ACETONE-SENSITIZED ANAEROBIC PHOTO-OXIDATION OF METHIONINE

G.GENNARI and G.JORI
Institute of Organic Chemistry, University of Padova, Via Marzolo, 1–35100 Padova, Italy

Received 1 August 1970

1. Introduction

The dye-sensitized photo-oxidation of methionine (Met) to methionine sulfoxide (MetO) has been studied extensively [1]. The reaction appears to proceed through an electrophilic attack on the thioether function of the Met side chain by singlet oxygen, which is produced via energy transfer from the photoexcited sensitizer [2, 3]. In attempting to develop new methods for the photochemical modification of amino acids, we have investigated the photosensitizing action of some water-soluble ketones. Although these compounds are widely employed as sensitizers for photoreactions involving nucleic acids and their constituents [4, 5], their effectiveness in promoting photoreactions of proteins has not yet been studied.

The results presented in this communication show that, in the presence of acetone, the photo-oxidation of Met to MetO also occurs under anaerobic conditions. The method is highly selective and can be usefully applied for probing the positions of the Met residues in the three-dimensional structure of proteins.

2. Materials and methods

L-Methionine (Fluka) and ribonuclease A (EC 2.7.7.16; grade V, Seravac Labs.) were used as received. The peptide *N*-benzyloxycarbonyl-L-methionyl-L-aspartic acid (Z-Met-Asp) was synthesized in this Institute. Acetone was a reagent grade product of Carlo Erba (Milano, Italy), and was distilled before use. Hydroquinone was purchased from Merck (Darmstadt, Germany).

Irradiations were performed at 20° by means of an Osram 150 W high pressure Hg lamp, using the same experimental arrangement as previously described [3]. In a typical experiment, 7 ml of a 1 mM solution of Met, Z-Met-Asp, or ribonuclease A in 4 M aqueous acetone were placed in quartz cuvettes of 2 cm light path and thoroughly deaerated by flushing with ultrapure nitrogen; a Balzers interference filter with peak transmission at 311 nm was placed in the front of the cell. In some experiments, hydroquinone in a 3:1 molar ratio to Met was added to the irradiated solution.

Column chromatograhy of native and irradiated ribonuclease A was performed on Amberlite CG-50 [6]. The procedures for determining the kinetics of Met photo-oxidation, as well as for assaying the enzymic activity and the amino acid content of ribonuclease A, have been reported elsewhere [6, 7].

3. Results

Thin layer and paper chromatography of the irradiated solutions of Met and Z-Met-Asp indicated that in both cases the original substrates were gradually destroyed and were converted to a single product. This product was identified as MetO and Z-MetO-Asp, respectively, on the basis of the R_f values in several solvent systems [7]. The photoreaction was complete within 2 hr or irradiation. Amino acid analyses of known amounts of irradiated material demonstrated that the yield of conversion of Met to MetO was over 95%.

The photoreaction appeared to be first-order with respect to Met, having a monomolecular rate constant

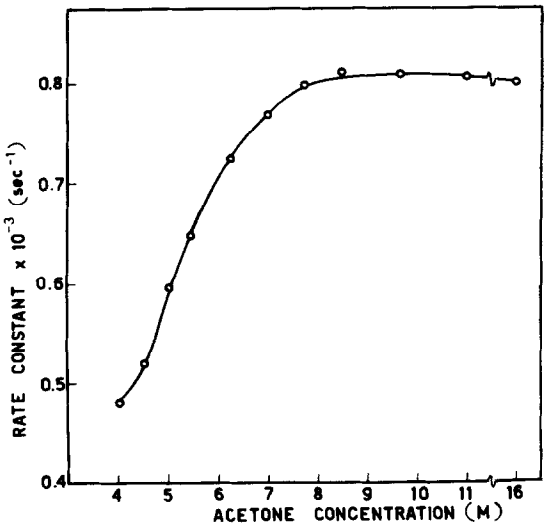

Fig. 1. The effect of the acetone concentration on the rate constant of the acetone-sensitized photo-oxidation of Met to MetO. Irradiations were performed at 311 nm in deaerated solutions. In all cases, there was total absorption of the incident light.

of 0.48 X 10^{-3} sec^{-1}. Increasing the concentration of acetone (fig. 1) up to about 8.5 M caused a significant enhancement of the rate constant, which levelled off to a value around 0.8 X 10^{-3} sec^{-1} at higher acetone concentrations. In particular, in the case of the peptide Z-Met-Asp, the kinetics of the Met photo-oxidation were essentially the same when the irradiation was done in 9 M aqueous as in anhydrous acetone. In all cases, the photoprocess was quantitatively inhibited by hydroquinone, and was about 1% as fast in oxygen-saturated solutions.

Finally, when ribonuclease A was illuminated for 2 hr in deaerated 4 M acetone solution, all four Met residues present in the enzyme were photo-oxidized to the sulfoxide (see table 1). None of the other amino acid residues was affected. The enzymic activity, as well as the spectroscopic and chromatographic properties of the irradiated protein, were identical to those of the tetrasulfoxide derivative of ribonuclease A, as obtained from the hematoporphyrin-sensitized photo-oxidation of the enzyme [8]. By contrast, when the irradiation of ribonuclease A was performed in 1.5 M acetone solution, only one Met residue was converted to MetO even after prolonged exposure to light. The monosulfoxide derivative appeared to be chromatographically homogeneous and possessed over 80% enzymic activity. Amino end-group analysis, after reaction of the monosulfoxide ribonuclease A with cyanogen bromide [8], showed Met-29 to be the selectively modified Met residue.

4. Discussion

The above results strongly suggest that acetone acts both as a sensitizer and as an oxygen-donor for the photo-oxidation of Met to MetO. Since the photo-oxidation kinetics for Z-Met-Asp in aqueous and in anhydrous acetone were identical, there does not seem to be any participation of water in the reaction. Now, acetone has a lowest-lying, n.π^* triplet state

Table 1
Amino acids analyses of native and irradiated samples of ribonuclease A.

Amino acid	Native	Irradiated +1.5 M acetone	Irradiated +4 M acetone
Histidine	3.8	3.9	4.0
Tyrosine	5.8	5.7	5.8
Half-cystine	8.0	7.8	7.7
Methionine*	3.9	3.1	0.0
Methionine-sulfoxide*	0.0	1.0	3.8

Amino acid analyses were performed by chromatography on a Carlo Erba 3 A 27 amino acid analyzer after 22 hr hydrolysis at 110° by 6 N HCl in evacuated sealed vials. The table includes only those amino acids which are usually susceptible to photochemical modification [1]. No change was found for all the other amino acids analyzed. The values in the table denote number of residues per mole of protein.

* Evaculated after 14 hr hydrolysis by 3.75 M NaOH at 100° [6].

which is formed with a quantum yield of nearly unity [9] and has a radical-like character [10]. Most probably, acetone in this triplet state carries out an electrphilic attack on the Met sulfur, which possesses two lone pairs of electrons. The mechanism by which the bond between oxygen and the central carbon atom of acetone is subsequently cleaved is not yet clear; however, the enhancement of the reaction rate constant with increasing sensitizer concentration indicates that more than one acetone molecule per molecule of Met is involved in the reaction. In any case, the presence of radical intermediates is demonstrated by the inhibitory action of hydroquinone and of molecular oxygen.

This novel photoreaction offers new possibilities in the field of protein modification. The approach of performing the irradiation under anaerobic conditions can be used to protect the other photosensitive amino acids from photo-oxidative attack, as shown in the irradiation experiments with ribonuclease A. Consequently, a new method is available for the selective modification of Met residues in polypeptide molecules, as a means of investigating their role in the biological function.

The results obtained with ribonuclease A also demonstrate that the number of modified Met residues can depend to a large extent on the acetone concentration. This is probably due to the denaturing effect of acetone on protein molecules. For example, at low concentrations of acetone, we obtained the specific photo-oxidation of Met-29; this residue has been shown by chemical [11] and photochemical [8] modification studies to be at least partially exposed at the surface of the native ribonuclease A molecule. Therefore, it is reasonable to assume that this Met residue was the only one which could interact with the photoexcited sensitizer as long as the tertiary structure of ribonuclease A was not perturbed. Increasing the concentration of acetone must have induced appreciable alterations in the three-dimensional conformation of the protein, thereby allowing the formerly buried Met residues to contact the sensitizer. By using this technique of performing the irradiation of protein in the presence of gradually increasing concentrations of acetone, one should therefore be able to estimate the number of Met residues which are fully accessible, partially buried, or deeply buried [12].

Finally, this photoreaction can be applied to probe the topography of proteins in solution. It is well known that acetone can be covalently linked to protein molecules, e.g. by reaction of selected functional groups of the protein with acetone [13]. Since the photocatalytic action of sensitizers occurs within a very restricted spatial range [12, 14], irradiation of the acetone-protein complex should produce the selective modification of only those Met side chains which are immediately adjacent to the sensitizers. If the position of the acetone moiety is known, once the modified Met residues have been identified, one can deduce their location in the tertiary structure of the protein. Further work is in progress in our laboratory to explore more fully the scope and the usefulness of this reaction.

Acknowledgements

We are indebted to Dr. Giulio Cauzzo and Dr. Martha L.Macknight for useful discussion.

References

[1] J.D.Spikes and R.Livingston, Advan. Radiation Biol. 3 (1969) 29.
[2] C.S.Foote, Accounts Chem. Res. 1 (1968) 104.
[3] G.Jori and G.Cauzzo, Photochem. Photobiol., in press.
[4] A.A.Lamola, Photochem. Photobiol. 8 (1968) 601.
[5] L.Kittler and G.Lober, Photochem. Photobiol. 10 (1969) 35.
[6] G.Jori, G.Galiazzo, A.Marzotto and E.Scoffone, Biochim. Biophys. Acta 154 (1968) 1.
[7] G.Jori, G.Galiazzo and E.Scoffone, Biochemistry 8 (1969) 2868.
[8] G.Jori, G.Galiazzo, A.M.Tamburro and E.Scoffone, J. Biol. Chem. 245 (1970) 3375.
[9] J.G.Calvert and J.N.Pitts, Photochemistry (Wiley, New York, 1966).
[10] P.de Mayo, J.B.Stothers and W.Templeton, Can. J. Chem. 39 (1961) 488.
[11] T.P.Link and G.R.Stark, J. Biol. Chem. 243 (1968) 1082.
[12] E.Scoffone, G.Jori and G.Galiazzo, Biochem. J. 117 (1970) 53 P.
[13] J.G.Beeley and H.Neurath, Biochemistry 7 (1968) 1239.
[14] E.Scoffone, G.Jori and G.Galiazzo, Biochem. Biophys. Res. Commun. 38 (1970) 16.