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A comparative kinetic study on the singlet molecular oxygen-mediated photoxidation of α- and β-chymotrypsins

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Dates:
Received 12 August 2002
Revised 7 October 2002
Accepted 22 February 2003

To cite this article:
Biasutti, M.A., Posadaz, A. & García, N.A.
A comparative kinetic study on the singlet molecular oxygen-mediated photoxidation of α- and β-chymotrypsins.
J. Peptide Res., 2003, **62**, 11–18.

ISSN 1397–002X

Key words: chymotrypsin; photodynamic effect; singlet molecular oxygen

Abstract: Kinetic aspects of the sensitized photooxidation of α- and β-chymotrypsins have been studied at pH 6 and 8. The sensitization, employing classical $O_2(^1\Delta_g)$-photogenerators, such as xanthene dyes, is a kinetically intricate process because of the presence of ground state dye–protein associations and to the simultaneous participation of superoxide ion and singlet molecular oxygen [$O_2(^1\Delta_g)$].Both proteins, that possess the same distribution pattern of photooxidizable amino acids, suffer a pure $O_2(^1\Delta_g)$-mediated photodynamic attack, using the carbonylic sensitizer Perinaphthenone. Overall and reactive rate constants for the $O_2(^1\Delta_g)$-quenching (in the order of 10^8 and 10^7/M/s, respectively), and rates of oxygen consumption determined by time-resolved, spectroscopic and polarographic methods indicate that α- and β-chymotrypsins are less photooxidizable at pH 6, as a result of an enhancement of the $O_2(^1\Delta_g)$-physical quenching component. In general terms, β-chymotrypsin exhibits the greater overall proclivity to interact with $O_2(^1\Delta_g)$, whereas structural factors, possibly evidenced by a higher exposure of the reactive tryptophan residues, impart an increased photooxidation degree to the proteins at pH 8, specially to the α-chymotrypsin.

Introduction

Photodynamic damages in living systems, caused by light and oxygen in the presence of dyes, has been the subject of intensive study in the last decades (for a review, see Ref. 1). Consequently, and along the same line, kinetic and mechanistic aspects of dye-sensitized photooxidations, in relation to substrates of relevance in photobiology and photomedicine, were increasingly investigated (2,3). In particular,

singlet molecular oxygen $O_2(^1\Delta_g)$-mediated reactions (Type II photooxidations) (4), which are known to play important roles in photoprocesses taking place in natural environments (5,6). The photochemical modifications of proteins, among Type II reactions on substrates of biological relevance, can provide useful information to topics concerning protein structure and particularly on the degree of photolability and accessibility of certain amino acids (1). It is known, that photodynamic alteration in proteins, in most of the cases proceeds from the degradation of some of the five photooxidizable amino acids, represented by histidine (His), tryptophan (Trp), methionine (Met), cysteine (Cys) and tyrosine (Tyr) (1,7). Nevertheless, kinetic results indicate that the $O_2(^1\Delta_g)$-mediated photooxidation rates of proteins, in aqueous solution, are lower than the corresponding ones for the direct mixture of the individual photooxidizable amino acid constituents (8–10). This simple and almost predictable result emphasizes on the influence of the protein structure, namely conformational factors and peptidic bonds among others, towards the permeability and further reactivity of complex biological assemblies (11,12).

As a result of the fact that the proteins are the main sites of attack by $O_2(^1\Delta_g)$ in the biological cell (13,14), the quenching of the oxidative species by proteins and peptides, under different experimental conditions constitutes a topic of paramount interest. In this context, the photosensitized oxidation of proteins and small peptides has been extensively reviewed (8,12,15–17) and the investigations have been focused on the mechanism, kinetics and identification of reaction products and their distribution.

Although, the photooxidation of α-chymotrypsin by singlet oxygen has previously been studied (18) in that case, the emphasis was on the 2,3-butanedione behaviour as a sensitizer and the comparison between the rates of photodestruction of free α-amino acids and α-chymotrypsin.

On the other hand, even when there are much more specific means of studying protein structure (19), the present results can contribute to establish possible relationships between substrate structure, environmental characteristics and the susceptibility to $O_2(^1\Delta_g)$-mediated photooxidation. In this context, we decided to embrace a comparative kinetic study including two related proteins, α-chymotrypsin (α-chymo) and β-chymotrypsin (β-chymo). These proteins, which belong to the group of major proteolytic enzymes in the pancreatic juice, only differ in their crystal habits (20) and both contain the same distribution of oxidizable amino acids, as follows: two His residues, seven Trp residues, two Met residues, four Tyr residues and half Cys residue.

Experimental Procedures

Chemicals

The proteins α-chymotrypsin (α-chymo; 50% units/mg solid, 55% units/mg protein) and β-chymotrypsin (β-chymo; 47% units/mg solid), Trp, and sodium azide (NaN_3) were purchased from Sigma (St. Louis, MO, USA). Perinaphthenone (PN), Rose Bengal (RB) and Eosine (Eos) were supplied by Aldrich Chemical Co. (Milwaukee, WI, USA). 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 6 and 8. D_2O (99.9%) was from Sigma. Freshly prepared solutions were used for all measurements.

Instrumentation and data handling

Ground state absorption measurements were carried out with a diode array spectrophotometer (HP 8452) (Waldbrom, Germany) or a Shimadzu 2401 apparatus. A Spex Fluoromax spectrofluorimeter (Edison, NJ, USA) was employed for the fluorescence measurements.

A Nd:YAG laser (Spectron, Rugby, Warwickshire, UK) was used as the excitation source for all time-resolved experiments. The frequency-tripled output at 355 nm was employed to excite the sensitizer. The laser kinetic spectrophotometer for time-resolved phosphorescence detection (TRPD) of $O_2(^1\Delta_g)$ has been described previously (21). In the present case PN was the sensitizer with $A_{355} = 0.3$ for TRPD measurements. The kinetic decays were first order in all cases. The rate constants k_t were evaluated from a simple Stern-Vömer treatment [eqn (1)],

$$1/\tau = 1/\tau_o + k_t\,[\text{chymo}] \qquad (1)$$

where τ and τ_o represent the $O_2(^1\Delta_g)$ phosphorescence lifetime in the presence and in the absence of chymo, respectively. Deuterated water was employed as a solvent for TRPD experiments because of the convenience of prolonging the lifetime of $O_2(^1\Delta_g)$, as compared with its lifetime in H_2O (22) given the relatively long time-response (ca. 3 μs for the available IR detector).

The reactive rate constants for $O_2(^1\Delta_g)$-mediated chymo photooxidation (k_r), obtained by employing PN, as a dye sensitizer ($Abs_{max} = 0.5$), 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 (23), eqn (2), which compares the slopes of the first-order plots for substrate and reference consumption against the irradiation time for the oxidizable substrate and a reference of known k_r:

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

The reference was Trp, with a reported $k_r = 3.4 \times 10^7$ M/s for pH 6 (24).

Substrate and reference consumption upon sensitized photoirradiation were spectrophotofluorimetrically determined. The excitation wavelength for Trp and chymo was 280 nm, whereas the emission wavelength was 350 nm. The rates of oxygen uptake (V_{ox}) were determined by evaluation of the initial slopes from the plots of oxygen consumption vs. irradiation times.

Either chymo consumption or oxygen uptake can monitor photooxidation rates. Nevertheless, in the second case, the precise stoichiometry of the photooxidation reaction in substrate and reference compound (moles of protein oxidized/moles of O_2 consumed) must be known in order to determine k_r values. For this reason we only determined the global rates of oxygen consumption, being the k_r values determined, as said, by substrate consumption.

The irradiation device for steady-state photolysis, including the specific oxygen electrode, has been described elsewhere (25). A cut-off filter for λ below 400 nm was employed in the photoirradiations.

Results

Sensitizer-protein association

The irradiation of air-equilibrated individual solutions of α- and β-chymo with visible light, in the presence of RB, Eos or PN in water, at pH 6 and pH 8, modifies the absorption and emission spectra of the proteins.

From the observed spectral characteristics of the mixture solutions of dye-chymo, under sensitizing work conditions, using the xanthene dyes RB and Eos as sensitizers, there arises clear evidences of a ground-state complex formation involving the proteins and the dyes. The absorption spectra of the mixture show a shoulder at 325 nm while the absorbance decreases at 275 nm (absorption band of the protein) and in the region of the main absorption band of the dye. This kind of association has been already reported for different amino acids with RB (24) and with other xanthene derivatives such as merbromin (26).

In order to quantify the magnitude of the complexation we employed the routine procedure of fluorescence quenching (eqn 3), to obtain the apparent association constants (K_{ass}). Rose Bengal (27) and Eos posses measurable fluorescence emission centred at 568 nm and 535 nm, respectively. Assuming that the fluorescence quenching by the proteins does not proceed *via* interaction with the excited state of the dyes, the apparent association constant values, for ground-state complexation can be calculated from the Stern-Volmer plots, according to eqn (3)

$$I_{fo}/I_f = 1 + K_{\text{ass}}\,[\text{chymo}] \quad (3)$$

where I_{fo} and I_f are the fluorescence intensities of the dyes solutions in the absence and presence of the quencher, respectively.

The possibility of an excited singlet quenching of the dyes should be disregarded: the reported lifetimes of the singlet excited RB and Eos in aqueous solutions are 0.82 ns and 3.6 ns respectively (28) indicating that the excited species cannot be intercepted by the proteins in the concentration range (6×10^{-6}–8×10^{-5} M) employed in these experiments. The respective K_{ass} values obtained for RB and Eos were 9600/M and 900/M (data not shown).

Protein photooxidation: absorption and fluorescence measurements

No ground-state complexation with chymo could be spectrophotometrically detected employing the known carbonylic $O_2(^1\Delta_g)$ sensitizer PN. For this reason, PN, that generates the oxidative species in water with a quantum yield of approximately 1 (29), was chosen for the remainder experiments on photooxidation. Figures 1 and 2 show the evolution of absorption spectra for α-chymo and the evolution of emission spectra for β-chymo in water at pH 6 and 8, respectively, upon aerobic PN-sensitized irradiation. No spectral changes were detected when the runs were performed in N_2-saturated solutions.

On this basis, the essential reactions and kinetic steps employed for the evaluation and discussion of the experimental results are shown in Scheme 1.

Scheme 1

$$\text{PN} + h\nu \xrightarrow{\text{Ia}} {}^1\text{PN}^* \xrightarrow{\text{Kisc}} {}^3\text{PN}^* \quad (4)$$

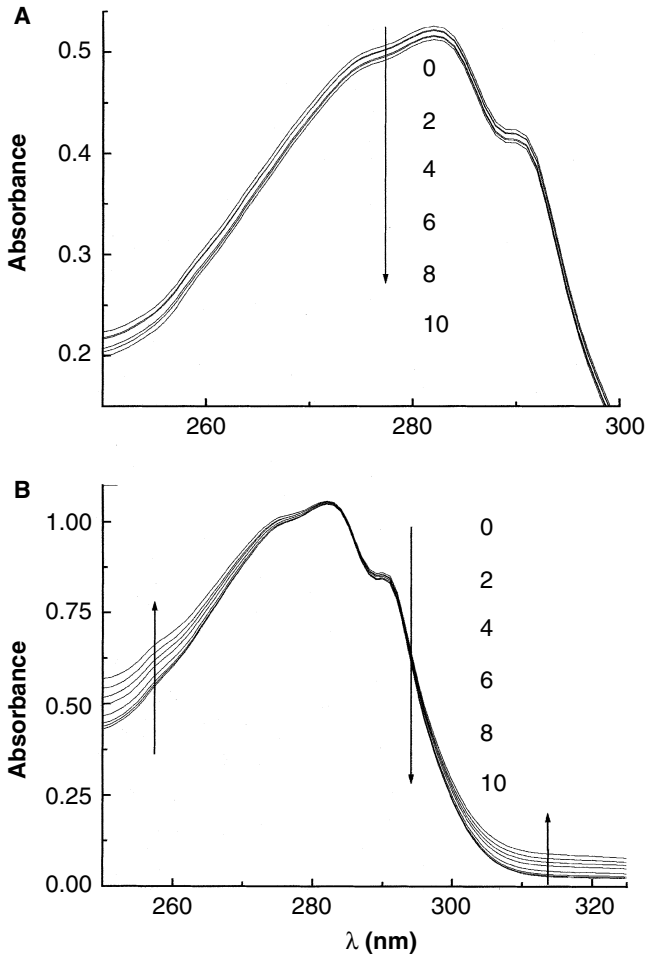

Figure 1. Time evolution of the absorption spectrum of α-chymo upon sensitized photoirradiation (sensitizer: PN) in water at pH: (A) 6 and (B) 8. Numbers indicate irradiation time in minutes. [α-chymo] = 1×10^{-5} M.

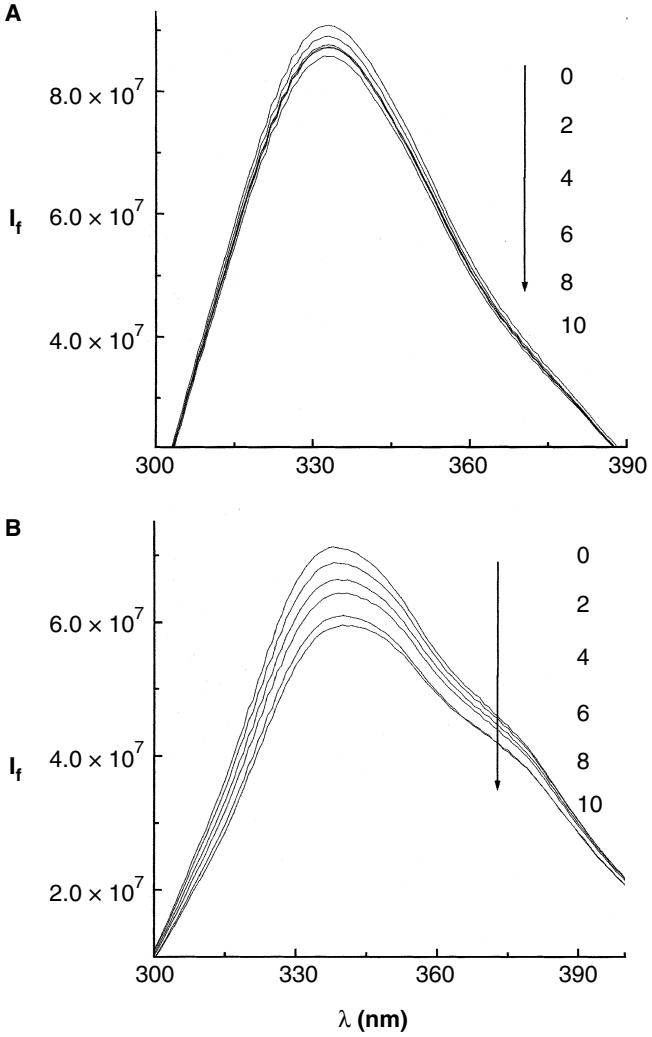

Figure 2. Time evolution of the emission spectrum of β-chymo on PN-sensitized photoirradiation in water at pH: (A) 6 and (B) 8. Numbers indicate irradiation time in minutes [β-chymo] = 1×10^{-5} M.

$$^3PN^* + O_2(^3\Sigma_g^-) \xrightarrow{k_{ET}} PN + O_2(^1\Delta_g) \quad (5)$$

$$O_2(^1\Delta_g) \xrightarrow{k_d} O_2(^3\Sigma_g^-) \quad (6)$$

$$O_2(^1\Delta_g) + \text{Chymo} \xrightarrow{k_q} O_2(^3\Sigma_g^-) + \text{Chymo} \quad (7)$$

$$O_2(^1\Delta_g) + \text{Chymo} \xrightarrow{k_r} \text{Products} \quad (8)$$

According to Scheme 1, the sensitizer, PN, absorbs the incident light and generates their electronically singlet and triplet excited states [reaction (4)]. An energy transfer reaction to the ground-state triplet molecular oxygen takes place producing the excited state oxygen species $O_2(^1\Delta_g)$ [reaction (5)]. It can decay by collision with surrounding molecules [typically the solvent, reaction (6)] or it can interact physically [reaction (7)] or chemically [reaction (8)] with chymo. According to Scheme 1, 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 ($k_t = k_q + k_r$).

Typical results for the determination of the rates of oxygen consumption by α-chymo, are shown in Fig. 3. The respective relative values for both proteins at pH 6 and 8 are collected in Table 1. Two general trends neatly arise from the data: (a) in all cases the rates of oxygen consumption are higher for β-chymo and (b) oxygen consumption is faster at pH 8.

The rate constants for reactive quenching of $O_2(^1\Delta_g)$ with chymo at pH 6 and pH 8 (Table 1), were graphically obtained from the respective first order plots, by monitoring protein consumption through fluorescence measurements (Fig. 4).

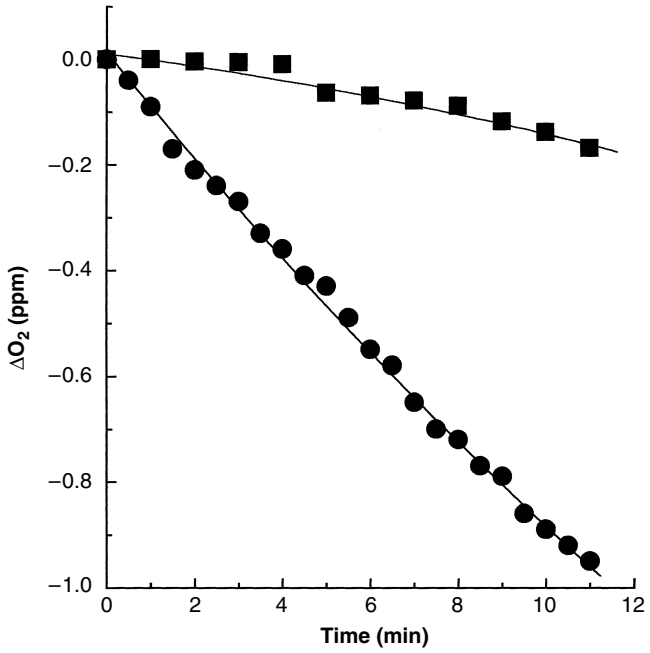

Figure 3. Profiles of oxygen uptake by α-chymo upon PN-sensitized photoirradiation at pH 6: (■) and pH 8: (●); [Chymo] = 1×10^{-5} M.

Oxygen uptake experiments

Oxygen uptake during RB-sensitized photooxidation was inhibited by NaN_3, a well-known selective $O_2(^1\Delta_g)$ physical quencher (30). The maximum effect (about 85% inhibition) was obtained with approximately 5 mM NaN_3. To a lesser extent the oxygen consumption was also inhibited by superoxide dismutase (SOD), a specific $O_2^{\cdot-}$ scavenger. This enzyme, which catalyses the dismutation of superoxide ion ($O_2^{\cdot-}$), has been employed reiteratively with identification and quantification purposes, as a quencher of the oxidative species (31–33). The inhibitory effect of SOD on the RB-sensitized photooxygenation of chymo corresponds to approximately 18% of oxygen consumption. This finding, employing RB as a sensitizer for the determination of total oxygen consumption, does not constitute a novel result. Lee and Rodgers (34), reported in 1987 that the quenching of the triplet excited state of RB by ground state molecular oxygen [$O_2(^3\Sigma_g^-)$] leads to approximately 75% $O_2(^1\Delta_g)$ and 20% $O_2^{\cdot-}$. Our results, employing the selective quenchers NaN_3 and SOD are very close to those quantities, demonstrating a typical behaviour of the sensitizer RB towards the photooxidation of chymo.

Oxygen uptake upon PN-sensitized photoirradiation was also monitored. This process was practically suppressed in the presence of 5 mM NaN_3. This experimental fact in addition to the absence of reactivity under N_2 atmosphere, and the observed quenching of $O_2(^1\Delta_g)$-emission by α- and β-chymo through the TRPD experiments (see below), largely demonstrate the involvement of $O_2(^1\Delta_g)$ as the main or exclusive oxidative agent in the PN-sensitized photooxidation of chymo.

Quenching of $O_2(^1\Delta_g)$ phosphorescence by the proteins

Results for the determination of the overall rate constants k_t (Table 1) by means of TRPD method are shown in Fig. 5. All data from Table 1 obeys the same pattern than that described for oxygen uptake experiments: the higher rate constant values belong to β-chymo and to the alkaline medium. A reproducible behaviour is observed (Fig. 5) in the case α-chymo at pH 8: the experimental points of the $O_2(^1\Delta_g)$ quenching do not neatly fall onto the plotted straight line. This apparent gradual change in the quenching rate constant appears to be related to a decrease in the accessibility of $O_2(^1\Delta_g)$ as the protein concentration increases (see Discussion section). The k_t value for α-chymo at pH 8 (Table 1) is the statistical mean value for the Stern-Volmer plot, including all experimental points.

Table 1. **Rate constants for overall (k_t, M/s) and chemical (k_r, M/s) quenching of $O_2(^1\Delta_g)$ by chymotrypsines, k_t/k_r ratios and relative rates for oxygen consumption (V_{ox}) upon Perinaphthenone sensitized photoirradiation of chymotrypsines in pH/pD 6 and pH 8 aqueous solutions. Literature k_t and k_r values for Trp and Tyr at diffrent pH values were included for comparative purposes**

	pH/pD 6				pH/pD 8			
Compound	$k_r \times 10^{-8}$	$k_t \times 10^{-8}$	k_t/k_r	V_{ox}	$k_r \times 10^{-8}$	$k_t \times 10^{-8}$	k_t/k_r	V_{ox}
α-chymo	0.15	1.56	10.2	0.05	0.54	2.01	3.75	0.67
β-chymo	0.60	4.35	7.2	0.18	0.75	4.84	6.41	1
Trp	0.34[a]				3.05			
Tyr		0.27[b]			0.38[c]			

[a]from reference (24); [b]at pH 7 and [c]at pH 10, from reference (25).

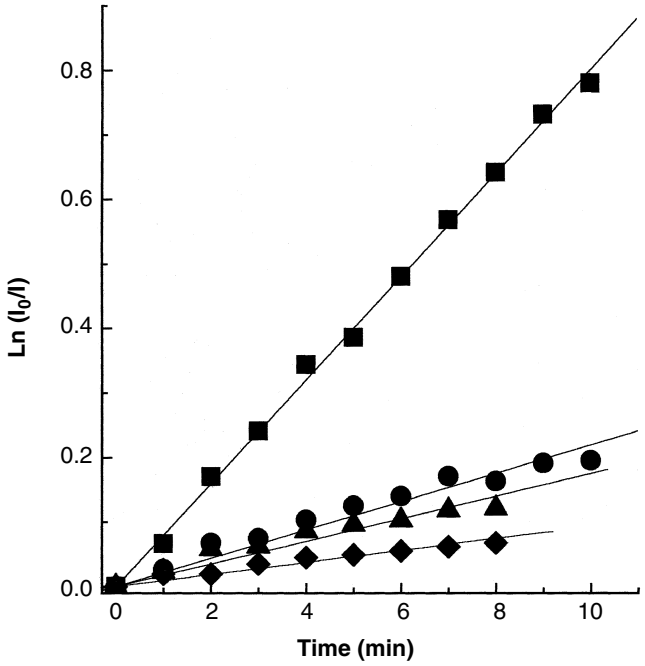

Figure 4. First order plot for β-chymo consumption upon PN sensitized photoirradiation at pH: (●) 8; (▲) 6 and Trp at pH: (■) 8 and (◆) 6. [Chymo] = 1×10^{-5} M.

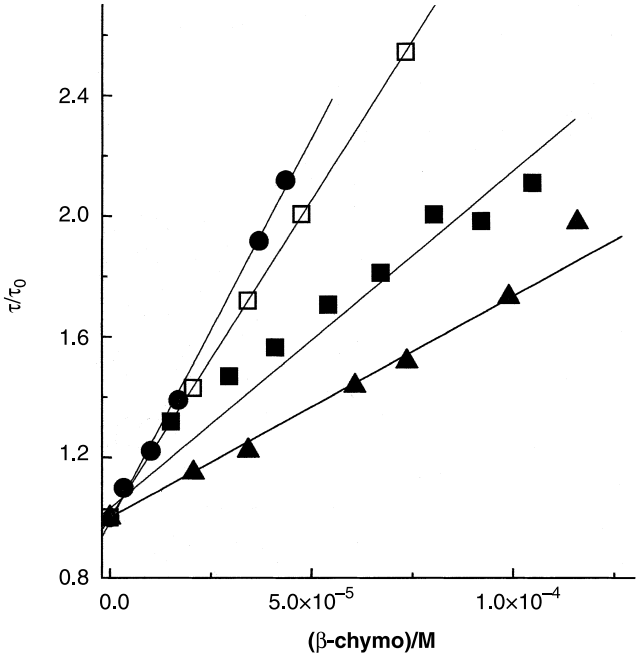

Figure 5. Stern-Volmer plots for the quenching of singlet molecular oxygen phosphorescence (TRPD) by chymo in D_2O, α-chymo at pH: (▲) 6 and (■) 8; β-chymo at pH (□) 6 and (●) 8.

Discussion

The kinetic determinations carried out in this work were performed at two pH values, namely pH 6 and pH 8. The objective of this procedure was to develop a comparative study on α- and β-chymotrypsins, stressing on the kinetic behaviour of Tyr and Trp, the two amino acid residues sensible to pH changes, among the photooxidizable amino acids' distribution in chymo. It is known that Tyr (1,26,35,36) is a moderate $O_2(^1\Delta_g)$ physical deactivator when the phenolic group is undissociated. The presence of the ionized phenolate form confer to Tyr characteristics of relatively strong $O_2(^1\Delta_g)$ quencher with chemical and physical components. Also it, is known that the photooxidation kinetics of His, Met and Cys residues are insensitive to pH changes in the range 6–8 (37,38). Finally, our present results (Table 1) indicate a considerable change in the k_r value for Trp by comparing the extreme values of the mentioned pH range.

From Table 1, it can be observed that both rate constants k_t and k_r and the rate of oxygen uptake (V_{ox}) for α-chymo and β-chymo show the same tendency, being the respective values for β-chymo, pH 8 > α-chymo, pH 8 and β-chymo, pH 6 > α-chymo, pH 6. These facts could be interpreted, in a simplistic fashion, as an enhanced reactivity of β-chymo with regard to the α-chymo. Nevertheless, as it was stressed elsewhere (39) no relevant information about the effectiveness of the actual photodegradation is obtained from the straightforward comparison of rate constants in a $O_2(^1\Delta_g)$-mediated photooxidation. Certainty, the rate constant k_t provides a helpful insight about the feasibility of an overall interaction of a given substrate with $O_2(^1\Delta_g)$. Even so, in order to evaluate the actual significance of the photooxidation process, the simultaneous effect of the physical and chemical interaction should be considered, being the first contribution usually interpreted, in practical terms as a form of self-protection against $O_2(^1\Delta_g)$-mediated photooxidation. This situation is absolutely contemplated in the expression of the photooxidation quantum efficiency [φ_r, eqn (9)].

$$\varphi_r = k_r[Q]/(k_d + k_t[Q]) \qquad (9)$$

Nevertheless, this expression is somewhat limited, because of the dependence on the concentration of the photooxidizable substrate ([Q]), which is particularly difficult to estimate in complex biological environments. A more simple and useful approach is the k_t/k_r ratio, which can be envisaged as the fraction of overall collisions substrate-$O_2(^1\Delta_g)$ that effectively leads to chemical reaction. Calculated k_t/k_r values accounting for the efficiency of effective photooxidation of chymo (Table 1) range from 3.8 to 10.2, and indicate that both proteins tested are fairly good candidates to suffer photodynamic action, specially in pH 8 water. The experiments at pH 6 exhibit the highest k_t/k_r quotients, interpreted as a better situation of self-protection against $O_2(^1\Delta_g)$-mediated oxidation in this medium (39,40).

The enhancement of the reactive component in the alkaline medium possibly reflects the contribution of the Trp residues in both proteins. Reactive rate constants for Trp (Table 1) increase approximately nine-fold going from pH 6 to pH 8 (Table 1). On the other hand, given that the pK value for the OH group of isolate Tyr is 10.1 (41), the reactive contribution of Tyr residues to the reactive interaction seems to be negligible, given that only approximately 1% of these residues should be in the phenolate form at pH 8.

It has been proposed by other authors (11), that structural factors, namely conformational and configurational characteristics restrict the access of $O_2(^1\Delta_g)$ to the interior of the protein and imposes steric (10) limitations to Type II photooxidations. In other words, tertiary and quaternary structures play an important role on the kinetics of protein photooxidation, obviously in addition to the pattern of photooxidizable/non-photooxidizable amino acids. In general terms, on the basis of k_t values, neatly arises that the conformation of β-chymo is more permeable to the interaction with $O_2(^1\Delta_g)$ practically in a pH-independent fashion, whereas the higher degree of effective photooxidation corresponds to both chymotrypsines at pH 8, possibly because of the contribution of the Trp residues.

On the basis of the k_t/k_r quotients and on the known pH dependence of the Trp photooxidation kinetics, we can say that at pH 8, Trp residues in α-chymo seems to be more exposed to $O_2(^1\Delta_g)$ attack. The k_t/k_r ratio for the α-chymo (Table 1) which is higher in the acidic pH region, accounting for an interesting protein self protection degree, becomes particularly small at pH 8, a medium in which the Trp reactive contribution becomes much more important.

As a conclusion we can say that both proteins, α- and β-chymotrypsin, at pH 6 and 8 suffer photodynamic oxidation under adequate photosensitizing conditions. Whereas β-chymo exhibits the greater overall proclivity to interact with $O_2(^1\Delta_g)$, the higher self-protection degree for both proteins is reached at pH 6, owing to an increase of the $O_2(^1\Delta_g)$ physical quenching contribution. At pH 8 the α-chymo becomes more photodegradable, possibly because of a higher accessibility of the Trp residues in this protein.

Acknowledgements: Thanks are given to CONICET, SECyT (UNRC), FUNDACION ANTORCHAS of Argentina, for financial support. A. Posadaz thanks CONICET for a research fellowship. M.A.B. holds a research position at CONICET.

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