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Sensitized photo-oxidation of amino acids in proteins: Some important biological implications

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The sensitized photo-oxidation of amino acids in proteins is a special case of chemical modification, which is used in particular to study buried and exposed groups in proteins. The general principles of the technique and various applications of it are discussed in this article.

Key terms: *buried and exposed groups; photodynamic action; riboflavin; sensitized photo-oxidation of amino acids.*
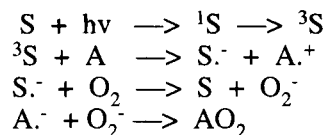
INTRODUCTION

The role of enzymes as catalysts of the reactions occurring in living organisms as well as their increasing use in biotechnology have stimulated the investigations devoted to provide information regarding the molecular details of the active site, as an approach to the understanding of their mode of action. The chemical modification method has been traditionally used to establish the amino acid residues that participate in the active site of enzymes, and the sensitized photo-oxidation of amino acids in proteins can be included as a particular case of this method.

The photodynamic action or sensitized photo-oxidation represents a process which originates a chemical change in the substrate by irradiation of the system under study with visible light in the presence of a sensitizer. The photodynamic action requires molecular oxygen, and the sensitizer in an excited triplet state seems to be the species responsible for the beginning of the reaction.
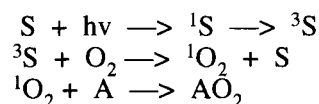
MECHANISM OF THE SENSITIZED PHOTO-OXIDATION

Two mechanisms have been proposed to explain sensitized photo-oxidation (Foote, 1968; Kramer & Maute, 1972; Nilsson & Kearns, 1973).

Type I Mechanism (Example):

$$S + h\nu \longrightarrow {}^1S \longrightarrow {}^3S$$
$${}^3S + A \longrightarrow S^{\cdot -} + A^{\cdot +}$$
$$S^{\cdot -} + O_2 \longrightarrow S + O_2^{\cdot -}$$
$$A^{\cdot +} + O_2^{\cdot -} \longrightarrow AO_2$$

Type II Mechanism:

$$S + h\nu \longrightarrow {}^1S \longrightarrow {}^3S$$
$${}^3S + O_2 \longrightarrow {}^1O_2 + S$$
$${}^1O_2 + A \longrightarrow AO_2$$

where S, ${}^1S$ and ${}^3S$, represent the sensitizer in the ground state and in the excited singlet and triplet states. A represents an oxidizable substrate.

In type I mechanism, the substrate initially reacts with the sensitizer in the triplet state and then with molecular oxygen (or one of its active species) via a radical intermediate. In type II mechanism, the excitation energy is transferred from the sensitizer in the triplet state to molecular oxygen, giving rise to ${}^1O_2$, which, in turn, reacts with the acceptor. In some cases, the two mechanisms can be present, depending on the sensitizer used (Kramer & Maute, 1972; Silva, 1979; Sconfienza *et al*, 1980; Tsai *et al*, 1985). It is also possible to initiate a photo-oxidative process by means of the enzymatic excitation of the sensitizer through a chemical reaction, in a process called 'photochemistry without light' (Durán *et al*, 1983).

Of the two types of singlet oxygen, $^1\Delta$ and $^1\Sigma$ (Fig 1) (Kasha & Branham, 1979), only the first species has an effective lifetime in aqueous systems (Rodgers & Snowden, 1982; Ogilby & Foote, 1983).

One of the methods used to demonstrate the participation of the $^1O_2$ in biological and chemical processes consists in the electronic relaxation of their excited states by the action of chemical agents. Hasty et al (1972) reported that sodium azide produces a significant decrease in the lifetime of the singlet oxygen; this was used by Nilsson et al (1972) to demonstrate the participation of $^1O_2$ in the sensitized photo-oxidation of the amino acids and in the photodynamic inactivation of alcohol dehydrogenase.

The measurement of chemical luminescence at a specific wavelength is frequently used as reliable evidence for the presence of $^1O_2$ in biological reactions (Cadenas et al, 1980). The emission of singlet oxygen dimol (chemoluminescence arising from simultaneous transitions in pairs of singlet oxygen molecules) takes place at 634 and 703 nm (Khan & Kasha, 1970). For the mono-molecular species, the emission is observed at 1,268 and 1,407 nm (Khan & Kasha, 1979).

Tertiary aliphatic amines have also been described as quenchers of the $^1O_2$ (Ouannes & Wilson, 1968). Another useful diagnostic method to test for the presence of singlet oxygen is based on the effect of deuterated water over the lifetime of the $^1O_2$ (Merker et al, 1972). The lifetime of $^1O_2$ has a value of 53-68 µs in D_2O (Linding & Rodgers, 1979; Ogilby & Foote, 1983), and of 4 µs in H_2O (Rodgers & Snowden, 1982). Thus the reaction rate, neglecting solvent isotope effects for photo-oxidation, is increased by a factor of 15 in going from H_2O to D_2O.

Just as the presence of $^1O_2$ can be considered as evidence for a type II mechanism, the type I mechanisms is suggested by the quenching effect of the electron acceptor $[Fe(CN)_6]^{-3}$ (Dewey & Stein, 1970; Rossi et al, 1981). Koizumi & Usui (1972) have demonstrated that in an aqueous medium the interaction between two molecules of sensitizer, one in the triplet state and the other in the basal state, leads to the formation of two radical ions (sens.+ and sens.-). These radical ions are then efficiently attacked by molecular oxygen, giving rise to superoxide ions (O_2-), a species that is highly reactive. Other studies of Zwicker and Grossweiner (1963) have pointed out that the sensitizer in the triplet state reacts with the substrate, producing radical ions, which in turn react with molecular oxygen, leading to the formation of oxidized derivatives of the substrate.

Tsai et al (1985) have demonstrated that for a series of enzymes, the photodynamic process is preceded by the binding between the enzyme and the sensitizers.

SELECTIVITY OF THE PHOTODYNAMIC EFFECT IN PROTEINS

The photochemical modification produced in proteins by the effect of the sensitized photo-oxidation is circumscribed to the specific modification of the side-chains of certain amino acid residues, no alterations being produced at the level of the peptide bonds (Weil et al, 1953; Ghiron & Spikes, 1965). Out of the twenty types of amino acid residues that can be part of a protein, only the side-chains of Cys, His, Met, Trp and Tyr residues are susceptible of being modified by the effect of sensitized photo-oxidation (Galiazzo et al, 1968; Silva et al, 1974).

In the case of Cys, photo-oxidation has been studied over a wide range of pH, and the only characterized photoproduct has been cysteic acid (Jori et al, 1969).

Tomita et al (1969) reported that the His sensitized photo-oxidation leads to the formation of aspartate and urea, which was subsequently confirmed by Tsai et al (1985).

By means of photo-oxidation, Met is converted into methionine sulfoxide, and after a long reaction period, into methionine sulfone, and apparently into homocysteic acid (Weil et al, 1953; Benassi et al, 1967; Jori & Cauzzo, 1970). When the determination of the amino acid composition of a protein is used with the purpose of quantifying the photo-oxidation of the Met residues present in it, an alkaline hydrolysis must be previously carried out, since -methionine sulfoxide is reverted to methionine during an acid hydrolysis (Jori et al, 1968a,b; Risi et al, 1973).

The products of Trp photo-oxidation have been the subject of numerous studies (Benassi et al, 1967; Asquith & Rivett, 1971; Creed, 1984 a). Saito et al (1979) have proposed three pathways starting with a common intermediate (hydroperoxiindolalanine) for the transformation of Trp in its photoproducts kinurenine and N-formylkinurenine, whose fluorescent characteristics have been reported by Fukunaga et al (1982). Work by Nakagawa et al (1985) has revealed that the pH not only exerts an influence on Trp oxidation velocity, but also influences the formation of other photoproducts, such as 5-hydroxy formylkynurenine at pH values > 7.0 and a trycyclic hydroperoxide in the pH range 3.6-7.1.

The Tyr photo-oxidation products have not been characterized yet (Creed, 1984b), and only the photoproducts of phenolic derivatives analogous to Tyr have been identified (Saito et al, 1975; Matsuura, 1977).

The photo-oxidation rate of the susceptible amino acids is strongly dependent on reaction conditions, as has been extensively discussed in the classic work of Spikes and Livingston (1969). At pH values smaller than six, the His and Tyr residues are not photooxidizable, owing to protonation of the imidazole ring and because of the weak activation effect of the hydroxylic substituent of the benzene ring (Sluyterman, 1962). Trp presents a wide range of photoreactivity; only the photoproducts obtained vary with the pH employed, as was mentioned before.

The methionyl residues can be selectively converted to the sulfoxide by irradiation in acetic acid solution at low temperatures, in the presence of rose Bengal or methylene blue sensitizers (Jori et al, 1968a,b); in the presence of hematoporphyrin, methionine can also be selectively photooxidized in aqueous solutions buffered at pH values lower than 6.5 (Jori et al, 1969).

Crystal violet acts selectively on cysteine both in aqueous solution over the pH range 2.5-9.0 and in 5 to 95% acetic acid (Bellin & Yankus, 1968; Jori et al, 1969).

The efficiency and selectivity of photosensitized reactions may depend on various factors (Foote, 1976; Spikes, 1977; Jori & Spikes, 1981). Binding (complexation) of sensitizer to the substrate may be one of the

most important questions to be raised (Grossweiner, 1969; Grossweiner & Kepka, 1972; Silva & Gaule, 1977; Jori & Spikes, 1981; Tsai et al, 1985). Most sensitizers can complex reversibly with nucleic acids, proteins, and polysaccharides (Amagasa, 1981). Sensitizer-substrate complexation may increase the population of the triplet state and decrease the collisional quenching of the triplet state (Bellin, 1968). Furthermore, the vicinity of the substrate and sensitizer may enhance the probability of a direct reaction of these counter-parts. Type I mechanism is thus favored. Type I and Type II reactions for important biomolecules have been reviewed (Foote, 1976; Spikes, 1977).

Tsai et al (1985) have shown that the rates of photo-inactivation are at least an order of magnitude faster with efficient sensitizers (methylene blue, hematoporphyrin, rose Bengal, erythrosin B, and eosin Y) than with inefficient ones (fluorescein, acridine orange, riboflavin and pyridoxal 5'-phosphate). No obvious correlation exists between sensitizing efficiency and dye structures. Although the physicochemical parameters that characterize sensitizing efficiency are yet to be defined, halogenated xanthines and thiazine with low triplet state energies are shown to be effective photosensitizers.

Higher quantum yields for photoinactivation of lysozyme sensitized by riboflavin in relation to those obtained in the presence of methylene blue (Shugar, 1952; Hopkins & Spikes, 1969, 1970; Silva et al, 1974; Silva & Gaule, 1977) have been interpreted through the photo-induced complex obtained on irradiating lysozyme in the presence of riboflavin (Silva & Gaule, 1977; Ferrer & Silva, 1981). In subsequent studies, it was demonstrated that the bond between riboflavin and the enzyme is specific, and that it is produced at the Trp residues present in lysozyme (Ferrer & Silva, 1985).

DETERMINATION OF BURIED AND EXPOSED GROUPS IN PROTEINS BY MEANS OF SENSITIZED PHOTO-OXIDATION

In the three-dimensional structure of a protein, the photo-oxidizable amino acid residues can be located either at the surface of the molecule in contact with the solvent or

in the interior at the hydrophobic regions. To these two groups, one should add those residues that are in an intermediate situation and therefore are neither completely exposed to the solvent nor correspond to the buried ones in the hydrophobic regions.

It has been found that photo-oxidizable amino acid residues are modified in a different manner depending on their location in the three-dimensional structure of the protein. For example, Weil *et al* (1965) found that only the His residues were photo-oxidized when they studied insulin in its native form, but the modification was extended to the Tyr residues when 8 M urea was added. RNAase suffers sequential photo-oxidation of its Met residues when it is irradiated in the presence of increasing concentrations of acetic acid, using hemato-porphyrin as sensitizer (Jori *et al*, 1970). The homologous proteins a-lactalbumin and lysozyme present different reactivity to sensitized photo-oxidation in their native and denatured forms (Edwards & Silva, 1985).

Ray and Koshland (1961, 1962) proposed a kinetic model to calculate the number of residues of amino acids exposed on the surface, and therefore of those located in the hydrophobic interior of the protein. They postulated that the amino acid residues exposed, owing to a higher accessibility to the reactive species, would be photo-oxidized in a more efficient form than the internal ones.

When studies tending to clarify the mechanism of sensitized photo-oxidation were carried out, it was found that in a very large number of cases sensitized photo-oxidation took place via singlet oxygen through a Type II mechanism (Jori & Spikes, 1981). This fact not only identified the reactive species participating in sensitized photo-oxidation, but also had implications in the interpretation of the kinetic analysis of Ray and Koshland (1961). In order to be able to apply this model, it was necessary to find an explanation for the difference in reactivity of the internal and external amino acid residues to singlet oxygen. An adequate explanation was not easy with the information available. The lifetime of the singlet oxygen is very much higher in an apolar medium than in a polar solvent like H_2O (Turro, 1978), a fact

which favors the existence of a reactive species in the hydrophobic interior of the protein. Besides, the diffusion velocity of molecular oxygen is much higher than that of any organic molecule, which would also favor the arrival of singlet oxygen to the hydrophobic interior of the proteins (Turro, 1978).

In 1973, Lakowicz and Weber performed a series of experiments in which they studied the intrinsic fluorescence quenching of proteins by the action of molecular oxygen under high pressure. They found that molecular oxygen is able to quench the fluorescence of the Trp residues, including those that are in the interior of the protein structure, which means that oxygen is able to diffuse and reach this type of residue formerly considered to be inaccessible.

More recently, several investigations provided the necessary information required to understand the behavior of oxygen in the interior of a protein. In 1982, Eftink and Jameson studied fluorescence quenching of alcohol dehydrogenase. This enzyme is characterized by having two Trp residues in its structure: Trp-15 that is exposed to the solvent, and Trp-314 that is in the hydrophobic interior of the protein. These authors found that quenching by oxygen was five times more effective for the Trp-15 residue. According to the most recent evidence available, the value of the quenching constant by oxygen for Trp-314 is the lowest that has been found for proteins. These results support the kinetic analysis of Ray and Koshland (1961) that is based on the fact that the residues of internal amino acids are less accessible to the reactive species (singlet oxygen) than the exposed residues that would be completely accessible and therefore more reactive. This is also in accordance with the work of Calhoun *et al* (1983a, b), who, using the oxygen and other small molecules as fluorescence quenchers, found that all of them were more efficient for the Trp residues accessible to the solvent.

In addition, Reddi *et al* (1984) found that in spite of the fact that the singlet oxygen has a longer lifetime in solvents less polar than water, the efficiency of photo-oxidation was smaller in apolar media. These results are also supported by the fact that the

internal residues are less reactive than the external ones, since they are in a less polar environment.

In summary, the methodology proposed by Ray and Koshland (1961) still retains its validity with the simplifications and limitations characteristic of any kinetic model.

Three modes of kinetic behavior can be expected in sensitized photo-oxidation:

(a) No effect is observed in spite of the use of adequate conditions to modify certain amino acid residues. This kind of result indicates that these residues are not accessible to the reactive species or that their reactivity is very low, and therefore they are located in the hydrophobic region of the protein.

(b) A straight line is obtained in a semilogarithmic plot of the remaining concentration of the amino acid as a function of irradiation time. This indicates that all the residues could be photo-oxidized with the same first-order rate constant (k).

$$\frac{C}{Co} = e^{-kt} \qquad (1)$$

This situation is clearly present when a protein has only one amino acid residue of a certain type and the photo-oxidation phenomenon takes place in this residue. Such is the case of the His-15 residue in lysozyme (Risi *et al*, 1973).

With the purpose of estimating the exposure of the photo-oxidizable residue to the solvent, the rate constant measured experimentally is compared with the one obtained for the amino acid in its free form (Jori *et al*, 1970). It is also possible to resort to small peptides that contain the amino acid under study, or to the same protein in its denatured form (Edwards & Silva, 1985).

(c) A non-linear semilogarithmic plot is obtained for the photo oxidation of the Trp residues in a-lactalbumin (Edwards & Silva, 1985). In this case, the shape of the observed curve for residue modification suggests that some of the Trp react with higher velocity than others. This difference in reactivity is attributable to their

location in the structure, and therefore to the polarity of their environment and their accessibility to singlet oxygen, as already discussed.

The expression for the overall rate of photo-oxidation will then be the sum of the photo-oxidation rates of the accessible and partially inaccessible residue groups, and will be given by equation (2), if two different degrees of reactivity are involved.

$$\frac{C}{Co} = f_1 e^{-k_1 t} + f_2 e^{-k_2 t} \qquad (2)$$

C/Co is the fraction of the total amino acid residues, which is obtained from the results of the amino acid analyses carried out at various times after irradiation; f_1 and f_2 are the fractions of the total residues corresponding to the fast and slow reacting groups; and k_1 and k_2 are the first-order rate constants for the photo-oxidation of the respective residues. The values of f and k are obtained from a semilogarithmic kinetic plot.

The situation described by equation (2) may be extended to cover cases with three or more sets of reactive groups.

In their original work, Ray and Koshland (1962) discussed in detail the advantages and limitations of their method. Among the advantages, it was stressed that no high specificity is required for the photo-oxidation, since the modification kinetics are analyzed in separate form for each amino acid residue. It is necessary, however, to point out that the presence of many residues of the same type can make the method more complex. In this way, the reaction of a single residue can be masked in the presence of many other residues of the same type that react with other reaction constants.

From the kinetic values obtained with this method, a correlation between the modification of amino acid residues and enzyme inactivation can be sought, which can lead to the determination of the number and type of residues that participate in the catalytic activity of an enzyme (Ray & Koshland, 1962). For this type of correlation to be valid, it is necessary to make sure that during the photo-oxidation process no conforma-

tional changes take place. Photo-physical methods have been of great usefulness in the determination of conformational changes (Edwards & Silva, 1985). In particular, the fluorescence techniques appear among the most convenient ones, because they are particularly sensitive to conformational changes (Burstein *et al*, 1973).

PHOTODYNAMIC ACTION USING SENSITIZER COVALENTLY BONDED TO THE PROTEIN

To obtain information about the location of certain residues in the spatial conformation of proteins, sensitizers covalently bound to them have been used. Jori's group, in Italy, pioneered this type of work (Scoffone *et al*, 1970; Galiazzo *et al*, 1972). The procedure involves the irradiation of proteins that contain one sensitizer covalently bound at a certain position in the molecule. The sensitizer can be present in the protein either in natural form, as it occurs with the hematoproteins, flavoproteins, or pyridoxal enzymes, or it can be artificially introduced by means of a chemical reaction, as for example the formation of the 41-DNP-RNAase A (Scoffone *et al*, 1970). The selective modification of those residues that are located in a close proximity to the dye can be achieved by irradiation of the protein-sensitizer complex.

To get reliable results, some precautions must be taken when applying this methodology:

(a) The insertion of the sensitizer into the molecule must bring about no significant alteration of the tertiary structure. A rigorous conformational analysis can be carried out, using the sensitive spectroscopic methods presently available (Jori *et al*, 1970; Burstein *et al*, 1973; Sun & Song, 1977).

(b) All photoreactions of intermolecular type must be avoided. With the purpose of minimizing this type of interaction, it is convenient to work with dilute solutions. In any case, the occurrence of intermolecular photosensitization can be easily detected by means of the irradiation of a mixture of labeled and unlabeled protein. If no intermolecular phenomena

are produced, the unlabeled protein must be recovered without damage after irradiation.

(c) The binding of the sensitizer to the protein must be carried out with a high degree of selectivity, in such a way that the photodynamic action of the introduced sensitizer operates in a very well defined and restricted area.

BIOLOGICAL IMPLICATIONS OF RIBOFLAVIN SENSITIZED PHOTO PRODUCTS OF TRYPTOPHAN

Most types of molecules of biological importance are relatively insensitive to direct effects of visible light since they do not absorb radiation in this wavelength range. However, a variety of biological systems are subjected to damage and destruction by light in the presence of appropriate photosensitizers and molecular oxygen. These dye-sensitized photooxidation reactions are commonly termed photodynamic action (Tsai *et al*, 1985; Roberts & Berns, 1989; Spikes, 1991).

The vitamin B_2, riboflavin (Rb), is a photosensitizer that, in water solution and in the presence of oxygen, leads to substrate oxidation through both Type I and Type II mechanisms (Yoshimura & Ohno, 1988; Silva *et al*, 1994). The role of free and protein-bound tryptophan in the light sensitivity of biological systems has prompted numerous studies of the photoprocesses of this essential amino acid, either following the direct absorption of light by the indole group or promoted by compounds acting as sensitizers (Creed, 1984a). Of the sensitized photoprocesses, those involving the riboflavin-tryptophan system are of particular interest due to the endogenous nature of both compounds. An exceptional efficiency for the photo-oxidation of Trp has been found when the vitamin riboflavin is used as sensitizer (Silva *et al*, 1974). This process is characterized by higher quantum yields than those observed with other sensitizers such as methylene blue or rose Bengal, which involve a Type II photo-oxidation mechanism, suggesting that riboflavin may act preferentially through a Type I mechanism.

This fact has been demonstrated by the lumiflavin-sensitized photo oxygenation of indole (Yoshimura & Ohno, 1988) and recently by the riboflavin-sensitized photo-oxidation of Trp (Silva et al, 1994). In the last case, it was found that the photodegradation of Trp in oxygen saturated aqueous solution sensitized by riboflavin is accompanied by the generation of the following reactive oxygen species: $^1O_2$, $.OH$, H_2O_2 and O_2. It was also demonstrated that the yield of the riboflavin-sensitized photodegradation of Trp via radical processes was much higher than that via $^1O_2$ processes, though the saturated oxygen aqueous solution favors the $^1O_2$ generation. In this same respect, it was previously reported (Silva & Gaule, 1977) that irradiation of lysozyme with visible light, in the presence of riboflavin and molecular oxygen, not only produced the photooxidation of some amino acid residues of that enzyme, but also generated a binding of this sensitizer to the protein. This riboflavin-lysozyme photo-binding can also be obtained in an anaerobic atmosphere, thus avoiding photo-oxidative Type II processes and allowing the Type II process in its first stage, that does not require the presence of molecular oxygen.

In subsequent studies, after obtaining peptides from lysozyme (Ferrer & Silva, 1981) it was demonstrated that a Trp residue of the enzyme was specifically involved in the binding between riboflavin and lysozyme (Ferrer and Silva, 1985). Through the irradiation of Trp in its free form in the presence of Rb in oxygen saturated aqueous solutions, it has been possible to isolate and characterize spectrophotometrically the following species: aggregate forms of riboflavin, indolic products associated to flavins, indolic products of molecular weight higher than tryptophan, formylkynurenine, and other tryptophan photodecomposition products (Silva et al, 1994). A vibrational interpretation of the structure of a pure fraction of a riboflavin-Trp adduct was performed on the basis of its infrared spectrum (Campos et al, 1994). The vibrational assignment suggests an important electronic redistribution in the structure of riboflavin and tryptophan when the adduct is formed. From this fact, at least

two ways of binding between the indole and isoalloxazine rings can be inferred. The effects of pH and ionic micelles on the rates of product formation following irradiation of riboflavin in the presence of tryptophan has also been investigated by absorption and fluorescence spectroscopy (Silva et al, 1991). The formation of riboflavin-tryptophan adducts is inhibited in acid solutions under anaerobic conditions and also by the addition of anionic (sodium dodecyl-sulfate) and cationic (cetyltrimethyl ammonium bromide) micelles. The oxidation of tryptophan photo-induced by riboflavin is considerably faster in basic solutions.

Riboflavin sensitized photoproducts of tryptophan and hepatic dysfunctions

The presence of tryptophan and riboflavin has been related to hepatic dysfunctions produced by parenteral nutrition (Farrel et al, 1982; Brawley et al, 1993). Studies in animals have suggested a role of the riboflavin photosensitized oxidation of tryptophan in the pathogenesis of hepatic dysfunction in neonatal gerbils (Bhatia & Rassin, 1985). Considering that the action of visible light on solutions containing tryptophan and riboflavin generated not only photo-oxidation products, as a consequence of the production of the active oxygen species O_2-, H_2O_2, $.OH$ and $^1O_2$ during irradiation, but also indol-flavin and indol-indol photoadducts (Ugarte et al, 1992; Silva et al, 1994), it was interesting to study the possible toxicity of these compounds. A decrease in the gain of weight and an increase in the activity of serum g-glutamyl transpeptidase was found in rats receiving intraperitoneally, both anaerobic and aerobic light-exposed tryptophan-riboflavin solutions for 12 days as compared to controls. Concentrations of g-glutamyl transpeptidase were higher in animals receiving the anaerobic irradiated solutions than in the other groups (Donoso et al, 1988). This enzyme is usually employed in clinical studies, as an indicator of hepatic canalicular membrane function and integrity; thus an increase of g-glutamyl transpeptidase at the plasma level is associated with hepatic dysfunctions.

Cytotoxic effect of the riboflavin sensitized photoproducts of tryptophan

Wang et al (1974) have reported that when mammalian cells in tissue-culture medium (Dulbecco's modified Eagle's medium) were irradiated with the near-UV light emitted by black-light tubes, the cells were killed both physiologically and reproductively. In another report, they showed that the killing effect was due to formation of toxic photoproducts from riboflavin, tryptophan, and tyrosine in the medium (Stoien & Wang, 1974). Cell damage does not occur when these components are withdrawn from the medium prior to irradiation (Nixon & Wang, 1977). Considering that under the experimental conditions described by these authors, tryptophan, riboflavin and indol-indol binding also occur, it was of interest to compare the cytotoxic effect of tryptophan-riboflavin solutions irradiated under either anaerobic or aerobic conditions. In the first case, the adducts are preferentially generated, and in the oxygenated medium these compounds are accompanied by products of tryptophan photooxidation. When the products of the anaerobic irradiation of a tryptophan-riboflavin solution were added to cell culture media seeded with F-9 teratocarcinoma cells, it was observed that these products inhibited both cellular adhesion to the substrate and the natural proliferation process (Silva et al, 1988; Edwards et al, 1994). This same effect was found in the presence of a mixture of the tryptophan photo-oxidation products and the adducts when using solutions previously irradiated with visible light in an O_2 atmosphere. A cytotoxic effect was also observed with embryos incubated in the presence of a tryptophan-riboflavin adduct; in the latter case, necrosis and embryo development arrest occurred (Silva et al 1988).

Riboflavin-photosensitized anaerobic modification of rat lens proteins

A number of chemical and/or structural modifications occur in lens proteins during aging or cataractogenesis: an increase in insoluble protein (Srivastava, 1988; Srivastava & Ortwerth, 1989); the formation of high-molecular-weight proteins (Harding, 1979; Swamy & Abraham, 1987); the production of a yellow to brown coloration (Mellerio, 1987; Zigman & Paxhia, 1988), and the formation of blue fluorescence (Rao et al, 1987). Several workers have attempted to associate the action of light with these changes on lens proteins. Furthermore, cross-linking of crystallins caused by photooxidation has been induced by photosensitizers, such as methylene blue (Goosey et al, 1980; Mandal et al, 1986; Kono et al, 1988), 8-methoxypsoralen (Lerman & Borkman, 1977; Bando et al, 1984), promazines (Merville, 1984), kynurenine derivatives (Mandal et al, 1986; Zigler et al, 1982; Bando et al, 1984; Dillon, 1984; Ichijima & Iwata, 1987; Balasubramanian et al, 1990) and riboflavin (Kono et al, 1988; Mandal et al, 1988).

Since riboflavin is normally present in the ocular lens, a tissue permanently exposed to light, the study of the behavior of lens proteins irradiated with visible light in the presence of riboflavin is of interest. Bose et al (1986) have presented evidence that the riboflavin-sensitized conformational changes of a-crystallin widely differ from those sensitized by methylene blue or N-formylkinurenine.

It was previously reported (Salim-Hanna et al, 1987) that photobinding between free tryptophan and riboflavin may also be obtained in an anaerobic medium where oxygen-mediated processes can be suppressed. Because the in situ lens has a low oxygen concentration (less than 10-5 M) (Dillon & Spector, 1980), it is likely that aerobic and anaerobic photoprocesses would contribute simultaneously to the photoinduced damage of lens proteins. When ^{14}C-riboflavin enriched lenses were exposed to visible light, a photo-induced binding between riboflavin and a water insoluble protein fraction of the lenses was observed (Salim-Hanna et al, 1988).

The irradiation of rat lens homogénate, or its soluble protein fractions, in the presence of riboflavin leads to a modification in the chromatographic elution pattern with an increase in the high-molecular-weight fraction (Ugarte et al, 1992). In a study with rats of different ages, it was shown that the

proportion of the high-molecular-weight protein fraction significantly increased with age, whereas the proportion of the low-molecular-weight protein fraction concomitantly decreased (Ugarte *et al*, 1992). It was postulated that aging produces an increase in the accessibility of the tryptophan residues of the lens proteins, as established from iodide-quenching experiments. In this way, these amino acids would be more susceptible to the interaction with excited riboflavin, with generation of radical species that could be responsible for the initiation of the aggregation processes.

ACKNOWLEDGMENTS

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