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On the $O_2(^1\Delta_g)$-mediated photooxidative behaviour of tripeptide glycyl-tyrosyl-alanine in alkaline medium A kinetic study

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Summary. The type II singlet molecular oxygen [$O_2(^1\Delta_g)$]-mediated photooxidation of the tripeptide gly-tyr-ala was studied. It has two non-oxidizable amino-acids (gly and ala) bonded to the oxidizable one, tyr. Overall (k_t) and reactive (k_r) rate constants for the interaction were determined by time-resolved methods (*IR* emission of $O_2(^1\Delta_g)$) and stationary photolysis, in water at pH 11.5 as well as in alkaline non-aqueous etOH–MeCN (80:20, v/v, 10 mM in KOH) solutions. An important solvent polarity effect on k_t was detected; the rate constant increasing one order of magnitude in going from the organic mixture to water (k_t $H_2O = 2 \times 10^9$ M^{-1} s^{-1}). Nevertheless, k_r does not parallel this trend; gly-tyr-ala being less photooxidizable in a more polar environment. The effective quantum yield ($\varnothing_r$) for *TPE* photooxidation is much higher in etOH–MeCN ($\varnothing_r = 0.056$) than in water ($\varnothing_r = 0.023$). Results are discussed on the basis of the formation of an exciplex with polar character between the TPE and $O_2(^1\Delta_g)$.

Two remarkable points should be taken into account: a) the rate costants for the interaction of $O_2(^1\Delta_g)$ with gly-tyr-ala are practically the same as for free tyr. b) New $-NH_2$ groups are generated upon sensitized irradiation. Both findings indicate that the peptide bonds in the TPE break as a result of the photooxidation. A thorough analysis with data for tyrosine and related dipeptides is undertaken.

Keywords: Amino acids – Singlet-oxygen – Peptides – Photooxidation – Kinetics

Abbreviations: *$O_2(^1\Delta_g)$* singlet molecular oxygen; *AA* amino-acid; *TPE* tripeptide; *gly-tyr-ala* glycyl-L-tyrosine-alanine; *tyr-gly* L-tyrosyl-glycine; *FFA* furfuryl alcohol; *tyr* L-tyrosine; *gly-tyr* glycyl-L-tyrosine; *DPE* dipeptide; *NaN_3* sodium

azide; *RB* rose bengal; *ZnTPP* Zinc tetraphenyl porphyrine; *MeCN* acetonitrile; *DMA* 9,10-dimethyl anthracene; *etOH* ethanol; *TRPD* time-resolved phosphorescence detection; *—NH_2 loss* loss of primary amine reactivity.

Introduction

Nilsson et al. (1972) and more recently Matheson and Lee (1979) demonstrated, through precise kinetic evidence, that in aqueous solutions only a few amino-acids (AAs) are susceptible to singlet molecular oxygen [$O_2(^1\Delta_g)$]-sensitized photooxidation. Tyrosine (tyr) belongs to this group, which is involved in the so called "photodynamic effect", whereas other amino-acid residues such as glycine (gly) and alanine (ala), typically found in proteins, are resistant to photooxidation (for a review see Straight and Spikes, 1985).

Tyr residues, among others, constitute important sites of biochemical lesion due to photooxidative damage in proteins (Spikes and Mac Knight, 1970; Jori, 1975; Straight and Spikes, 1985). It is a crucial objective in this area to establish possible relationships between substrate structure and susceptibility to photodynamic action. In complex biological systems, one possible approach to this study is the analysis of the reaction kinetics and mechanisms involved in the photooxidation of a given assembly. For this reason Bertolotti et al. (1991) studied the dye sensitized photooxidation of tyr and their simplest dipeptides (DPEs) such as glycyl-tyrosine (gly-tyr) and tyrosyl-glycine (tyr-gly). These selected DPEs involved the bonding of carboxyl and amino groups of the oxidizable AA (tyr) to a non-oxydizable one (gly). We discussed the influence of the peptide bond on the bimolecular rate constant for the overall interaction between $O_2(^1\Delta_g)$ and tyr derivatives, and its effect on the distribution between physical and reactive quenching, mechanism which is represented as follows as reported by Wilkinson and Brummer (1981):

$$O_2(^1\Delta_g) + \text{DPE} \xrightarrow{k_t} \text{overall quenching} \quad (1)$$

$$O_2(^1\Delta_g) + \text{DPE} \xrightarrow{k_q} O_2(^3\Sigma_g) + \text{DPE} \quad (2)$$

$$O_2(^1\Delta_g) + \text{DPE} \xrightarrow{k_r} \text{products} \quad (3)$$

$k_t = k_q + k_r$ and $^3O_2(^3\Sigma_g)$ being the ground state of oxygen.

Those results indicated a similar photooxidative behaviour in tyr and tyr-gly, whereas photooxidation was hindered when the amino group of tyrosine was chemically bonded. In all cases a significant pH effect on the photooxidation rate constants operated.

On this basis, and looking for systems structurally more complex, a tyr tripeptide (TPE) was chosen as a photooxidizable substrate. The study was undertaken in order to investigate the role of a double peptide bond in the kinetics and photodynamic effect of glycyl-tyrosyl-alanine (gly-tyr-ala). For this compound, where tyr is bonded to the non-oxidizable AAs gly and ala, certain unexpected results were obtained. Basically, the findings for gly-tyr and tyr-gly and tyr itself cannot be related in a straightforward manner to understand the kinetics of photooxidation in the TPE.

Material and methods

Materials

Gly-tyr-ala, and fluorescamine were purchased from Sigma and used as received. The 9,10-dimethyl anthracene (DMA), and the sensitizers (rose bengal (RB) and Zinc tetraphenyl porphyrine (ZnTPP)) were from Aldrich and recrystallized twice from methanol and methanol-benzene, respectively, before use.

Mixtures of HPLC grade acetonitrile (MeCN) and ethanol (etOH) (80:20, v/v; 10 mM in KOH) were used as one of the solvents, water triply distilled being the other one. Buffer solutions were prepared employing Na_2HPO_4–NaOH for pH 11.5 (Hodgman et al., 1963), editors).

In both solvents, the presence of the ionized form (tirosinate) as the only species of TPE was confirmed through spectroscopic evidence.

Methods

Kinetic measurements

Time resolved determinations

The $O_2(^1\Delta_g)$ phosphorescence detection (TRPD) method used to obtain the total rate constants, k_t [reaction (1)], for the interaction of gly-tyr-ala with $O_2(^1\Delta_g)$ in MeCN–H_2O has already been described (Bertolotti et al., 1991). Briefly, it consisted in a Laser Optics nitrogen laser with pulses of 5 mJ and 7 ns halfwidth (337 nm) as the excitation source. The emitted radiation was detected at right angles (using an amplified Judson J16/8Sp germanium detector) after having passed through appropriate filters. The output of the detector was coupled to a digital oscilloscope and to a personal computer to carry out the signal processing. Usually sixteen shots were needed for averaging, in order to get a good signal/noise ratio, from which the decay times were calculated. The absorbance of the sensitizers (either RB or ZnTPP) for the dynamic measurements, in etOH–MeCN (80:20, v/v) were 0.5 at 337 nm.

The $O_2(^1\Delta_g)$ lifetimes were evaluated in the absence (τ^0) and in the presence (τ) of the quencher, and their ratio plotted as a function of quencher concentration, [TPE], according to a simple Stern-Volmer treatment [eq. (4)]:

$$\tau^0/\tau = 1 + k_t\tau^0 \quad [\text{TPE}] \tag{4}$$

Static determinations

The irradiation device, including the specific oxygen electrode has been described elsewhere (Palumbo et al., 1988). A cut off filter below 400 nm was employed.

i) Determination of k_t in water: From the intrinsic characteristics of the IR detector (Nonell, 1988) in our experimental set up for TRPD, it could not be employed for k_t determinations in water where the $O_2(^1\Delta_g)$ lifetime is lower than 8–10 μs. In this case the methodology described by Wilkinson and Brummer, 1981, was employed. If the unique source of oxygen consumption is given by reaction (3), the reciprocal of the initial rates (R) of substrate (TPE) consumption can be written as:

$$R^{-1} = (I_a\varnothing)^{-1}(k_t/k_d + k_d/k_r \quad [\text{TPE}]) \tag{5}$$

where I_a is the intensity of light absorbed by the sensitizer and $\varnothing$ is the quantum yield for $O_2(^1\Delta_g)$ generation. If R^{-1} is plotted vs. [TPE], from the intercept/slope ratio, k_t can be obtained provided a value for k_d is available. The literature value of 2.5×10^5 M^{-1} s^{-1} (Rodgers, 1988) for k_d (the reciprocal of τ^0 in water) was employed in the calculations.

ii) Determination of k_r: The reactive rate constant for the interaction of gly-tyr-ala with $O_2(^1\Delta_g)$ was determined by different actinometric methods, depending on the solvent. In all

cases the knowledge of the reaction rate constant $k_r A$ for the photooxidation of a reference compound A [reaction 6] is required:

$$O_2(^1\Delta_g) + A \xrightarrow{k_r A} \text{products} \quad (6)$$

In water the reference was furfuryl alcohol (FFA) with $k_r A = 1.2 \times 10^8$ M^{-1} s^{-1} (Scully and Hoigne, 1987) and the sensitizer RB (Abs. 560 = 0.5). We used, as previously reported (Mártire et al., 1991), a modification of the method employed by Scully and Hoigne (1987). Parallel experiments of oxygen consumption vs. irradiation time in solutions containing the sensitizer plus either FFA or TPE were run. Assuming that the reaction of $O_2(^1\Delta_g)$ with the quencher is the only way for oxygen consumption, and under conditions of excess of substrate, the ratio of the first order slopes of oxygen uptake by the reference compound and substrate yields the ratio of the reactive rate constants from which k_r TPE can be obtained.

In etOH–MeCN the method for k_r determination was basically the same as in water, except that in the latter the loss of substrate and reference compound instead of oxygen consumption was monitored because the electrode available (Orion 97-08) has a membrane which can be used only in aqueous media with a high percent of water (typically, more than 95%).

The reference was 9,10-dimethylanthracene (DMA), with a $k_r A = 8.2 \times 10^8$ M^{-1} s^{-1}, determined by TRPD. The loss of DMA was monitored fluorometrically (excitation and emission wavelengths were 395 and 450 nm respectively) using an Aminco SPF 125 spectrophotometer and the loss of gly-tyr-ala was followed with an HP 8452A diode array spectrophotometer measuring the initial decrease (conversions lower than 7%) in the absorbance at 245 nm.

iii) Determination of loss of primary amine reactivity: The methodology employed was that described by Straight and Spikes (1978). The rate of loss of primary amine reactivity ($-NH_2$ loss) can be monitored fluorometrically in a given substrate during the course of a sensitized photooxidation. Fluorescamine reagent was excited at 390 nm and its fluorescence observed at 475 nm. The sensitizer was RB.

Results

It is known that the rate of tyr photooxidation with most photosensitizers shows a very sharp pH dependence, the optimum being located around 10 where, as long as type II photooxidations are concerned, the phenolic anion is the reactant (Straight and Spikes, 1985). For this reason all the experiments were carried out exclusively in the alkaline range of pH.

Sensitized irradiation of gly-tyr-ala with either RB or ZnTPP as photosensitizers, resulted in oxygen uptake, substrate consumption and $-NH_2$ loss. Figure 1 shows the absorption spectra of gly-tyr-ala after different irradiation times at wavelengths higher than 400 nm where it is evident the consumption of the substrate in etOH–MeCN at around 250 nm; the other peaks (225, 275 and 330 nm respectively) resulting from an increase in the concentration of the photoproducts. The same qualitative results were observed when aqueous solutions were photolyzed. The reaction was suppressed either in the presence of 1 mM NaN_3 or in argon bubbled solutions. These evidences (C. S. Foote, 1984) together with the TRPD results confirmed the occurrence of a Type II $O_2(^1\Delta_g)$-mediated photooxidation mechanism.

In order to establish the behaviour of gly-tyr-ala under aerobic sensitized irradiation, the overall and reactive $O_2(^1\Delta_g)$ quenching rate constants, k_t and k_r respectively, and the $-NH_2$ loss were determined.

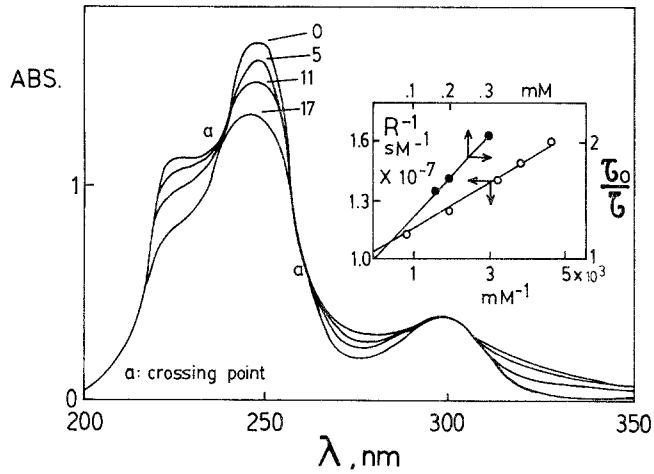

Fig. 1. Absorption spectra of gly-tyr-ala, in alkaline etOH–MeCN (80:20, v/v), after and before irradiation in the presence of RB as a sensitizer. Numbers correspond to irradiation time (min.). Inset: Stern-Volmer plot for the $O_2(^1\Delta_g)$ quenching, determined by TRPD, for gly-tyr-ala in etOH–MeCN, and reciprocal plot (eq. 5) for gly-tyr-ala in H_2O, pH 11.5

The overall rate constant ($k_t = k_q + k_r$) for the quenching process was evaluated from the decay lifetimes of $O_2(^1\Delta_g)$ phosphorescence through the slopes of the Stern-Volmer plots according to eq. (4) (Fig. 1 inset, upper and right scales). The value of τ measured by the IR emission decay of $O_2(^1\Delta_g)$ in the mixture etOH–MeCN (80:20, v/v) was 19 μs, in agreement with reported data (Palumbo et al. (1990a)). No detectable quenching of excited states of the sensitizer occurred under the experimental conditions; the initial amplitude of the TRPD signal being the same in the absence and in the presence of the tripeptide within experimental error.

Also included in the inset of Fig. 1 (lower and left scales) are the experimental data for the determination of k_t in water, pH 11.5, according to eq. 5.

The reactive rate constants for $O_2(^1\Delta_g)$ quenching by the TPE were determined, as described earlier, by substrate consumption and oxygen uptake in etOH–MeCN and H_2O respectively. In Fig. 2 the first order kinetic representations are shown for the two reference compounds as well as for gly-tyr-ala using both methods.

Results for both, overall and reactive rate constants for $O_2(^1\Delta_g)$ quenching by gly-tyr-ala as well as their respective k_t/k_r ratios, are presented in Table 1. For comparative purposes the available information on related rate constants for tyr and DPEs are included in Table 1. An overview of the data shows several important differences in the kinetic behaviour of gly-tyr-ala. This fact constitutes, in principle, an unexpected finding, basically because the main oxidizable substrate is common for all the compounds. In order to have additional information on probable side effects in the TPE, some other experiments were made. Even though the free AAs gly and ala have been largely reported as non reactive towards $O_2(^1\Delta_g)$ (Straight and Spikes, 1985), experimental runs of time resolved $O_2(^1\Delta_g)$ quenching (in the organic solvent mixture) and oxygen uptake (in H_2O) by equimolar mixtures of tyr plus ala, tyr plus gly and tyr alone

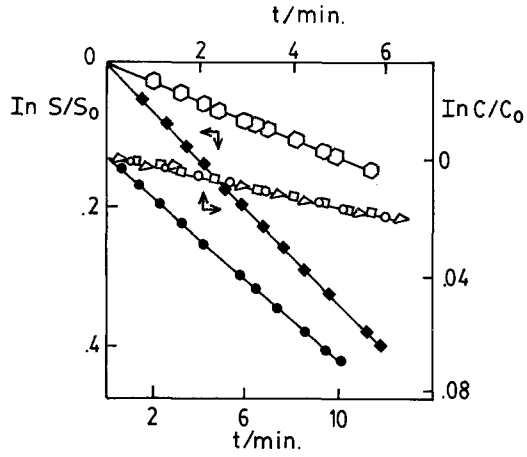

Fig. 2. First order plots for oxygen and substrate consumption. Water at pH 11.5: ● FFA, ○ tyr, □ tyr plus ala, △ gly-tyr-ala (all compounds in concentration 0.2 mM). Alkaline etOH–MeCN: ■ DMA; ⬡ gly-tyr-ala. C and C_0 represent the oxygen concentrations at times $t = t$ and $t = 0$, respectively. The same for the symbols S and S_0 as referred to substrate concentrations

Table 1. Rate constants for the overall ($k_t/M^{-1}\ s^{-1}$) and reactive ($k_r/M^{-1}\ s^{-1}$) quenching of $O_2(^1\Delta_g)$ by gly-tyr-ala and k_t/k_r ratio in etOH–MeCN (80:20, v/v) and water, at room temperature

Compound	$k_r \times 10^{-8}$	$k_t \times 10^{-8}$	k_t/k_r
	etOH–MeCN alkaline		
gly-tyr-ala	0.30	2.0	6.7
tyr	0.30	1.8	~6
tyr-gly	0.10	1.2	12
gly-tyr	0.027	0.94	~35
	water, pH 11.5		
gly-tyr-ala	1.0	20	20
tyr	0.52		
tyr-gly	0.43		
gly-tyr	0.19		

Data for tyr and their DPEs [Bertolotti et al. (1991)] are included for comparison purposes

were carried out. We then aimed either to discard or to confirm a possible effect of $O_2(^1\Delta_g)$ quenching by the free AAs in the presence of tyr. The experiments demonstrated, as expected, that the only photooxidizable substrate, when free, was tyr; the results for the mixtures being totally coincident with those for the aromatic AA alone. In Fig. 2 are also included the experimental data for oxygen consumption by tyr and tyr plus ala. The lack of an additional kinetic effect in the mixture due to the presence of ala is clearly demonstrated.

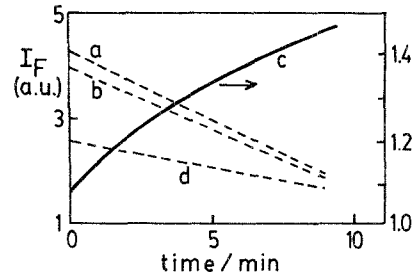

Fig. 3. Rate of NH_2 loss or production upon sensitized irradiation for tyr-gly (**a**); tyr (**b**); gly-tyr (**d**) and gly-tyr-ala, pH 11.5 (**c**). Sensitizer, RB. Experimental points were omitted in order to clarify the plot. Data in dotted lines belong to Bertolotti et al. (1991)

The $-NH_2$ loss for the TPE determined in aqueous solution is represented by the solid line in Fig. 3. Gly-tyr-ala showed an increase in the $-NH_2$ group with photooxidation. Dotted lines show the published data for tyr and their DPEs. In these cases a loss of $-NH_2$ with the sensitized photolysis is observed. It should be noted that the same experimental conditions (pH, sensitizer and method for $-NH_2$ dosage) were employed for all, TPE, tyr and DPEs (Bertolotti et al., 1991).

In summary, a broad general conclusion arises from the observation of the results in Table 1 and the plots for $-NH_2$ loss: the photooxidative behaviour of gly-tyr-ala cannot be predicted by a straightforward extension of those results obtained for tyr and related DPEs.

Discussion

The discussion of results in the $O_2(^1\Delta_g)$-mediated photooxidation of gly-tyr-ala can be divided into two parts: a) solvent effect on rate constants and b) comparative analysis between the results for tyr and their DPEs and TPE.

Solvent effect

There is an important increase (ten fold) in the value of k_t going from the alkaline organic mixture to water, at pH 11.5. At the same time, the reactive rate constant only increases by a factor of three, with the same solvent change. Consequently, the k_t/k_r ratio, which certainly represents a degree of autoprotection of the substrate against photodynamic action, suffers a considerable increase with the enhancement of solvent polarity. In other words, even when the absolute value of k_r is increased in water, the photooxidability of gly-tyr-ala diminished. This fact can be better visualized if the quantum yields of photooxidation ($\varnothing_r$) for the TPE in the organic mixture and water are considered (eq. 7). This parameter, as it was exhaustively discussed (Palumbo et al., 1990b), constitutes a realistic measure for the potential degree of photooxidative degradation for a given substrate.

$$\varnothing_r = \varnothing k_r\,[\mathrm{TPE}]/(k_d + k_t\,[\mathrm{TPE}]) \qquad (7)$$

Employing a value of 0.75 for $\varnothing$ of RB in water, 0.86 in etOH–MeCN (Neckers,

1989) and a concentration of TPE 0.2 mM, as utilized in the experiments, $\varnothing_r$ (etOH–MeCN) renders a value of 0.056, while the corresponding $\varnothing_r(H_2O)$ is 0.023. On this basis, it can be concluded that gly-tyr-ala becomes less photooxidizable in a highly polar environment.

The bimolecular reactions of $O_2(^1\Delta_g)$ with different substrates such as phenols have been postulated to occur through a mechanism involving an exciplex (Gorman et al., 1988):

$$^1O_2 + \text{TPE} \underset{k_{-a}}{\overset{k_a}{\longleftrightarrow}} [^1O_2\text{—TPE}] \xrightarrow{k_r} \text{Products}$$

$$[^1O_2\text{—TPE}] \xrightarrow{k_{ISC}} [^3O_2\text{—TPE}] \longrightarrow {}^3O_2 + \text{TPE}$$

According to this scheme, the physical quenching process arises from a spin-orbit-induced intersystem crossing (ISC) within the exciplex. As previously discussed for DPEs (Bertolotti et al. (1991)) the increase of k_t with solvent polarity can be explained also for the present case in terms of an increase in the polar character of the exciplex as a whole. The relative small change in k_r shows that the balance between k_r and k_{ISC} is not equally affected by solvent polarity. This fact is probably explained in terms of the respective polarities of the intermediates in the $O_2(^1\Delta_g)$-mediated oxidation. In fact, only a moderate polarity in intermediates or products, i.e., a moderate change in polarity, going from reactants to products, should be expected in the case of gly-tyr-ala.

Comparative analysis between results for tyr, their DPEs and gly-tyr-ala

One of the aims in the study of the photooxidative behaviour of small peptides is the possibility to extend the results to structurally more complicated biochemical systems, trying to simulate, finally, the proteinaceous environment. But the present case, for a simple tyr tripeptide, calls our attention in the sense of being extremely cautious before making generalizations: the results for TPE are totally different from those reported (Bertolotti et al., 1991) for the related peptides tyr-gly and gly-tyr.

One general point is common for all tyr derivatives (Table 1): the tyr moiety suffers $O_2(^1\Delta_g)$-mediated photooxidation, independently of the type of peptide bond in which it is involved. It is well known that photooxidation of tyr produces the cleavage of the aromatic ring (Weil, 1965). In the case of tyr, tyr-gly and gly-tyr, this fact was accompanied (Bertolotti et al. (1991)) by a considerable loss of the $-NH_2$ group (Fig. 3) in contrast to the case of the TPE where a very strong increase of the $-NH_2$ signal was obtained upon sensitized photolysis. This means that at least one, or possibly two new primary amino groups are being generated in the photooxidative process.

As seen in Table 1, the rate constants are practically the same as the reported ones (Bertolotti et al. (1991)) for free tyr. Regarding the k_t/k_r ratio, tyr and gly-tyr-ala are much less protected against photooxidation than the DPEs. In both, organic and aqueous media, the higher k_r values belong to the TPE and free tyr. These results are probably connected with that of $-NH_2$ loss: the tendency

of the TPE to acquire the structure of free Tyr, upon regeneration of primary amino groups from the peptide bonds, could explain the similar behaviour of the peptide and the free AA towards sensitized photooxidation.

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