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Influence of the pH on the photodynamic effect in lysozyme A comparative kinetic study with the sensitized photooxidation of isolated amino acids

A. T. Soltermann, M. A. Biasutti, A. Senz, and **N. A. García**

Departamento de Química y Física, Universidad Nacional de Río Cuarto, Río Cuarto, Argentina

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Summary. The kinetics of the eosin-sensitized photooxidation ($[O_2(^1\Delta_g)]$-mediated) of the protein lysozyme (Lyso) was investigated under two different pH conditions (pH 7 and pH 11). Rates of oxygen consumption and the fade in the protein fluorescence spectrum upon sensitized irradiation were monitored. Parallel studies on both denatured Lyso (absence of the four −S−S− bridges in the protein) and different mixtures of the photooxidizable amino acids of Lyso were also carried out. The mixtures maintained the same molar ratio as in the native protein, and were selected just in order to throw into relief the preferential amino acids that were being photooxidized at both pH values.

Under work conditions Lyso was only photooxidizable at pH 7, whereas the opposite accounted for the denatured protein: only measurable oxygen consumption was detected at pH 11. Nevertheless, Lyso at pH 11, evidenced an important physical quenching of $O_2(^1\Delta_g)$ due to the Tyr and Trp residues.

The results for the native protein were interpreted on the basis of a previously described dark complex Eosin-Lyso, which selectively favours the photooxidation of the bounded protein. The Trp residues were the main reactive entities in the native protein. The photodinamic effect in denatured Lyso was characterized by the prevalence of Tyr residues as photooxidizable targets.

In the discussion of the results, a comparisson with the photooxidation kinetics of the mixtures of free amino acids was made.

Keywords: Amino acids – Eosin – Lysozyme – Photodynamic effect – Photooxidation – Singlet oxygen

Abbreviations: $O_2(^3\Sigma_g^-)$: ground state triplet oxygen; $O_2(^1\Delta_g)$: singlet molecular oxygen; Lyso: lysozyme; LysoD: denatured lysozyme; Eos: eosin; FFA:

furfuryl alcohol; Trp: tryptophan; Tyr: tyrosine; Cys: cysteine; Cis: cystine; Met: methionine; His: histidine; AA: amino acid; a.u.: arbitrary units.

Introduction

The singlet molecular oxygen [$O_2(^1\Delta_g)$]-mediated photooxidation of several enzymes has been the subject of an intense study in the last two decades (Spikes and Mc Night, 1970; Jory, 1975; Straight and Spikes, 1985). Photodynamic alteration in proteins results, in the great majority of the cases, from the degradation of the side chains of the five susceptible amino acids (cysteine (Cys), histidine (His), methionine (Met), tryptophan (Trp) and tyrosine (Tyr). The kinetics of the sensitized photooxidation of some of the mentioned isolated amino acids is known to be highly dependent on the conditions of the reaction medium, especially pH and polarity and, in some cases, independent on the characteristics of the sensitizing agent (Wilkinson and Brummer, 1981; Straight and Spikes, 1985). This is only partially true when the photooxidation of proteins is considered. Peptide bonds and conformational factors in the protein greatly affect the nature of the amino acid residue exposed to photooxidation (Hopkins and Spikes, 1969; Churakova et al., 1973; Risi et al., 1973). Hence, the photodynamic effect in proteins can not be straightforwardly predicted from *a priori* evaluation of the kinetic behaviour of individual isolated AAs. Furthermore, it is known that dark and photoinduced binding of dye molecules to proteins play a dominant role in the kinetics of photooxidation (Kepka and Grossweiner, 1973). As a difference with photooxidation of simple molecules (such as isolated AAs), the choice of the sensitizer in proteins, stablishes in advance some characteristics of the photooxidative event to be considered.

Lysozyme (Lyso) is one of the most widely investigated proteins in relation to photodynamic effect. Most of the kinetic studies compare the degree of photooxidative damage with the maintenance of enzymatic action (Risi et al., 1973; Churakova et al., 1973; Hopkins and Spikes, 1970) under a variety of experimental conditions, including the effect of pH, employing eosin (Eos) as a dye sensitizer (Kepka and Grossweiner, 1973). We have considered an interesting task to go deeply in the kinetics of the Eos-sensitized photooxidation of Lyso under different pH conditions. In the present contribution, a parallel study was carried out on the native and denatured protien (LysoD). In the latter the four disulfide bridges of Lyso (highly responsible for the stability of the protein (Stryer, 1988)) are broken. The strategy of the work was to stablish a comparisson between the kinetic behaviour of a series of different mixtures of free amino acids in solution with that of native and denatured Lyso, under identical experimental conditions, upon Eos-sensitized areorbic irradiation. These mixtures, assayed at different pH values, contained selected combinations of the photooxidizable amino acids of Lyso, mantaining the same molar ratio as in the native protein. Obviously the aim for the employment of these AA mixtures was not to mimic the complex entity represented by the protein. The mixtures were utilized for

comparative purposes, in order to throw into relief the possible AAs that suffer the photooxidative damage, under different pH conditions, in the protein.

The selection of the Lyso as a photooxidizable target and Eos as the sensitizer, in this work, was motivated mainly by the following reasons: a) It was known the effect of photodynamic damage and pH on the enzymatic activity of the protein (Kepka and Grossweiner, 1973; Hopkins and Spikes, 1970; Rosenkranz et al., 1978). Nevertheless, the kinetics of the photooxidative process under different pH conditions, and the evolution of the implicated AA residues have not been closely connected. b) The association between the dye and Lyso has been perfectly characterized, as a function of the pH (Kepka and Grossweiner, 1973).

Materials and methods

Materials

The chemicals lysozyme (Lyso) from chicken egg white 3 × cristallized, dialized and lyophilized, L-tryptophan (Trp), L-tyrosine (Tyr), methionine (Met), histidne (His), cystine (Cis) and cysteine (Cys) were purchased by Sigma. Furfuryl alcohol (FFA) and Eos were from Aldrich. Water was triply distilled. For all measurements freshly prepared solutions were used. Buffers Na_2HPO_4/NaOH and NaH_2PO_4/NaOH were employed for pH 11 and 7 respectively (Hodgman et al., 1963).

Four different AA mixtures were employed. They were prepared maintaining the same molar proportion as in the native protein. Lyso posses, among others, one residue of His, six of Trp, two of Met, eight of Cys, and two of Tyr (Stryer, 1988) (only the photooxidizable AAs or those relevant for the purposes of the paper were mentioned). The so called Mixture 1 (M1) contained His (1); Trp (6); Met (2); Cys (8) and Tyr (2). Mixture 2 (M2) contained His (1); Trp (6); Met (2); Cis (4) and Tyr (2). Mixture 3 (M3) contained His (1), Trp (6), and Tyr (2). Finally, Mixture 4 (M4) contained His (1) and Tyr (2). The numbers in parenthesis represent the molar proportion of each AA in the mixture.

Methods

The irradiation set-up (cut-off filter at 400 nm) including the specific electrod (Orion 97-08) has been already described (Gsponer et al., 1987). All experiments were carried out under air-saturated conditions and at room temperature. The irradiation was carried out employinf a 150 W quarz-halogen lamp.

Rates of oxygen consumption were calculated from the ratio of the initial rates of oxygen uptake (see Fig. 2). The amount of absorbed photons by the sensitizer was kept constant for all experimental conditions.

In order to compensate the possible variation of the quantum yield for $O_2(^1\Delta_g)$ generation by Eos going from pH 7 to 11, the rates of oxygen consumption for the runs of Lyso, LysoD and the AA mixtures, were normalized by doing the quotient with those of FFA under identical experimental conditions. It is known that the rate constant (k_r) for the $O_2(^1\Delta_g)$-mediated photooxidation of FFA is pH-independent (Hagg and Hoigne', 1986). In all cases (for Lyso, LysoD and for the different AA mixtures) the concentration of either the protein or His in the AA mixtures was 0.01 mM and the absorbance of the sensitizer (Eos) was 0.5 at 514 nm.

The rate constants (k_r) for some individual AAs (Table 1) were obtained through oxygen consumption experiments, as described elsewere (Miskoski and García, 1993)

Table 1. Relative rates of oxygen consumption (V_{ox}) during the Eos-sensitized photooxidation of Lyso, LysoD and several mixtures of isolated AAs (a) at pH 7 and 11 (b). Are also included the values of the reactive rate constants (k_r, $M^{-1}s^{-1}$) for the interaction of several individual AAs with $O_2(^1\Delta_g)$

Compound	V_{ox}		$k_r \times 10^{-7}$	
	pH 7	pH 11	pH 7	pH 11
Lyso	0.4	<0.001		
LysoD	<0.004	0.13		
M1	0.29	1		
M2	0.06	0.14		
M3	0.27	0.73		
M4	0.004	0.09		
His			9.0(c)	10.5(d)
Trp			3.2(e)	29.5(d)
Tyr			NR(f)	9.5(g)
Met			2.1(c)	
Cys			0.78(d)	8.87(d)
Cis			NR(f)	0.37(d)

(a) M1: His (1); Trp (6); Met (2); Cys (8); Tyr (2).
M2: His (1); Trp (6); Met (2); Cis (4); Tyr (2).
M3: His (1); Trp (6); Tyr (2).
M4: His (1); Tyr (2).
Numbers in parenthesys represent the molar proportion for each AA in the mixture.

(b) The reaction mixture was 0.05 mM in the photooxidizable substrate and Eos, absorbance 0.5 at 514 nm in the presence of phosphate buffers.

(c) Miskoski and García, 1993; (d) This work; (e) Bertolotti et al., 1991; (f) non reactive; (g) Criado et al., 1994.

employing FFA as a reference compound. The k_r value utilized for FFA was $1.2 \times 10^8 M^{-1}s^{-1}$ (Hagg and Hoigné, 1986).

Fluorescence measurements were made using an Spex FluoroMax fluorometer. Both, the protein and the different AA mixtures were excited at 290 nm, in order to ensure that both fluorescent components of Lyso (Tyr and Trp) absorb the incident light.

The kinetic evidence obtained through fluorescence measurements was employed to qualitatively confirm the determinations of oxygen consumption in the different experimental media. Fluorescence emission in Lyso is due to the presence of six tryptophyl and two tyrosyl residues in the molecule. It is important to keep in mind the fact that the fluorescence quantum yield of Tyr (pK = 10.05 for the phenolic −OH in Tyr, from Rizzuto and Spikes, 1977) is dramatically decreased in the alkaline range of pH (Lakowicz, 1983).

Ground state absorption spectra were recorded with a Hewlett Packard 8452A diode array spectrophotometer.

The denaturation of Lyso was carried out by performic acid oxidation, according to the technique described by Ozolos, 1990.

Results

It has been demonstrated that $O_2(^1\Delta_g)$ is the major oxidizing entity in the photosensitized degradation of Lyso, employing Eos as dye sensitizer (Kepka and Grosswiener, 1973). Hence, the following reaction scheme applies in the present case:

$$\text{Eos} + h\nu \longrightarrow {}^*\text{Eos}^1 \xrightarrow{k_{ISC}} {}^*\text{Eos}^3 \quad (1)$$

$${}^*\text{Eos}^3 + O_2(^3\Sigma_g^-) \xrightarrow{k_{ET}} \text{Eos} + O_2(^1\Delta_g) \quad (2)$$

$$O_2(^1\Delta_g) \xrightarrow{k_d} O_2(^3\Sigma_g^-) \quad (3)$$

$$O_2(^1\Delta_g) + \text{Lyso} \xrightarrow{k_q} O_2(^3\Sigma_g^-) + \text{Lyso} \quad (4)$$

$$O_2(^1\Delta_g) + \text{Lyso} \xrightarrow{k_r} \text{Products} \quad (5)$$

Eos absorbs the incident light and generates its electronically singlet and triplet excited states (reaction (1)). From the latter, an energy transfer reaction to ground state-triplet molecular oxygen $[O_2(^3\Sigma_g^-)]$ can take place, producing the excited state oxygen species $O_2(^1\Delta_g)$ (reaction (2)). It can decay by colission with sorrounding molecules (typically the solvent, reaction (3)) or interact physically (reaction (4)) or chemically (reaction (5)) with Lyso.

Three different chemical entities (Lyso, LysoD and a series of mixtures of selected AAs) at two values of pH (7 and 11) were evaluated in the present work.

The mentioned pH values were chosen due to the fact that, in some cases, the rate of photooxidation greatly depends on the ionization of determined functional groups in the molecule of the isolated photooxidizable AAs of Lyso, as follows: a) For Tyr it is known that the photooxidative reaction occurs with the phenolic ring in the anionic form (pK of the phenolic −OH approximately 10). This AA is a relatively strong physical quencher of $O_2(^1\Delta_g)$at neutral and acidic pH (Bertolotti et al., 1991). b) The kinetics of photooxidation of Trp varies from low rates at low pH (typically pH 4) to higher rates at pH values superior than 8 (this work and Straight and Spikes, 1983). c) The rate of photooxidation of Met with several sensitizers (including Eos) is practically independent of pH over the range of 4 to 10 (Straight and Spikes, 1983). d) For His it is known a marked increase of the photooxidation rate going from pH 3 to 11 (being the reactant the unprotonated imidazole ring (pK = 5.8)). For Met, the same as for His, the interaction with $O_2(^1\Delta_g)$ is enterely reactive regardless of the pH and sensitizer employed (Miskoski et al., 1993 and references therein). e) Finally, for Cys it is known that the Eos-sensitized photooxidation occurs mainly by a $O_2(^1\Delta_g)$-mediated mechanism at pH values higher than 7, being cystine the major reaction product (Straight and Spikes, 1983).

We evaluated reaction (5) by monitoring both the rate of oxygen uptake and rate of fluorescence loss, in the protein or the AA mixtures upon sensitized irradiation. It was made on the asumption that reaction (5) was the only source of Lyso and oxygen consumption (Kepka and Grossweiner, 1973). In Fig. 2 typical runs for the mentioned determinations are shown, under different pH conditions. In all cases the spectral modifications and oxygen consumption upon sensitized irradation are totally suppressed when assayed in the presence of 10 mM of NaN_3 or in N_2-saturated solutions. The spectral changes were much higher in magnitude when D_2O was replaced by H_2O. According to currently accepted criteria (Wilkinson and Brummer, 1981), these experimental findings indicate that both, the observed spectral modifications and oxygen consumption obey, in a greater extent, to a $O_2(^1\Delta_g)$-mediated reaction.

The V_{ox} for Lyso, LysoD and the four different sets of AA mixtures are shown in Table 1. Also are included, for comparative purposes, some values of reactive rate constants (k_r, reaction (5)) either, extracted from the literature or determined in this work, as described in the experimental section.

Sensitized photooxidation of native Lyso

In Fig. 1 the changes in the fluorescence spectrum of Lyso, at pH 7, upon Eos-sensitized irradiation can be observed. No changes at all were detected when the same experiment was carried out at pH 11. In Fig. 2 is shown the profile of oxygen uptake at both pH values. Again in this case results indicate non detectable reactivity in the alkaline medium and relatively fast oxygen consumption in the neutral medium. Nevertheless, in the alkaline range of pH, the presence of native lysozyme exerts a considerable delay on the rate of oxygen uptake of FFA due to the presence of Lyso. This was stablished by comparisson of the respective rates of oxygen consumption by FFA in the absence and in the presence of the protein, upon Eos sensitized irradiation (Fig. 2, inset). This effect was interpreted as an effective $O_2(^1\Delta_g)$ physical quenching by the native protein (reaction (4) in the kinetic scheme).

Sensitized photooxidation of denatured Lyso

Lyso is a very stable protein. It posses four cross-linked disulphide bridges that highly contribute to the mentioned stability (Stryer, 1988). In contrast to ultraviolet irradiation, peptide bonds and disulfide bonds in proteins are not broken by photodynamic treatment (Hopkins and Spikes, 1970; Michaeli and Feitelson, 1994). The aim for the study of LysoD was to obtain information towards the influence of the disulphide bridges on the kinetics of photooxidation of the protein. Unfortunately, most of the denaturation procedures does not prevent the spontaneous renaturation processes (i.e. the reversibility in the formation of the $-S-S-$ bridges). Nevertheless, denaturation by the method of performic acid converts Cis and Cys to cysteic acid and converts Met to Met sulfoxide, derivatives that are stable toward acid hydrolysis.

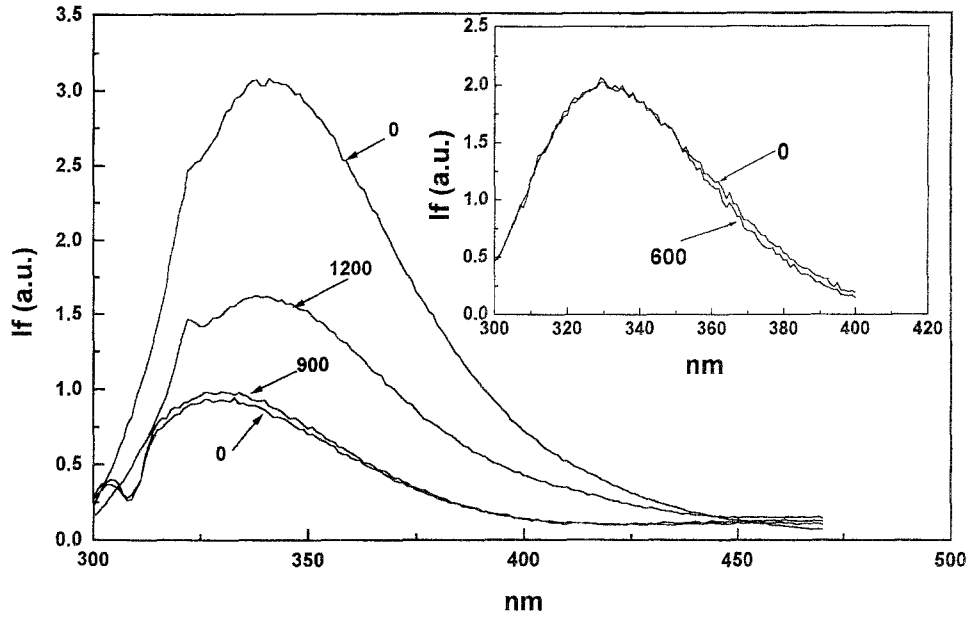

Fig. 1. Fluorescence spectra of Lyso and LysoD at pH 7, after and before eosin-sensitized irradiation under air-saturated conditions. Inset: Fluorescence spectra fo M4 after and before Eos-sensitized irradiation. Numbers indicate minutes of photolysis

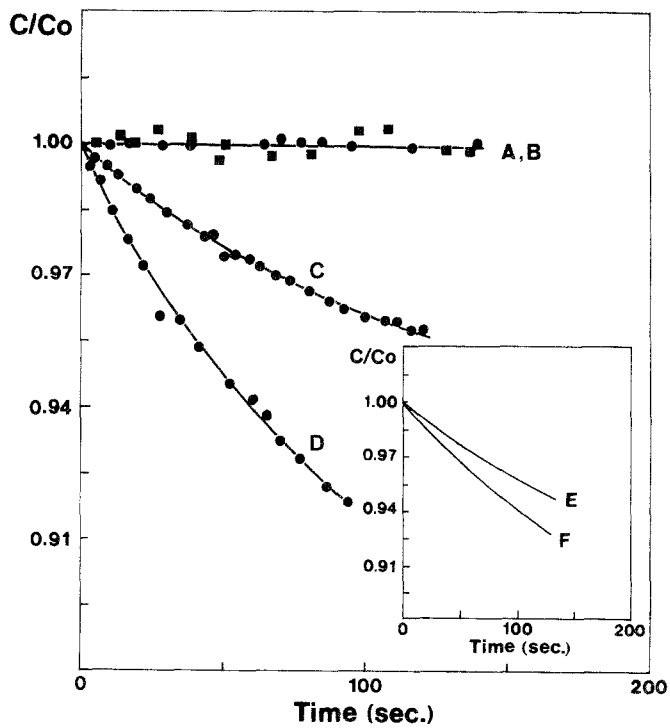

Fig. 2. Normalized (see *Methods*) profiles of oxygen consumption upon eosin-sensitized irradiation (air saturated solutions) of Lyso and LysoD at different pH. *A*: Lyso at pH 11 (●); *B*: LysoD at pH 7 (■); *C*: LysoD at pH 11; *D*: Lyso at pH 7. Inset: The same normalized profiles for *E*: FFA plus Lyso 0.06 mM and *F*: FFA, both at pH 11. Concentrations of the reactants were described in the *Materials* section. Co and C represent the oxygen concentrations at time cero and t respectively

Frequently performic acid oxidation destroys Trp and/or Tyr residues, in a variable extent, depending on the experimental conditions. This was a secondary undesirable effect in our case. After the denaturation procedure, we could detect only that fluorescence characteristic of Tyr residues. The typical fluorescence due to Trp residues (one of the main quenchers of Tyr fluorescence) was totally absent.

The kinetic behaviour of LysoD photooxidation was totally opposite to that determined for Lyso. No photoreaction could be detected by either fluorescence or oxygen uptake monitoring at the neutral pH. These results are shown in Figs. 1 and 2 respectively. In the alkaline medium a very efficient photooxidative process was observed for the denatured protein.

Sensitized photooxidation of amino acids mixtures

Four different mixtures of free AAs were assayed as reactive $O_2(^1\Delta_g)$ quenchers. As puntualized, the already described mixtures contained some of the photooxidizable AAs present in Lyso, maintaining the same molar ratio as in the protein. Excluding M4 (containing only His and Tyr) at pH 7, all the mixtures exhibited measurable rates of oxygen consumption upon sensitized aerobic irradiation (see also the constancy in M4 fluorescence spectrum, after sensitized irradiation, in Fig. 1, inset). In Fig. 3 is shown the evolution of the fluorescence spectra of M3, upon Eos-sensitized irradiation, at two different pH values. It can be clearly appreciated the difference in V_{ox} between both runs.

The V_{ox} for the mixtures reaches the maximun value for M1 at pH 11. As

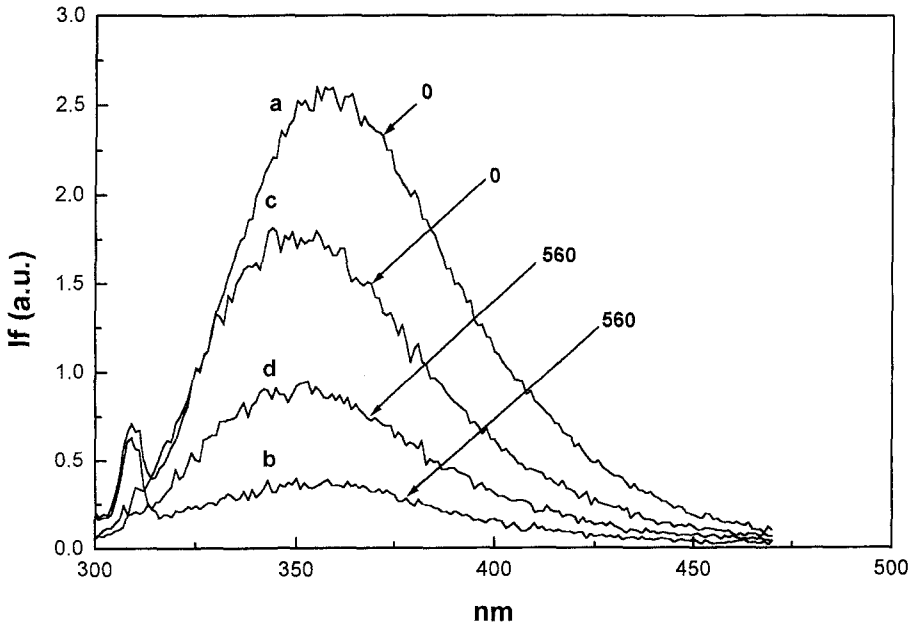

Fig. 3. Fluorescence spectra of M3, at pH 7 and 11, after and before Eos-sensitized irradiation. Numbers indicate minutes of photolysis. *a* and *b* pH 11; *c* and *d* pH 7

a general behaviour, for all four mixtures the V_{ox} were much higher in the alkaline range of pH than those values obtained in neutral solutions.

The AA mixture M2 was included in the present work in order to compare the effect of four $-S-S-$ groups with that of eight -SH groups (M1) on the rate of oxygen uptake. The V_{ox}, at both pH values, are much lower (between 5 and 7 times) for the cystein-containing mixture. When this AA is taken away from the mixture (M3) the V_{ox} return to values comparable with those excibited by M1.

It has been stablished (for a review see Straight and Spikes, 1985) that Cis is not susceptible to $O_2(^1\Delta_g)$-mediated photooxidation at the neutral pH. Nevertheless, we found, that at pH 11 the AA suffers a relatively slow Eos-sensitized photooxidation. We determined a value of $3.7 \pm 0.2 \times 10^6 M^{-1}s^{-1}$ for k_r (reaction (5)) as measured by oxygen uptake (see *Methods*). Besides, no traces of oxygen consumption could be detected at pH 7.

Another interesting information on the interaction Cis-$O_2(^1\Delta_g)$ arises from the comparison of the respective V_{ox} (Table 1) of M2 and M3. On the basis that Met only quenches $O_2(^1\Delta_g)$ by a reactive pathway (reaction (5)) (Miskoski et al., 1993), the difference between V_{ox} must be attributed to a physical quenching (at both pH values) exerted by free Cis.

Discussion

Photooxidation of Lyso

Lyso exhibits a relatively fast profile for oxygen uptake at the neutral pH, whereas at pH 11, only the physical quenching $O_2(^1\Delta_g)$ could be detected. Tyr residues are normally non emissive in proteins, due to a number of factors (mainly represented by energy transfer to Trp residues and quenching by nearby groups in the protein (Lakowikz, 1983)). Thus, is not a surprising result the fact that lyso fluorescence at pH 7 tends to completely desappear, upon prolonged sensitized irradiation, even when it was clearly stablished that tyrosine is not photooxidizable at the neutral pH.

In contrast to the kinetic profiles obtained for Lyso, the different AA mixtures assayed, without exception, exhibited the higher V_{ox} values at pH 11, (see also this trend in k_r values, from Table 1, for the individual AAs).

The first temptation, in order to rationalize this unexpected pH-dependent behaviour in the photodynamic effect of Lyso, is the occurrence of structural modifications in the protein, due to the abrupt change in the pH conditions. It is known that the enzymatic activity of Lyso drops sharply on either side of the optimal pH (approximately 5). The decrease in the alkaline side is due to the ionization of glutamic acid 35, whereas the decresase in rate on the acid side reflects the protonation of aspartate 52 (Stryer, 1988). Nevertheless, it is also known that none of these AAs participate in the photodynamic damage of the protein (Hopkins and Spikes, 1970; Kepka and Grossweiner, 1973). The first authors suggest that the aerobic dye sensitized (including Eos-sensitized) inactivation of Lyso, could be related to alterations of tertiary and secondary structure of the protein during photodynamic treatment.

Our results, by monitoring the rates of oxygen uptake can be analyzed by comparison with those of Kepka and Grossweiner (1973) on the correlation between pH and the loss of Lyso enzymatic activity upon Eos-sensitized photooxidation. The authors demonstrated that the protein, by means of a ground-state complex, binds one eosin molecule from pH 4 to 12, without inhibition of the enzymatic activity. The presence of Trp residues play a dominant role in the mentioned dark association. The fraction of Lyso bound to Eos decreases as the pH increases, and the main photooxidative inactivation takes place with the complexed fraction of Lyso molecules.

The origin of this effect was not totally clear for Kepka and Grosswiner (1973). They advanced the very interesting possibility that the interaction Eos-Lyso could introduce non-inactivating conformational changes in the protein. As a result different photooxidizable targets could be exposed to $O_2(^1\Delta_g)$ attack as a function of the degree of complexation.

The open question is why the reactive channel, which implies oxygen consumption (reaction (5)), remains practically closed in the alkaline range.

From the comparisson of the relative rates (V_{ox}) of all three M1, M2 and M3 it arises that the presence of Cis exerts a considerable protection (antioxidant effect) in the AA mixtures due to an effective $O_2(^1\Delta_g)$-physical quenching (reaction (4)). This effect could be even much more intense in a compact protein such as Lyso.

This fact could also explain the particular sensitivity of the Eos-complexed Lyso to photooxidation. Assuming that the dye, due to the complexation, should occupate inner positions within the protein structure, the diffusion of $O_2(^3\Sigma_g^-)$ could generate $O_2(^1\Delta_g)$ nearby the photooxidizable targets. On the other hand, in the absence of the Eos-Lyso complexation (in the alkaline range) the externaly generated $O_2(^1\Delta_g)$ should be physically quenched by the efficient $-S-S-$ bridges during the difussion process.

At pH 7, even when Met, Cys and His residues should reactively contribute to the observed oxygen uptake (Fig. 1), the evidence provided by fluorescence experiments demonstrate that Trp residues are possibly the main impicated entities in the photodynamic dammage of Lyso. This result is in agreement with those obtained by Hopkins and Spikes (1970) and Kepka and Grossweiner (1973) by monitoring the loss of enzymatic ativity of Lyso during the course of the photodynamic treatment.

Photooxidation of LysoD

In contrast to ultraviolet irradiation, peptide bonds and disulfide bonds in proteins are not broken by photodynamic treatment. Nevertheless, specially the $-S-S-$ groups are susceptible to suffer attack from free radicals generated in primary, singlet oxygen-mediated-photoprocesses (Straight and Spikes, 1985). By this way, a moderately prolonged sensitized irradiation of Lyso can produce the breaking of a certain amount of disulphide bridges. It should cause conformational changes, and as a consequence, the exposure of a different fashion of photooxidizable targets to photodynamic damage. We

found an interesting topic the evaluation of the photooxidative kinetics of LysoD, representing the extreme situation where the AA residues, responsible for the −S−S− bridges, have been transformed to cysteic acid. As already puntualized, the Trp and Met residues in LysoD have been also transformed by the denaturation procedure. For this reason, in principle, it is possible to compare the kinetic behaviour of oxygen uptake by LysoD with that of M4. As shown in Table 1, they were totally coincident. The absence of reactive interaction at pH 7, in both cases, can be understood by considering that Tyr is the main remaining photooxidizable AA residue. It exerts only a moderate physical quenching of $O_2(^1\Delta_g)$ at the neutral pH. In the alkaline range, the phenolic AA is highly reactive due to the ionization of the −OH group (Miskoski and García, 1993; Criado et al., 1994).

Concluding remarks

The present work provides additional evidence in the sense that pH greatly affect the rates of the Eos-sensitized photooxidation of Lyso. The dark association Lyso-Eos and the protein structure govern the kinetics of photooxidation. The parallel studies on LysoD, in the absence of the disulfide bridges and Trp residues of the native Lyso turns the photooxidative behaviour comparable to that of the free remaining photooxidizable AAs. Trp residues are the dominant photooxidizable targets in Lyso at the neutral pH. In the alkaline range, the $O_2(^1\Delta_g)$-physical quenching effect exerted by −S−S− bridges in Lyso, protect the protein against photodynamic degradation.

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Authors' address: Dr. N. A. García, Departamento de Química y Física, Universidad Nacional de Río Cuarto, 5800 Río Cuarto, Argentina.

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