

Drug-Protein Interaction: 8-Methoxy Psoralen as Photosensitizer of Enzymes and Amino Acids

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For a better insight of the molecular basis of the photobiological effects of furocoumarins, the relevance of proteins oxydation by singlet oxygen produced by these substrates under irradiation with long u. v. light was studied.

Complex oligomeric as well as simple monomeric purified enzymes with high or low molecular weight and different properties and simple amino acids were irradiated under oxygen in presence of 8-methoxy-psoralen. The effects on both proteins and amino acids were compared with those obtained under similar conditions with typical photosensitizers as methylene blue and Rose Bengal. The results indicated that the photooxydation of proteins, although possible, appears to play a minor role, if any, in the mechanism of action of furocoumarin.

It is generally accepted that the molecular basis of the photobiological effects produced by furocoumarins is a covalent linkage (by means of a photo-cyclo-addition reaction) to the pyrimidine bases of nucleic acids and in particular of DNA [1, 2]. However, it was recently reported [3–5] that 8-methoxy-psoralen (8-MOP) and other analogous furocoumarins, under irradiation with long wavelength UV light, may be able to produce singlet oxygen, by means of energy transfer from their excited triplet states; singlet oxygen, in turn, may be involved in oxidations of the biological substrates, among which proteins appear to be very important. In particular, it was reported [3] that lysozyme may be inactivated in water, and to a larger extent in deuterated water, through this mechanism.

Moreover, very recently Bensasson *et al.* [6], studying some properties of the triplet excited state of furocoumarins, reported evidence that a covalent photobinding of furocoumarins to amino acids may be possible, other than to pyrimidine bases. In this connection results obtained with fluorescence microscopy have been also published, which seem to indicate a localization of 8-MOP in the cytoplasm and in the membrane rather than in the nucleus of the cutaneous cells [7].

Therefore, different factors appear to be involved in determining the biological effects of furocoumarins; an investigation on the relevance of these single factors is of great importance, considering the wide use of these drugs in the photochemotherapy of

psoriasis and other cutaneous diseases [8] and the potential risk of carcinogenicity connected with any chemical modification of important cell component [9]. With the experiments here reported we wanted to verify if under the conditions of irradiation known to produce photobinding to DNA, proteins and amino acids may also undergo photodynamic oxydation by 8-MOP as a general rule, as the above reported example of lysozyme [3] seems to suggest. An extensive modification of proteins by photodynamic oxydation would in part allow an interpretation of the mechanism of action of this substances which, from a genetic point of view, would be certainly less dangerous than a damage to DNA.

In the present paper we describe the results of our studies on the photodynamic inactivation of several enzymes and the photooxidation of amino acids in the presence of 8-methoxy-psoralen (8-MOP). Studies on the covalent photobinding of 8-MOP to proteins are still in progress and will be communicated later.

Materials and Methods

Enzymes and chemicals – enolase from rabbit muscle, lysozyme and bovine glutamate dehydrogenase were obtained as ammonium sulfate suspension from Boeringer (Mannheim, Germany). 6-phosphogluconate dehydrogenase from yeast was a gift of Prof. M. Rippa, Ferrara University, Italy. Thermolysin was from Daiwa Kosei K. K. (Osaka, Japan). Ribonuclease A from Worthington. Tuna liver glutamate dehydrogenase was from our

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laboratory [10]. Amino acids were obtained from Fluka (Fluka AG, Switzerland). 8-methoxy-psoralen was purchased from Chinoin (Milano) and purified by means of vacuum sublimation and crystallization from ethyl alcohol. All other biochemicals were of the highest purity from Boeringer; salts and solvents from Merck (Darmstadt, Germany).

Enzyme assays

Thermolysin activity was evaluated on the basis of decrease in absorbance at 345 nm following hydrolysis of phenylacryl-glutamyl-leucylamide (15 mg/50 ml) in Tris-HCl 0.1 M buffer, 10 mM CaCl_2 , pH 7.2. Ribonuclease A activity was evaluated from the increase in absorbance at 287 nm following hydrolysis of the ester bond of cyclic cytidin-2'-3'-phosphate (10 mg/25 ml) in 3,3'-dimethylglutaric acid 0.05 M HCl buffer, 0.1 N NaCl, pH 6. Enolase activity was evaluated from the increase in absorption at 230 nm following the formation of the double bond of 2-phosphoenol-pyruvate from 2-phosphoglycerate 20 mM in Tris-HCl 0.05 M buffer, 10^{-3} M MgSO_4 , pH 7.5. Yeast 6-phosphogluconate dehydrogenase activity was evaluated from the increase in absorbance at 340 nm following reduction of NADP, 0.3×10^{-3} M, in Tris-HCl buffer, 0.3×10^{-3} M 6-phosphogluconic acid, pH 8. Bovine and tuna liver glutamate dehydrogenase activity was evaluated by the increase in absorption at 340 nm following reduction of NAD, 10^{-4} M, in phosphate buffer 0.03 M, 5×10^{-3} M glutamic acid, pH 8. Lysozyme activity was estimated from the decrease in absorbance at 450 nm following solubilization of *Mycrococcus luteus* cells due to the lysis of cell walls. The assay mixture contained a suspension of cells which gave an approximate OD of 0.500 at 450 nm in phosphate buffer 0.05 M, pH 7.5. All enzyme assays were made by continuous recording of the optical density signal following addition to the assay mixture of a proper amount of enzyme as last component.

Amino acid analysis — These were performed on a Jeol Mod. JLC-6 H amino acid analyzer using the single column procedure. Amino acid mixtures were also analyzed by high voltage paper electrophoresis at pH = 1.9. After drying the paper the compounds were revealed under UV light and by ninhydrin staining.

Irradiation — The irradiation of proteins or amino acids were performed in pyrex tubes, maintained at 18 °C by circulating water, with UV light

from an high pressure mercury lamp Phillips mod. HP W 125 emitting mainly at 365 nm plus small amounts of radiation at 313, 334, 390 and 404 nm, placed at 14 cm from the tubes [1–2] or with visible light from an Osram mod. HVI 500 W tungsten lamp placed at 28 cm. The distances between the light sources and the tubes were calculated to reach the solutions with approximately the same amounts of energy. During irradiation the solutions of the tubes were maintained under N_2 or O_2 by bubbling the gas previously purified through a solution of water for O_2 and through successive solutions of 10% $\text{Na}_2\text{S}_2\text{O}_4$, 2% anthracinon sulfonate in 7% NaOH (W/V), 30% NaOH and finally water for N_2 .

Results

Fig. 1 reports the time course of inactivation of bovine and tuna liver glutamate dehydrogenase, rabbit muscle enolase, yeast 6-phosphogluconate dehydrogenase, thermolysin, ribonuclease A and lysozyme by irradiation at 365 nm in the presence of 2.8×10^{-4} M 8-methoxypsoralen. Fig. 2 A and B reports the inactivation rates of the same enzymes by irradiation with visible light in the presence of

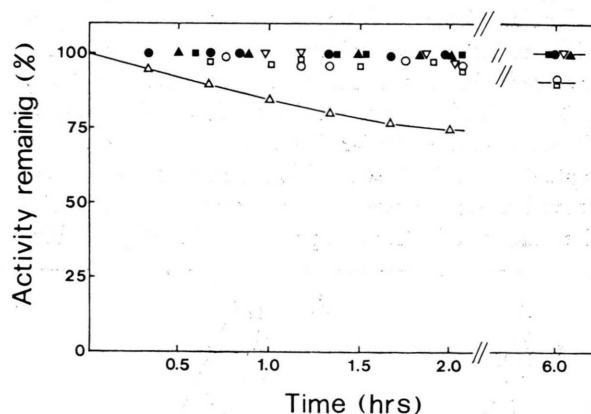


Fig. 1. Photodynamic inactivation of enzymes in presence of 2×10^{-4} M 8-MOP under oxygen. Bovine (\square — \square) or tuna (\blacktriangle — \blacktriangle) liver glutamate dehydrogenase, 2.8×10^{-6} M in phosphate buffer 0.05 M, 0.9% NaCl, pH 7.4. Rabbit muscle enolase (\blacksquare — \blacksquare), 1.2×10^{-6} M in Tris-HCl buffer 0.05 M, 0.9% NaCl, 10^{-3} M 2-mercaptoethanol, pH 7.5. Yeast 6-phosphogluconate dehydrogenase (\circ — \circ), 1×10^{-6} M, in Tris-HCl buffer 0.1 M, 5×10^{-5} M EDTA, 10^{-3} M 2-mercaptoethanol, pH 7.4. Thermolysin, (\bullet — \bullet), 2.2×10^{-5} M, in Tris-HCl buffer 0.1 M, 10^{-2} M CaCl_2 , pH 7.2. Hen egg white lysozyme (\triangle — \triangle), 5×10^{-5} M, in phosphate buffer 0.01 M, 0.9% NaCl, pH 7.2. Ribonuclease A (∇ — ∇) 8×10^{-5} M, in ammonium acetate buffer 0.05 M, pH 5.

Rose Bengal and methylene blue 0.5×10^{-4} M and 1.3×10^{-4} M respectively.

No inactivation was observed either by irradiation in the absence of photosensitizer (with the exception of lysozyme, see later) or by irradiation under N_2 in the absence of photosensitizer, or by bubbling O_2 without irradiation.

Glutamate dehydrogenase from two sources was studied as a model of complex oligomeric enzyme in which several amino acid residues sensitive to oxydation are involved in maintaining enzyme activity [11]. During irradiation the enzyme, 1 mg/ml, was dissolved in phosphate buffer 0.05 M, 0.4% NaCl, pH 7.4. Phosphate buffer was used as solvent since it is known to stabilize glutamate dehydrogenase towards denaturation, furthermore in this buffer thiol groups are less exposed to solvent and may be stable to oxygen exposure [12]. When the enzymes of the two sources are irradiated by UV light in presence of 8-MOP no significant loss of enzyme activity is observed for two h, some loss, less than 10%, being observed in the bovine enzyme after 6 h (see Fig. 1). On the other hand when Rose Bengal, Fig. 2 A or methylene blue, Fig. 2 B are used as photosensitizers, a rapid loss of glutamate dehydrogenase activity does occur, 50% inactivation being reached after 8 and 20 min respectively.

Enolase from rabbit muscle is a dimeric enzyme with amino acids residues sensitive to oxydation involved in maintaining the catalytic activity [13–14]. The enzyme, 0.1 mg/ml, was dissolved in Tris-HCl 0.05 M buffer 0.9% NaCl, 10^{-3} M 2-mercaptoethanol, 10^{-3} M $MgSO_4$, a mixture which was proved useful to protect the enzyme from dissociation and inactivation. In presence of methylene blue

or Rose Bengal, see Fig. 1 B, it is rapidly inactivated as it was already reported by other Authors [14–15]. In our experience in presence of both photosensitizers some activation occurred in the first minutes of irradiation. On the other hand no appreciable inactivation did occur following 6 h irradiation with 8-MOP as photosensitizer (see Fig. 1).

Yeast 6-phosphogluconate dehydrogenase is a dimeric-SH dependent dehydrogenase [16] in which, in addition to cysteine [17], other oxydizable residues were reported to be involved in the activity [18–19]. The enzyme, 0.1 mg/ml, was dissolved in Tris-HCl 0.1 M buffer, 10^{-5} M EDTA, 10^{-3} M 2-mercaptoethanol a solvent mixture which protects the enzyme from an easy inactivation probably related to $-SH$ oxydation. In this solvent the enzyme was found very stable to photooxydation in presence of 8-MOP, see Fig. 1 some loss of activity occurring only after long irradiation whereas in presence of Rose Bengal, rapid inactivation with biphasic kinetic did occur as it was already reported by others [18]. Also methylene blue acts as a strong photodynamic sensitizer towards the enzyme since 50% inactivation in less than 10 min was observed.

Thermolysis is a monomeric proteolytic calcium and zinc dependent enzyme [20] with several amino acids, some of them sensitive to oxydation, present at the active site [21]. The enzyme, 1 mg/ml, dissolved in Tris-HCl 0.1 M buffer, 10^{-2} M $CaCl_2$, pK 7.2 was found quite stable to photooxydation in presence of 8-MOP for over 8 h whereas inactivation occurs (50% in about 1 h) with Rose Bengal as well with methylene blue.

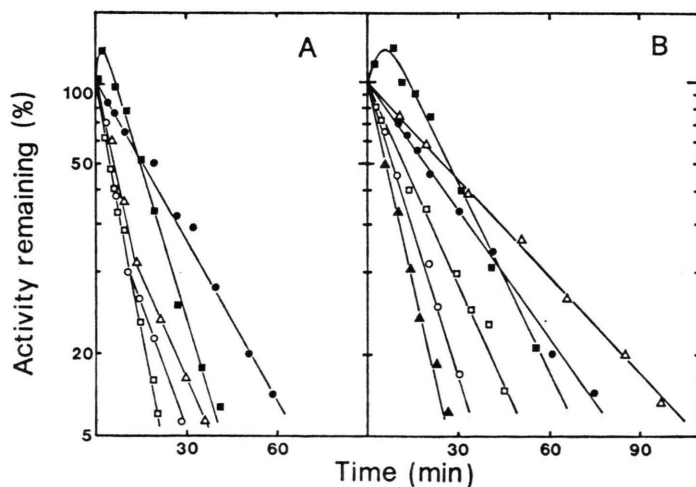


Fig. 2. Photoinactivation of enzymes in presence of A. 0.5×10^{-4} M Rose Bengal or B. 1.3×10^{-4} M methylene blue under oxygen. The buffer conditions and symbols are those reported in Fig. 1.

Ribonuclease A, monomeric, small molecular weight enzyme, dissolved in concentration of 1 mg/ml in ammonium acetate buffer 0.05 M, pH 5, was also quite stable for over 6 h to photooxydation in presence of 8-MOP.

Lysozyme is a monomeric, small molecular weight enzyme with an high amount, about 20%, of oxydizable amino acids, many of them involved in enzyme catalysis or in maintaining the proper conformation of the enzyme [22–24]. When lysozyme (0.1 mg/ml, in 0.01 M phosphate buffer, 0.9% NaCl, pH 7.2) was irradiated under oxygen with a low pressure mercury lamp, an appreciable loss of activity occurred after 2 h that could be reduced to about 5% using a tryptophan solution as low wavelengths radiation scavenger. In presence of 8-MOP inactivation occurred, 25% after two h a figure that however may be considered negligible if compared to the total loss of activity found in less than 60 min with Rose Bengal as sensitizer or 80% loss with methylene blue, see Fig. 2 A and B.

Amino acid decomposition by photosensitization

As a further control of the photodynamic activity of 8-MOP its effect on amino acids modification was studied. Table I reports the analysis of a standard mixture of amino acids, performed after prolonged irradiation under oxygen in phosphate buffer 0.01 M pH 7.2 in presence of Rose Bengal or 8-MOP.

Table I. Per cent recovery of amino acids evaluated by amino acid analyzer after 6 h irradiation under oxygen of an amino acid mixture 1.5×10^{-4} M in phosphate buffer 0.02 M, 0.9% NaCl, pH 7 in presence of 5×10^{-4} M 8-MOP or 0.5×10^{-4} M Rose Bengal as photosensitizers. The table reports the lower and upper values obtained from 4 experiments. The conditions of irradiation are reported in the text.

Amino acid	In presence of Rose Bengal	In presence of 8-MOP
Aspartic acid	97–102	97–101
Threonine	100–102	99–101
Serine	98–101	99–100
Glutamic acid	99–100	100–102
Proline	98–102	98–102
Glycine	100	99–101
Half cystine	66–70	95–99
Valine	100	100
Methionine	0	89–94
Isoleucine	100	100
Leucine	100	100
Tyrosine	75–80	97–100
Phenylalanine	90–95	100–102
Lysine	98–101	98–102
Histidine	0	88–97
Arginine	99–101	100–101
Tryptophan	0	96–100

With 8-MOP as photosensitizer limited decomposition of 5–10% was found with half cystine, methionine and hystidine, only after long irradiation, 6 h, under oxygen. In these conditions tyrosine, phenylalanine and tryptophan did not appear modified or, if any decomposition did occur this was within the experimental error of the analyzer.

On the other hand with Rose Bengal as sensitizer the amino acids most sensitive to oxydation, methionine, hystidine and tryptophan [25] are completely oxydized whereas for half cystine and tyrosine decomposition values of about 35 and 25% were observed. A minor oxydation of phenylalanine was also found.

The amino acid mixture, following irradiation in presence of 8-MOP was also analyzed by high voltage paper electrophoresis: no new ninhydrin reactive or fluorescent spot was observed in addition to those of the starting amino acids. This result, in agreement with those obtained by the analyzer, seems to exclude or minimize, in these experimental conditions, the formation of new compounds from amino acids and 8-MOP during irradiation under oxygen.

Discussion

From the finding that 8-methoxy-psoralen and other analogous furocoumarins derivatives are able to generate little amounts of reactive singlet oxygen under irradiation with long wavelength UV light [3–5], the suggestion arised that in the production of the biological effects of furocoumarins the substrate oxydation, in particular of proteins, may play a role. This possibility was ascertained by Poppe and Grossweiner [3], which found that lysozyme may be partially inactivated by photodynamic oxydation in the presence of 8-methoxy-psoralen.

In order to collect elements useful for evaluating the relevance that protein oxydation may have in the mechanism of action of the pharmacologically active furocoumarins, we carried out an investigation about the possibility to photooxydize various other enzymes by irradiation in the presence of 8-methoxy-psoralen. In these experiments the conditions of irradiation for what source of light, distance from the source, 8-MOP concentration, were those generally used for studying the photobinding to DNA. Complex oligomeric, as well as simple monomeric, purified enzymes with high or low molecular weight and different properties were chosen as protein models. In any case, the extent of inactivation *via*

photodynamic oxydation was also checked irradiating the same enzymes under comparable conditions with visible light in the presence of Rose Bengal and of methylene blue, which are known to be very active in producing singlet oxygen by energy transfer in their excited state.

The buffer conditions under which the irradiation was carried out were carefully studied for each enzyme in order to prevent both denaturation during the time of experiments as well as inactivation by exposure to molecular oxygen, which easily occurs with the two glutamate dehydrogenases, 6-phosphogluconate dehydrogenase and enolase. Lysozyme, on the other hand, presented some problem for its easy inactivation by oxygen under UV irradiation also in the absence of photosensitizers. This could be prevented to a large extent using an appropriate scavenger for UV radiations of the lamp.

The results obtained in our experiments indicated that these enzymes are rather stable to irradiation with UV light in presence of 8-MOP as photosensitizer, whereas they are easily inactivated by irradiation with visible light in presence of Rose Bengal or methylene blue. Inactivation to some degree with 8-MOP was observed only with lysozyme; the extent of this inactivation was very similar to that reported by Poppe and Grossweiner [3], but it appears very low if compared with that found with photosensitizers as Rose Bengal or methylene

These results were confirmed by the experiments of photooxydation performed on a mixture of amino acids; they indicated again that if some oxydation of the most sensitive amino acids, methionine, half cystine and histidine did occur, it is very small using 8-methoxy-psoralen as photosensitizer. From the data here reported it seems pertinent to conclude that the photooxydation of proteins by furocoumarins, although possible, appears to play a minor role, if any, in the mechanism of action of these compounds, if compared to that generally accepted [1–2], which is based on the covalent linking to the pyrimidine basis of nucleic acids.

The data here presented although at a first instance may appear as negative results, they are instead of great interest in the context of the still opened and debated problem of the interpretation of the mechanism of action involved in the photochemotherapeutic use of furocoumarins. Actually these results stress again the action of these substances through the photoaddition to the pyrimidine bases than to modification of protein components.

This interpretation may receive further support if one considers the rapid turnover of most proteins in the living cells, in comparison to that of nucleic acids.

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