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Kinetics of the photosensitized oxidation of chymotrypsin in different media

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Summary. The kinetic aspects of the Perinaphthenone-sensitized photooxidation (singlet molecular oxygen $[O_2(^1\Delta_g)]$-mediated) of α-chymotrypsin (α-Chymo) have been studied at pH 8 and pH 11 as well in reverse micelles (RMs) of sodium 1, 4 bis (2-ethylhexyl) sulfosuccinate (AOT) in n-heptane.

The rate constant values for both overall (k_t) and chemical (k_r) quenching of $O_2(^1\Delta_g)$ by α-Chymo in homogeneous media were higher at pH = 11 than at pH = 8, indicating that the OH-ionized tyrosine (Tyr) residues, clearly dominate the quenching process. Besides, the rate constants in water were higher than those determined in RMs, demonstrating that the organized medium protects the protein against photooxidation, probably due to a diminution in both, the accessibility towards oxidizable amino acid residues and the polarity inside the aggregate, as compared to water. The protection effect of α-Chymo against the attack by the oxidative species $O_2(^1\Delta_g)$ in RMs of AOT seems to be due to the increase of protein stability by the encapsulation within the micellar structure.

The effect of both, surfactant concentration and variation of the ratio $([H_2O]/[AOT]) = W$ on the reactive rate constant was also investigated. The process does not depend significantly on micelles concentration while the k_r values increase as W increases. Furthermore, at W = 30, the highest W studied, k_r tends to the value obtained in aqueous medium.

Keywords: AOT – α-Chymotrypsin – Photooxidation – Reverse micelles – Singlet molecular oxygen

Introduction

Singlet molecular oxygen ($O_2(^1\Delta_g)$)-mediated oxidative damage in relevant biomolecules such as proteins and lipids, has been the subject of recent studies (Morgan et al., 2002; Davies, 2003, 2004; Nagaoka et al., 2005; Otsu et al., 2005; Wenli and Yaping, 2005; Bhattacharya et al., 2007). Proteins are major targets in photodynamic effect due to their high reaction rates with $O_2(^1\Delta_g)$ and oxygenated radicals (Agon et al., 2006) through the degradation of the side chains of the five oxidizable amino acids (AAs): histidine (His), tryptophan (Trp), methionine (Met), cysteine (Cys) and tyrosine (Tyr) (Michaeli and Feitelson, 1994, 1995, 1997; Soltermann et al., 1995).

Chymotrypsins belong to the group of major proteolytic enzymes in the pancreatic juice and they participate in the hydrolysis of peptidic bond and esters (The Merck Index 1989). The $O_2(^1\Delta_g)$-mediated-photooxidation of the protein α-Chymotrypsin (α-Chymo) was studied (Fliss and Viswanatha, 1979), using 2,3-butanedione as sensitizer. More recently, the kinetic aspects of the sensitized photooxidation of α and β-Chymo have been investigated by ourselves (Biasutti et al., 2003), employing perinaphthenone (PN), rose bengal (RB) and eosine (Eos) as dye-sensitizers. Both studies demonstrate a high susceptibility of the protein towards photodynamic effect, in water.

Nevertheless, aqueous homogeneous medium is not always the better choice to evaluate the potentiality of degradative events that occur in biological environments. Organized media such as reverse micelles (RMs), represent an attractive model system for biomembranes since they mimic a number of important and essential features of these biological assemblies, although lacking much of the complexity associated with these systems (Chattopadhyay et al., 2002). RMs are aggregates of surfactant molecules with their polar groups concentrated in the interior, while their hydrophobic moieties extend into and surrounded by the bulk non-polar solvent (Silber et al., 1999). They provide a very large interfacial region where the amphipatic essence is preserved. Among the anionic surfactants that can form RMs, without co-surfactant in different non polar media, the best well known is sodium 1,4-bis-2-ethylhexylsulfosuccinate (AOT).

α-Chymo, a hydrophilic and globular enzyme, is totally associated to the micelles and it has been shown that

the enzyme is entrapped in the water pool of RM (Hirai et al., 1995). A very interesting fact is that its enzymatic activity is more efficient in RM of AOT than in aqueous solution showing the higher catalytic efficiency around $W(=[H_2O]/[AOT]) \sim 12$, 13 (Hirai et al., 1995; Falcone et al., 2004).

These results clearly demonstrate the influence of the microenvironment on the biological activity of α-Chymo, and encouraged us to investigate its potential effects on the photodynamic process, which constitutes the main objective of the present contribution.

In this paper we report a kinetic study on the $O_2(^1\Delta_g)$-mediated photoxidation of α-Chymo in RMs of AOT, emphasizing on the respective influence of pH in homogeneous and in organized media.

Materials and methods

α-chymotrypsin (α-Chymo), molecular weight 24.00 kDa, from bovine pancreas (50% units/mg solid, 55% units/mg protein), Tryptophan (Trp) and Furfuryl alcohol (FFA) were purchased from Sigma. Perinaphthenone (PN) was supplied by Aldrich Chem. Co. Ultrapure water was obtained from Labonco equipment model 90901-01. KH_2PO_4/NaOH buffers were employed to adjust aqueous solutions to pH or pD 8 and 11. D_2O (99.9%) was from Sigma. Sodium 1,4-bis (2-ethylhexyl) sulfosuccinate (AOT) from Sigma (99%), was dried under a reduced pressure and kept under a vacuum over P_2O_5 until its use, to minimize H_2O absorption. n-heptane (Sintorgan HPLC quality) was used as received. The stock solutions of AOT were prepared by mass and volumetric dilution. In reverse micelles, it is known that the pH cannot be measured inside the water pool of the aggregate (Falcone et al., 2004). A meaningful approximation to the pH within the aqueous pseudophase of the RMs can be made using a pure source of AOT under conditions of sufficient buffer capacity in the bulk solutions. In this sense, the value of the pH inside the water pool is referenced to homogeneous buffer solution used to form the water pool and called pH_{ext}. Freshly prepared solutions were used for all measurements.

Instrumentation and data handling

The absorption spectra were measured employing Shimadzu 2401 equipment while Spex fluoromax equipment was used for the fluorescence measurements. A Nd:YAG laser (Spectron) was the excitation source (frequency-tripled output at 355 nm) for all time-resolved experiments. The laser kinetic spectrophotometer for time-resolved phosphorescence detection (TRPD) of $O_2(^1\Delta_g)$ has been described previously (Miskoski et al., 1993). In the present case PN was the sensitizer with $A^{355} = 0.3$ for TRPD measurements. The kinetic decays of the phosphorescent signals were first order in all cases. The rate constants k_t were evaluated from a simple Stern-Völmer treatment (Eq. (1)):

$$1/\tau = 1/\tau_0 + k_t[\alpha\text{-Chymo}] \quad (1)$$

where τ and τ_o represent the $O_2(^1\Delta_g)$ phosphorescence lifetime in the presence and in the absence of α-Chymo, respectively. Deuterated water was employed as a solvent for TRPD experiments in homogeneous medium due to the convenience of prolonging the lifetime of $O_2(^1\Delta_g)$, as compared with its lifetime in water (Criado et al., 1997) given the relatively long time-response (ca. 3 μsec) for the available IR detector. For the TRPD experiments in RMs, D_2O was used to form the water pool.

The reactive rate constants for $O_2(^1\Delta_g)$-mediated α-Chymo photooxidation, k_r, obtained by employing PN, as a dye sensitizer ($Abs^{355} = 0.5$), were determined by comparative methods. In all cases, the knowledge of the reactive rate constant for the photooxidation of a reference compound (R) was required. We used the method described by Foote and Ching (1975), Eq. (2), which compares the first-order slopes of the plots for substrate and reference consumption as a function of irradiation time for the oxidizable substrate and a reference of known k_r:

$$\text{slope}_{(\alpha\text{-Chymo})}[R]/\text{slope}_{(\text{Reference})}[\alpha\text{-Chymo}] = k_{r(\alpha\text{-Chymo})}/k_{r\ \text{Reference}} \quad (2)$$

The reference used in water was Trp, with a reported $k_r = 3.4 \times 10^7\ M^{-1}\ sec^{-1}$ at pH = 8 (Criado et al., 1996). For pH = 11, the reference was furfuryl alcohol (FFA), with a rate constant $k_r = 6.87 \times 10^7\ M^{-1}\ sec^{-1}$, determined in this work.

The rates of oxygen uptake were determined by evaluation of the initial slopes from the plots of oxygen consumption vs. irradiation times and relative rates (V_{ox}) referred to the highest value obtained in homogeneous medium. The oxygen electrode (Orion-97-08) used to monitoring oxygen consumption, has a membrane which can be used in media containing a high percentage of water. For this reason, in RM of n-heptane/AOT/water the photooxidation kinetics was monitored by protein consumption through fluorometric measurements.

Either protein consumption or oxygen uptake can be employed to determine photooxidation rate constants. Nevertheless, in the second case, the precise stoichiometry of oxygen consumption per mole of protein or reference consumed in the photooxidative reaction must be known, in order to determine k_r values. For this reason, in RM medium we report the global rates of oxygen consumption. Protein and reference consumption upon sensitized photoirradiation were spectrophotofluorimetrically determined ($\lambda_{exc} = 280$ nm; $\lambda_{emiss} = 330$ nm). The irradiation device for steady-state photolysis, including the specific oxygen electrode has been described elsewhere (Bertolotti et al., 1991). Stationary photolysis of the solutions containing α-Chymo and PN were carried out, under aerobic conditons, in a PTI unit provided with a high pass monochromator and 150 W Xe lamp, irradiating with 440 ± 10 nm, or in a home-made photolyser for non-monochromatic irradiation (150 W quartz-halogen lamp). In this case, cut-off filters (330 nm) ensured that the light was absorbed only by the sensitiser.

Results and discussion

Sensitized photo-oxidation in homogeneous medium

The photoirradiation of air-equilibrated aqueous solution of α-Chymo with wavelength higher than 330 nm, in the presence of PN modifies the absorption spectrum of the protein, as it was previously shown at pH values 6 and 8 (Biasutti et al., 2003). No ground state association with the protein occurs when this carbonylic photosensitizer was used. Besides, clear evidence of ground-state complexation with xanthene dyes was found. This factor precludes the simple kinetic analysis of the photodynamic effect and for this reason, PN, that generates $O_2(^1\Delta_g)$ in water with a quantum yield of approximately 1 (Nonell et al., 1993) was employed in the present contribution.

The photooxidation produces significant changes in the fluorescence intensity of α-Chymo, as shown in Fig. 1. This effect could not be detected when the experiments

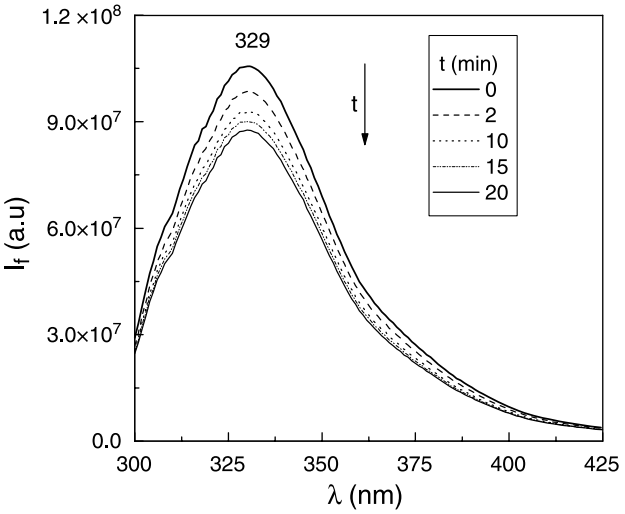

Fig. 1. Decrease of α-Chymo fluorescence on PN-sensitized photoirradiation in Buffer pH = 8, [α-Chymo] = 1×10^{-5} M, $\lambda_{exc} = 280$ nm

were performed in N_2-satured solutions. In the present work we employed pH values 8 and 11, in order to have, in the second case, a significant fraction of OH-ionized Tyr molecules in the protein, (pK = 10.1, according to Albert and Serjeant, 1984).

The distribution of oxidizable AAs residues in α-Chymo, is the following: two His, seven Trp, two Met, four Tyr and half Cys (The Merck Index, 1989) and the decrease in fluorescence intensity of the protein is assumed to be due to the photo-oxidation of the fluorescent amino acid residues Trp (mainly) and Tyr.

The reaction scheme employed for the evaluation and discussion of the results are given in Scheme 1.

According to Scheme 1, the sensitizer S absorbs the incident light and generates their electronically singlet and triplet excited states (reaction (3)). An energy transfer reaction to ground state triplet molecular oxygen takes place producing the electronically excited oxygen species $O_2(^1\Delta_g)$ (reaction (4)). It can decay by collision with surrounding molecules, typically the solvent (reaction (5)) or it can interact physically (reaction (6)) or chemically (reaction (7)) with α-Chymo. The rate constant k_t for the overall process of $O_2(^1\Delta_g)$ quenching, is given by the addition of the respective rate constants for the physical and chemical (reactive) events ($k_t = k_q + k_r$).

$$S \xrightarrow{h\upsilon} {}^1S^* \xrightarrow{k_{isc}} {}^3S^* \quad (3)$$

$${}^3S^* + O_2(\Sigma_g^-) \xrightarrow{k_{ET}} S + O_2(^1\Delta_g) \quad (4)$$

$$O_2(^1\Delta_g) \xrightarrow{k_d} O_2(\Sigma_g^-) \quad (5)$$

$$O_2(^1\Delta_g) + \alpha\text{-Chymo} \xrightarrow{k_q} O_2(\Sigma_g^-) + \alpha\text{-Chymo} \quad (6)$$

$$O_2(^1\Delta_g) + \alpha\text{-Chymo} \xrightarrow{k_r} \text{Products} \quad (7)$$

Scheme 1

Typical results for the determination of the rates of consumption of α-Chymo in buffer are shown in Fig. 2, at pH values 8 and 11. The rate constants for reactive quenching k_r of $O_2(^1\Delta_g)$ by α-Chymo (process (7), by monitoring protein consumption through fluorescence measurements, are shown in Table 1. In aqueous solu-

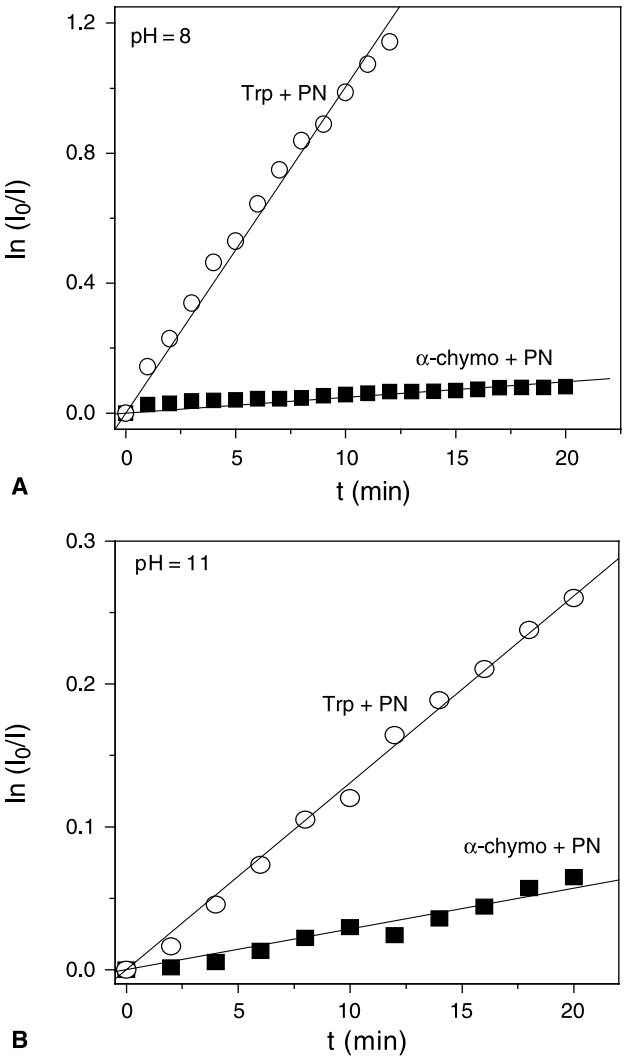

Fig. 2. First order plot consumption of α-Chymo upon PN-sensitized photoirradiation for (○) Trp and (■) α-Chymo in Buffer: (**A**) pH = 8 and (**B**) pH = 11. $\lambda_{em} = 330$ nm and 334 nm, respectively. I_0 and I represent the values of the respective maxima intensity in the fluorescence spectra at different irradiation times. $\lambda_{exc} = 280$ nm

Table 1. Rate constantes for overall (k_t, $M^{-1}\,sec^{-1}$) and chemical quenching (k_r, $M^{-1}\,seg^{-1}$) of $O_2(^1\Delta_g)$ by α-Chymo, k_r/k_t ratios and relative rates for oxygen consumption (V_{ox}) upon PN sensitized photoirradiation of α-Chymo, in buffer and in RMs of n-heptane/AOT/water, [AOT] = 0.1 M, W = 13

Medium	k_r and $k_{r,exp}\times10^{-7}$ ($M^{-1}\,seg^{-1}$)	k_t and $k_{t,app}\times10^{-7}$ ($M^{-1}\,sec^{-1}$)	k_r/k_t and $k_{r,exp}/k_{t,app}$	V_{ox}
Buffer				
pH/pD = 8	0.526	19.7	0.03	0.73
pH/pD = 11	1.55	80.9	0.02	1
RMs				
$pH/pD_{ext} = 8$	0.065	3.94	0.015	
$pH/pD_{ext} = 11$	3.27	5.71	0.6	

k_r and k_t for homogeneous medium; $k_{r,exp}$ and $k_{t,app}$ for RMs

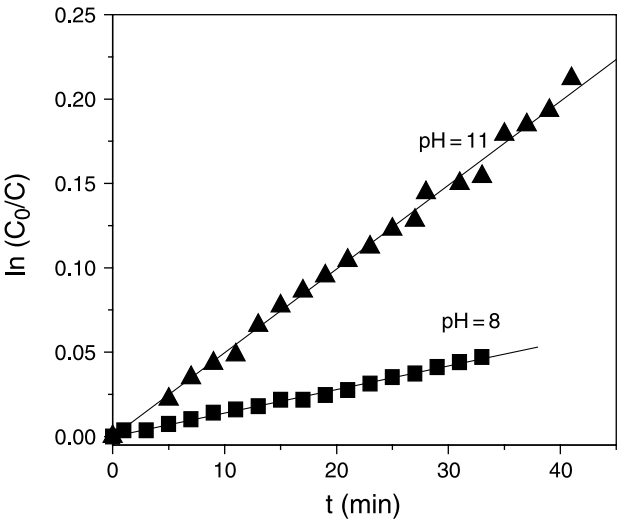

Fig. 3. Effect of the pH on the profiles of oxygen uptake by α-Chymo upon PN-sensitized photoirradiation, in buffer at (■) pH = 8, (▲) pH = 11 [α-Chymo] = 1×10^{-5} M

tions, the pH effect was also investigated by measuring rates of oxygen consumption (Fig. 3) and the relative values (V_{ox}) are collected in Table 1.

$O_2(^1\Delta_g)$ phosphorescence quenching by α-Chymo was investigated in the different media and a typical result is shown in Fig. 4. This experiment unambiguously demonstrate the interaction of the protein with $O_2(^1\Delta_g)$. The rate constants k_t are shown in Table 1.

From the data summarized in Table 1 it can be observed that the values for all three, the rate constants of overall and chemical quenching of $O_2(^1\Delta_g)$, and the relative rates of oxygen consumption, are higher at pH 11 than at pH 8. Additionally, the reactive contribution to the overall quenching only represents, in both media, 2–3% of the overall process of $O_2(^1\Delta_g)$ deactivation.

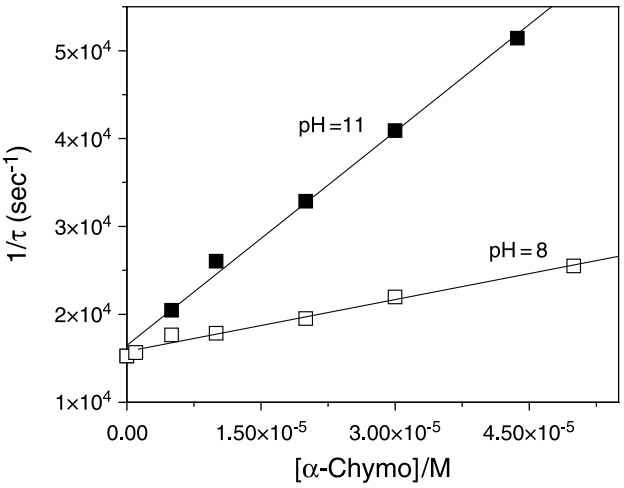

Fig. 4. Stern-Völmer plots for the quenching of singlet molecular oxygen phosphorescence by α-Chymo in D_2O at (□) pH = 8 and (■) pH = 11. Abs^{355} (PN) = 0.3

In homogeneous medium, experimental evidence demonstrates a clear pH dependence on the $O_2(^1\Delta_g)$-mediated photooxidation of α-Chymo. The fraction of ionized phenolate group from tyrosine (Tyr) residues, at pH = 11, affects the photoxidation process, in a similar kinetic fashion that the already reported ones for the cases free Tyr or Tyr residues from small peptides (Bertolotti et al., 1991; Miskoski et al., 1993; Criado et al., 1998).

Sensitized photo-oxidation in reverse micelles of AOT

In RMs of water/AOT/n-heptane, at W = 13 and pH_{ext} = 8, PN-sensitized photoirradiation of α-Chymo produces

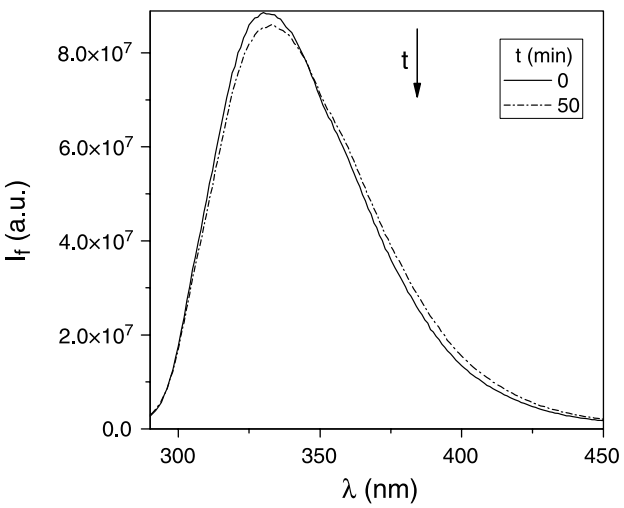

Fig. 5. Time evolution of the emission spectrum of α-Chymo on PN-sensitized photoirradiation in Reverse Micelles of water/AOT/n-heptane, [AOT] = 0.1 M, W = 13. [α-Chymo] = 2×10^{-5} M, pH_{ext} = 8, λ_{exc} = 280 nm

$$S_{int} \xrightarrow{h\upsilon} {}^1S_{int}^* \xrightarrow{k_{isc}} {}^3S_{int}^* \quad (8)$$

$${}^3S_{int}^* + O_2(\Sigma_g^-)_{int} \xrightarrow{k_{ET}} S_{int} + O_2({}^1\Delta_g)_{int} \quad (9)$$

$$O_2({}^1\Delta_g)_{int} \underset{k_-}{\overset{k_+}{\rightleftharpoons}} O_2({}^1\Delta_g)_{ext} \quad (10)$$

$$O_2({}^1\Delta_g)_{int} \xrightarrow{k_{d,int}} O_2(\Sigma_g^-)_{int} \quad (11)$$

$$O_2({}^1\Delta_g)_{ext} \xrightarrow{k_{d,ext}} O_2(\Sigma_g^-)_{ext} \quad (12)$$

$$O_2({}^1\Delta_g)_{int} + \alpha\text{-Chymo}_{int} \xrightarrow{k_{q,int}} O_2(\Sigma_g^-)_{int} + \alpha\text{-Chymo}_{int} \quad (13)$$

$$O_2({}^1\Delta_g)_{int} + \alpha\text{-Chymo}_{int} \xrightarrow{k_{r,int}} \text{Product} \quad (14)$$

Scheme 2

the emission changes, in intensity and spectral shape, shown in Fig. 5.

As α-Chymo is totally associated to the micelles and entrapped in the water pool of RMs at W $\sim$12, 13 (Hirai et al., 1995; Falcone et al., 2004), the lowest W value employed in this work was 13, and the experimental results were interpreted in terms of the kinetic Scheme 2, considering the protein as exclusively solubilized in the disperse pseudophase.

In Scheme 2 S_{int}, ${}^1S_{int}{}^*$ and ${}^3S_{int}{}^*$ represent the ground, excited singlet and triplet states of PN in the interior phase (water + surfactant). $({}^1\Delta_g)_{int}$, $({}^1\Delta_g)_{ext}$ and Σ_{int}, Σ_{ext} represent $O_2({}^1\Delta_g)$ and ground state oxygen ($O_2(\Sigma_g^-)$ in the interior and in the exterior phase (organic solvent), respectively. k_- and k_+ are the unimolecular entrance and exit rate constants; $k_{d,int}$ and $k_{d,ext}$ are the decay rate constants in the interior and in the exterior phases whereas $k_{q,int}$ y $k_{r,int}$ are the physical and the reactive quenching rate constants, respectively. α-$Chymo_{int}$ is the quencher concentration in the interior phase. The sum of the rate constants ($k_{r,int} + k_{q,int} = k_{t,int}$) is the overall rate constant accounting for $O_2({}^1\Delta_g)$ interaction with the protein.

In a previous work on phenolic compounds solubilised in RMs (Borsarelli et al., 1996), and on the basis of the same kinetic scheme for RMs of AOT, we pointed out several conclusions, which apply to the present study, as follows:

a) A single exponential behaviour should be observed for the $O_2({}^1\Delta_g)$ decay in W/O microemulsions. Therefore, under these conditions, both $({}^1\Delta_g)_{int}$ and $({}^1\Delta_g)_{ext}$ species decay with the same lifetime and the apparent total rate constant $k_{t,app}$ for $O_2({}^1\Delta_g)$ quenching by each α-Chymo, can be determined by evaluation of the $O_2({}^1\Delta_g)$ lifetimes in the absence ($\tau_0 = 1/k_{d,\ app}$) and in the presence ($\tau = 1/k^0_{d,app}$) of α-Chymo, using Stern-Völmer treatment (Eq. (1)) which can be rewritten as:

$$k_{d,app} = k^0_{d,app} + k_{t,app}[\alpha\text{-Chymo}]_{int} \quad (15)$$

where $k_{d,app}$ and $k^0_{d,app}$ are the unimolecular rate constants for the $O_2({}^1\Delta_g)$ deactivation in the presence and in the absence of α-Chymo, respectively, and being

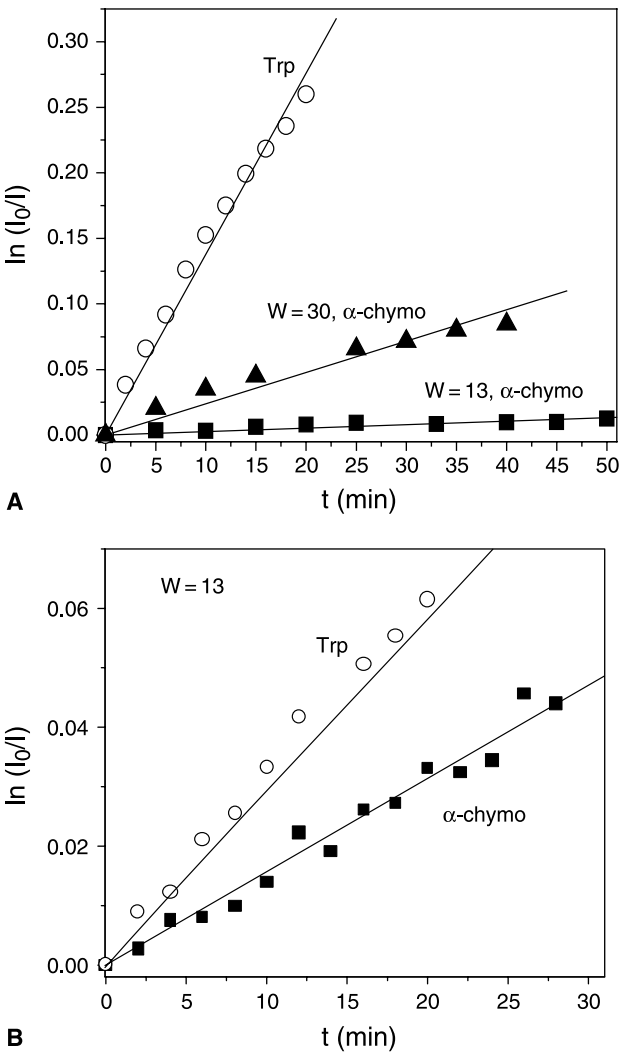

Fig. 6. First order plot consumption of α-Chymo upon PN-sensitized photoirradiation for (○) Trp and (■) α-Chymo in Reverse Micelles of water/AOT/n-heptane, at pH_{ext}: (**A**) 8 at W = 13 and 30 and (**B**) 11, W = 13. I_0 and I represent the values of the respective maxima in the fluorescence spectra at different irradiation times. [AOT] = 0.1 M, [α-Chymo] = 2×10^{-5} M

$[\alpha\text{-Chymo}]_{int} = [\alpha\text{-Chymo}]_T$. The subscript T denotes the bulk concentration.

b) Under steady-state irradiation, α-Chymo consumption is given by Eq. (16):

$$-d[\alpha\text{-Chymo}]_T/dt = k_{r,int}[(^1\Delta_g)_{int}][\alpha\text{-Chymo}]_T = k_{r,exp}[\alpha\text{-Chymo}]_T \quad (16)$$

being $k_{r,exp}$ the observed rate constant for the reactive interaction of α-Chymo with $O_2(^1\Delta_g)$. According to this rate law, the photooxidation process of α-Chymo should follow first-order kinetics.

Figure 6 shows typical results for the determination of the rate of α-Chymo consumption in water/AOT/n-heptane, at different pH_{ext} and W values.

The rate constants for overall ($k_{t,app}$) and reactive ($k_{r,exp}$) quenching of $O_2(^1\Delta_g)$ by the protein were determined at W = 13, at pH_{ext} 8 and 11, and the obtained values gathered in Table 1. A similar pH dependence, already found in homogeneous medium, is observed in RMs at constant micellar concentration and water content.

The values of overall and reactive quenching are smaller in RMs than those obtained in homogeneous media. These facts are probably due to a diminution on the accessibility of the AAs residues which seem to be more protected in this organized medium, against the attack by the oxidative species, $O_2(^1\Delta_g)$. The protection effect, as due to encapsulation of the protein, is in agreement with the increase in the stability of α-Chymo in RMs, which was already observed (Serralheiro and Cabral, 1999; Andrade and Costa, 2001) and attributed to an absence of S–S bond cleavage of its cystine residues and also to absence of auto-hydrolyses of the protein structure (Serralheiro and Cabral, 1999).

Moreover, a decreased polarity in the protein microenvironment could affect its susceptibility to photooxidation. It was already established that the $O_2(^1\Delta_g)$-mediated oxidation of some compounds (Q), such as phenols and indoles, including Tyr, and Trp (Garcia, 1994; Criado et al., 1995; Luiz et al., 2002, 2004) occurs through an excited encounter complex $[Q\cdots O_2(^1\Delta_g)]$ through a charge-transfer mediated mechanism, and concomitantly, the respective values for the rate constants k_r and k_t decrease as the solvent polarity decreases.

Regarding the extent of the photooxidative process, the most relevant information about actual photodegradation efficiency is obtained from photooxidation quantum efficiency values (φ_r) (Eq. (17)), as already stressed elsewhere (Garcia, 1994).

$$\varphi_r = k_r[Q]/(k_d + k_t[Q]) \quad (17)$$

where [Q] represents [α-Chymo]. Expression (17) takes into account the simultaneous effect of the physical and chemical interaction, being the first contribution usually interpreted in practical terms as a form of self-protection against mediated photooxidation. Nevertheless, this expression is some limited due to the dependence on the concentration of the photooxidizable substrate ([α-Chymo]) which is particularly difficult to estimate in complex biological environments. A more simple and useful approach is the k_r/k_t ratio, which can be envisaged as the fraction of overall collisions substrate-$O_2(^1\Delta_g)$ that effectively leads to chemical reaction. In order to compare this fact in homogeneous as well in RMs, calculated values k_r/k_t and $k_{r,exp}/k_{t,app}$ for W = 13, were also included in Table 1. The lower values for the quotient were obtained for pH = 8 and 11 aqueous solution and for $pH_{ext} = 8$ in RMs, likely as a consequence of the high component of physical deactivation of $O_2(^1\Delta_g)$ by the AAs residues. It can be seen that in micellar medium the overall rate constant drastically decreases, as compared to the respective values in water, possibly due to the already mentioned polarity effect. Nevertheless, the k_r value in RMs at $pH_{ext} = 11$ does not parallel the trend, resulting in a significant higher value $k_r/k_t = 0.6$. We attribute this behaviour to the phenolic tyr residues of the protein, as has been already observed by ourselves for a series of substituted phenols in alkaline solvents of different polarity: the overall rate constant was favoured by a solvent polarity increase, whereas the reactive component increased, for a given phenolate, as the polarity of the medium decreased. The peculiarity is congruent with both, the partial charge transfer character within the excited encounter complex and a further destruction of neat charges along the reaction pathway (Bocco et al., 1994).

On the other hand, the influence of micelles concentration and W value on the reactive quenching at $pH_{ext} = 8$, was investigated by varying: a) [AOT] at constant W and b) W at fixed [AOT]. $k_{r,exp}$ values obtained in both con-

Table 2. Rate constants for chemical quenching ($k_{r,\ exp}$ $M^{-1}\,seg^{-1}$) of $O_2(^1\Delta_g)$ by α-Chymo in RMs of n-heptane/AOT/water, varying: (A) surfactant concentration at fixed W(=13) and (B) water content (W) at constant [AOT] = 0.1 M. In all cases $pH_{ext} = 8$

(A)		(B)	
[AOT]/M	$k_{r,exp} \times 10^{-7}$ ($M^{-1}\,seg^{-1}$)	W	$k_{r,exp} \times 10^{-7}$ ($M^{-1}\,seg^{-1}$)
0.1	0.065	13	0.065
0.2	0.167	20	0.109
0.3	0.216	30	0.406

ditions are presented in Table 2. By increasing [AOT] from 0.1 M to 0.3 M at constant W (=13) a small variation on $k_{r,exp}$ was found as compared to the one obtained with variation in W. This effect of independence of the photooxidation rate on micelles concentration, has been already reported for other solutes totally incorporated into RMs of AOT, such as anthracenecarboxylic acid and ethyldimethyl[3-(9-antracenyl)propyl] ammonium bromide (Lissi et al., 1993).

By varying the water content of the RMs from $W = 13$ to 30, the reactive rate constants increase with the size of the water pool by a factor 6, being the $k_{r,exp}$ value at the highest W very close to that one obtained in aqueous solution, confirming the presence of free ''normal'' water at $W > 20$ (Silber et al., 1999). In conclusion, at a given pH_{ext}, the reactive photooxidation process is sensitive to the water content inside the RMs. The value for $k_{r,exp}$ at $W = 30$ is very close to the corresponding one in homogeneous medium.

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