Ae. (1962), *Biochim. Biophys. Acta 60*, 557.
Smillie, L. B., and Neurath, H. (1959), *J. Biol. Chem. 234*, 355.
Smolelis, A. M., and Hartsell, S. E. (1949), *J. Bacteriol. 58*, 731.
Spikes, J. D., and Glad, B. W. (1964), *Photochem. Photobiol. 3*, 471.
Spikes, J. D., and Straight, R. (1967), *Ann. Rev. Phys. Chem. 18*, 409.
Tallan, H. H., and Stein, W. H. (1953), *J. Biol. Chem. 200*, 507.
Tanford, C., Pain, R. H., and Otchin, N. S. (1966), *J. Mol. Biol. 15*, 489.
Troll, W., and Cannan, R. K. (1953), *J. Biol. Chem. 200*, 803.
Vernon, L. P. (1961), *Acta Chem. Scand. 15*, 1639.
Veronese, F., Boccu', E., Benassi, C. A., and Scoffone, E. (1969), *Z. Naturforsch. 24b*, 234.
Veronese, F., Fontana, A., Boccu', E., and Benassi, C. A. (1967), *Gazz. Chim. Ital. 97*, 321.
Weil, L. (1965), *Arch. Biochem. Biophys. 110*, 57.
Weil, L., Gordon, W. G., and Buchert, A. R. (1951), *Arch. Biochem. Biophys. 33*, 90.
Williams, E. J., Herskovits, T. T., and Laskowski, M., Jr. (1965), *J. Biol. Chem. 240*, 3524.