SELECTIVE AND QUANTITATIVE PHOTOCHEMICAL CONVERSION OF THE TRYPTOPHYL RESIDUES TO KYNURENINE IN LYSOZYME

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Dye-sensitized photooxidation is increasingly used as a tool for the elucidation of the structure-activity relationship in proteins. However, the similarity in the relative rates of oxidation of the susceptible amino acids is a serious limitation on the method. In a recent paper (Benassi et al., 1967), we showed that, in formic acid solution, only tryptophan and methionine are photoreactive, if proflavine is used as the sensitizer: the former is converted to kynurenine, the latter to methionine sulphoxide. Since methionine sulphoxide is easily reverted to methionine by reaction with mercaptans (Hofmann et al., 1966; Jori et al., 1968), the possibility exists to perform selective photooxidation of the tryptophyl residues. The method was successfully applied to model peptides (Benassi et al., 1967), however, its feasibility in the case of proteins could only be evaluated through actual application.

This report details our findings about the photodynamic action of proflavine on lysozyme (N-acetylmuramide glycanohydrolase, EC 3.2.1.17). Lysozyme was chosen since its threedimensional configuration has been elucidated (Blake et al., 1967), and the chemistry of the enzyme has been thoroughly investigated.

EXPERIMENTAL

Lysozyme (Worthington, Lot LYSF 6 JA) was irradiated in a vessel made by a water-cooled jacket surrounding a high pressure Hg-lamp (Hannovia

Q 1200/PL 357). The wavelenghths under 320 nm were cut off by a Pyrex filter. The optical path was 0.5 cm. In one experiment 64 mg of lysozyme were dissolved in 300 ml of 98-100% formic acid (analytical grade Merck product), added in the dark with 0.6 mg of proflavine, and exposed to light. During irradiation a stream of purified oxygen was slowly fluxed through the solution. The temperature was kept at 1^0-2^0 C. At the end, the solvent was removed by lyophilization, the residue was taken up with 0.1 M acetic acid, and freed of the dye by gel filtration on a column (1 cm x 60 cm) of Sephadex G-25 (Pharmacia, Uppsala, Sweden), using 0.1 M acetic acid as eluent.

In order to revert the methionine sulphoxide to methionine the reduction of the photooxidized lysozyme was performed by dissolving the enzyme in 5% aqueous 2-mercaptoethanol, at a concentration of 0.1 mg/ml. The reaction was allowed to run for 24 hr. at 20^0 in a nitrogen atmosphere. Under these conditions, some out of the four -SS- bonds of the molecule are disrupted (Azari, 1966). Therefore, the reduced product was air-oxidized by the procedure of Epstein and Goldberger (1963). The number of -SS- bonds, cleaved in the reaction of lysozyme with 2-mercaptoethanol, was estimated by the method of Sela et al. (1959).

The samples for amino acid analysis were hydrolyzed in 6 M HCl in evacuated sealed tubes at 110^0 for 22 hours. The content of methionine sulphoxide was evaluated after alkaline hydrolysis, as already described (Jori et al., 1968). The analyses were performed on a Technicon Autoanalyzer.

Spectra were recorded by means of an Optica CF 4 DR spectrophotometer.

RESULTS AND DISCUSSION

Photooxidation of lysozyme was attended by a gradual disappearance of the absorption maximum at 282 nm, which is mainly due to the indole chromophores. As shown in Fig. 1, after 1 hr. irradiation, the photooxidized product presented two maxima, which were located at 258 nm and at 360 nm, and are characteristic of kynurenine (Benassi et al., 1967). Prolonging the irradiation caused no further change in the extinction at both wavelengths.

The extent of the conversion of tryptophan to kynurenine was determined by amino acid analysis, after acid hydrolysis. The data are reported in Table I. All the amino acids were examined, and the table includes only those which are

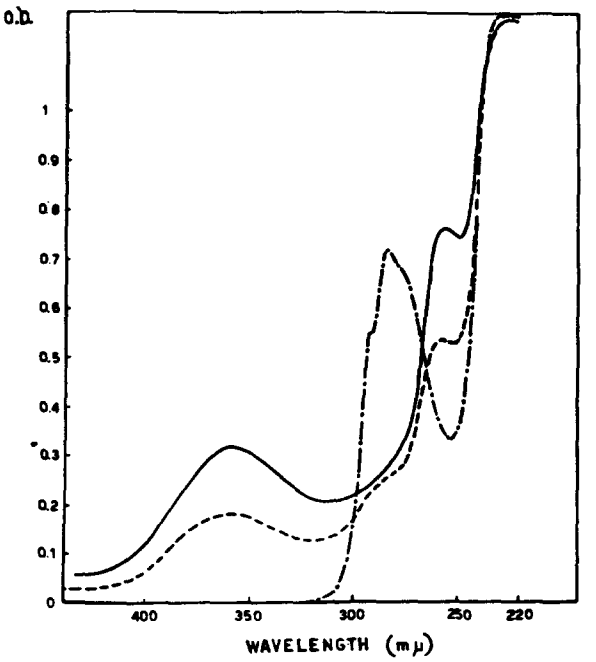

Fig. 1 - Absorption spectra of lysozyme 2.1×10^{-5} M in water at different stages of photooxidation:

—·—·— 0 min., — — — 30 min., —— 60 min.

known to be susceptible of photooxidation. It appears that all the six tryptophanyl residues were converted to kynurenine, all the other amino acids were unaffected. However, alkaline hydrolysis showed that 1.9 methionyl residues were oxidized to the sulphoxide. The homogeneity of the photooxidized product was also checked by chromatographic analysis on Amberlite CG-50, using 0.2 M phosphate buffer, pH 7.18, as eluent. As shown in Fig.2, the oxidized lysozyme emerged from the column as a main peak, ahead of the native protein.

Photooxidation caused an extensive inactivation of lysozyme. Activity measurements, performed according to the method of Smolelis and Hartsell (1949), pointed out that, after 30 min. irradiation, the enzymic activity was

TABLE I

Amino Acid Analyses of Lysozyme (Residues/molecule)

Amino Acid	Native	Photooxidized	Reduced-Reoxidized
Half-cystine	7.9	7.7	7.8
Methionine (a)	2.0	0.0	1.8
Methionine sulphoxide (a)	0.0	1.9	0.0
Histidine	1.0	0.9	0.9
Tyrosine	2.8	2.7	2.7
Tryptophan (b)	6.0	0.0	0.0
Kynurenine	0.0	6.1	5.9

(a) determined after alkaline hydrolysis

(b) determined by the absorbance at 282 nm ($E^{1\%}_{282\ nm}$ = 26.1)

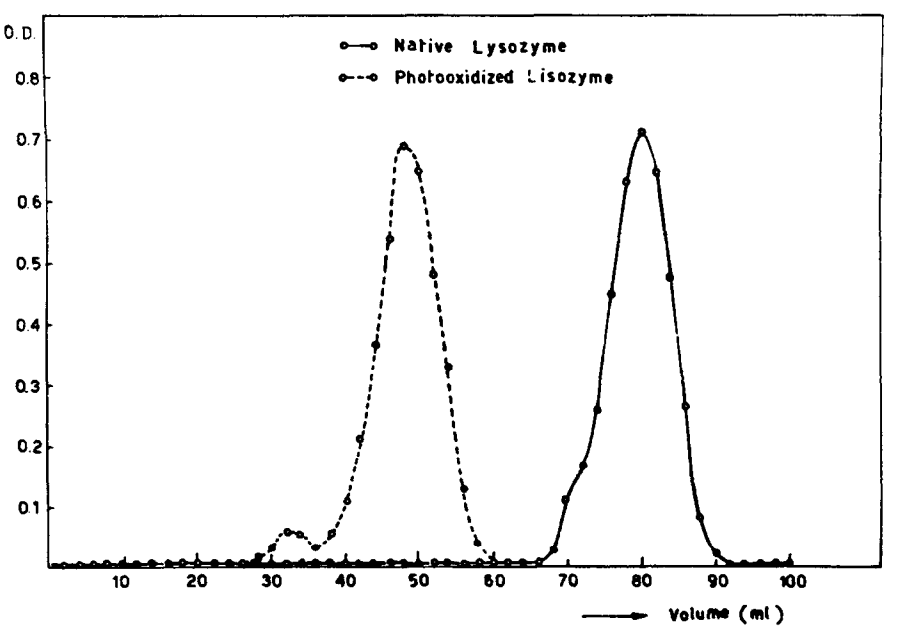

Fig. 2 - Chromatography of native and photooxidized lysozyme on Amberlite CG-50, 200-400 mesh (0.9 cm x 60 cm), eluent 0.2 M phosphate buffer pH 7.18.

less than 2%. At this point, the oxidation of the tryptophanyl residues was not completed, as indicated by the shoulder around 280 nm in the absorption spectrum (see Fig. 1). This would suggest that only some of the six tryptophanyl residues, which are present in the lysozyme molecule, are associated with the catalytic activity of the enzyme. The effect of the conversion of the methionyl residues to sulphoxides is, probably, of minor importance since, under the described conditions, methionine is oxidized at a much slower rate than tryptophan (Benassi et al., 1967).

In order to reduce the methionine sulphoxides, the photooxidized product was allowed to react with 2-mercaptoethanol and subsequently air-oxidized (see experimental). Amino acid analysis of the end-product, after acid and alkaline hydrolysis, showed that six kynurenine residues per protein molecule, but no sulphoxides, were present. The stability of kynurenine to chemical reduction with mercaptans was further supported by the coincidence in the extinction at 360 nm of an aqueous solution of photooxidized lysozyme and of reduced-reoxidized lysozyme.

The reoxidized sample was devoid of enzymic activity. This lack of reactivation may be the consequence either of the presence of the kynurenyl residues at the place of the tryptophyl residues, or of an incorrect pairing of the half-cystine residues: actually, two out of the four -SS- bonds were cleaved in the reaction with 2-mercaptoethanol. However, even if an equal statistic probability is assigned to the possible conformations, which may derive from the recombination of four -SH groups, none of them appears to possess catalytic activity. Therefore, the conversion of tryptophan to kynurenine seems to be the major cause of the loss of activity of the reduced-reoxidized lysozyme.

The oxidation of tryptophan to kynurenine in a polypeptide chain was previously performed by ozonization (Previero and Bordignon, 1964; Galzigna et al.,1964). However, the yields in kynurenine did not exceed 70%, owing to a partial overoxidation. On the contrary, the proflavine-sensitized photooxidation allows a quantitative preparation of kynurenine-containing polypeptides from the corresponding tryptophyl compounds. Owing to the lack of side reactions and high selectivity, this method provides a useful procedure for investigating the importance of tryptophan in biologically active polypeptides. Moreover, the method may be employed for the non-enzymic cleavage of a peptide chain in

correspondance of the tryptophyl residues, by lactonic cyclization after electrochemical (Scoffone et ala, 1966) or chemical (Previero et al., 1966) reduction of the carbonyl group of kynurenine. Further studies concerning such applications are in progress.

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