DYE-SENSITIZED PHOTO-OXIDATION AS A TOOL FOR MAPPING SOME AMINO ACID RESIDUES IN PROTEINS

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Summary. The irradiation of proteins containing a sensitizer covalently bound in known positions of the molecule (e.g. 41--DNP-RNase A) results in the selective modification of the amino acid residues which are adjacent to the sensitizer within the molecule. In this way, the location of the modified side chains in the three-dimensional network of the protein can be deduced.

The dye-sensitized photo-oxidation of proteins is widely used for the modification of specific amino acid residues in order to elucidate their importance for the biological function (1). New routes of application of this photochemical technique are being developed. The possibility of correlating the data of dye-sensitized photo-oxidation with the three--dimensional conformation of proteins was first suggested by Ray and Koshland (2).

In this paper we propose a method which should allow a deeper insight into the problem of the elucidation of the tertiary structure of proteins. The method involves the irradiation of proteins containing the sensitizer covalently linked in known positions of the molecule. In this way, the selective modification of the photo-oxidizable residues which can be contacted by the sensitizer should be achieved and, therefore, it should be possible to deduce the location of the modified side chains in the three-dimensional network of the

protein molecule.

For this aim, a suitable sensitizer can be bound to specific functional groups of the protein. Actually, we ascertained that many group-specific reagents, containing nitro or keto substituents, are able to promote the photo-oxidation of the CysH, Met, His, Tyr and Trp residues in proteins, provided that the irradiations are carried out in slightly alkaline solutions (pH 7.5-9). In this paper, we report some preliminary results, obtained with N_{ε} -41-dinitrophenyl-ribonuclease A (41-DNP--RNase A), which appear to support the validity of the above exposed principles, showing that, by this method, the amino acid residues in a protein can be photo-oxidized with a high spatial selectivity.

EXPERIMENTAL

41-DNP-RNase A was prepared from RNase A (grade V, Seravac Labs.) by the method of Hirs (3). Crystal violet and 2-mercapto-ethanol were reagent grade products of Fluka.

Photo-oxidations were performed at 25° by exposing 8 ml of a 1 mM protein solution to the light of a high pressure mercury lamp (Hanovia Q 1200/PL 357). The wavelengths below 320 nm were cut off by a Pyrex filter. Denaturation of 41-DNP-RNase A was achieved by disrupting the disulphide bridges with 2-mercapto-ethanol (4); recombination of the half-cystine residues was prevented by converting them to cysteic acid through crystal violet-sensitized photo-oxidation of the reduced protein in 16% acetic acid solution: under these conditions, no other constituents of a protein molecules are affected (5).

Amino acid analyses were carried out on a Carlo Erba 3A27 automatic analyzer according to the procedure of Spackmann et al. (6). The content of methionine sulfoxide was evaluated after alkaline hydrolysis (7). Chromatographic analyses of 41-DNP-RNase A were performed on a column (58 x 0.9 cm) of

Amberlite CG-50, according to the procedure of Hirs et al. (8).

RESULTS

The amino acid analysis of 41-DNP-RNase A after 10 min. irradiation at pH 8.2 is reported in Table I. It appears that one Met, one His, and one Tyr residue per protein molecule were photo-oxidized. Prolonging the irradiation for 120 min. yielded identical results. Chromatography of the irradiated samples on Amberlite CG-50 (Fig. 1) showed that they were constituted by one peak. Moreover, gel filtration on Sephadex G-75 showed that no aggregation of the photo-oxidized samples had occurred.

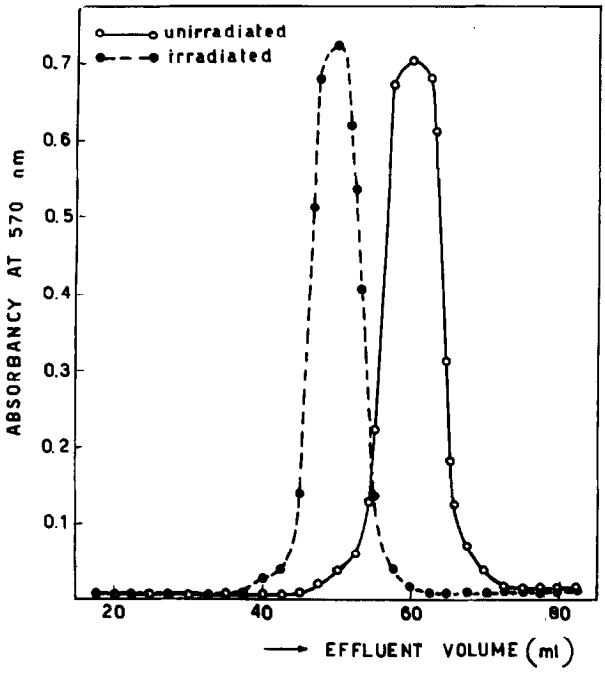

Fig. 1 - Chromatography of unirradiated and photo-oxidized 41-DNP-RNase A (0.5 micromoles) on a column (0.9 x 58 cm) of Amberlite CG-50. The eluent was 0.2 M sodium phosphate buffer, pH 6.44.

TABLE I

AMINO ACID CONTENT OF UNIRRADIATED AND PHOTO-OXIDIZED 41-DNP-RNase A

The amino acids were determined with a Carlo Erba 3A27 analyzer after 22-hours hydrolysis in 6 N HCl at 110°. Only the potentially photo-oxidizable amino acids are reported. No change was found in the other amino acids. The values in the table denote number of residues per molecule. All irradiations were carried out in pH 8.2 buffered solutions.

Amino acid	Unirradiated	Irradiated for 10 min.	Irradiated for 120 min.	Denatured and irradiated for 120 min.
Histidine	3.9	2.9	3.1	0.0
Tyrosine	5.8	5.0	4.9	0.0
Half-cystine	7.8	7.8	7.7	8.0 (a)
Methionine	4.1	3.1	2.8	0.2
Methionine sulfoxide	0.0	1.1	1.1	3.7

(a) Recovered as cysteic acid after crystal violet - sensitized photo-oxidation of the reduced protein (see experimental section).

On the other hand, the denatured 41-DNP-RNase A, upon irradiation, underwent total loss of Met, His and Tyr (Table I, column 4).

In some experiments, the irradiation of 41-DNP-RNase A was run in the presence of variable amounts (3 to 10 mg) of unlabelled RNase A. After chromatographic separation on Amberlite CG-50 (3) only two peaks were isolated, corresponding to irradiated 41-DNP-RNase A and to RNase A. The recovered RNase A displayed enzymic activity, rotatory power at 366 nm, and amino acid analysis coincident with those of the native enzyme.

DISCUSSION

On the basis of our results, the highly limited number of amino acid residues modified by photo-oxidation in 41-DNP-RNase A can be ascribed to the concerted action of two factors: i) the fixed location of the sensitizer within the protein molecule; ii) the restrictions imposed by the spatial network of proteins to the interaction between the excited sensitizer and the potentially photo-oxidizable side chains. Actually, when the tertiary structure of the protein was disrupted, so that the mobility of the DNP group was no longer hindered, the total loss of the photoreactive amino acids did occur. Therefore, the photo-oxidized residues in the native proteins must be those which are located in close proximity to the sensitizer as a consequence of the three-dimensional folding of the molecule.

Alternative interpretations of the data do not appear to be possible. The observed number of photo-oxidized residues cannot be the result of the partial modification of different residues of a given amino acid within one molecule, or of the chance modification of different residues in different molecules. If this were the case, more than one product should have been detected by chromatographic analysis, and the amount of the photo-oxidized amino acids should have been increased by prol-

onging the time of irradiation. Moreover, the progress of the reaction was not inhibited by the formation of aggregates as a consequence of irradiation, as shown by the gel filtration experiments.

Furthermore, the data obtained from photo-oxidation reflect the actual location of the modified residues in the region of the protein molecule surrounding the sensitizer.

First of all, the possibility of intermolecular photo-sensitization can be discarded, since the unlabelled enzyme was not affected when irradiated in the presence of the labelled protein. Moreover, since the attachment of the DNP chromophore to the ϵ-NH_2 group of Lys-41 brings about no significant changes of the conformation of RNase A (9), the modified residues must be actually located close to Lys-41 in the native protein.

In conclusion, the described photo-oxidative technique appears to be quite suitable for the three-dimensional mapping of protein molecules in solution. Work is in progress in order to identify the position of the modified residues in the amino acid sequence of RNase A. Moreover, this method should be appliable to proteins naturally containing a chromophoric group which can act as sensitizer for the photo--oxidation of amino acids, such as the cytochromes or the pyridoxal phosphate depending enzymes.

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