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Original Communication

Kinetic and mechanistic behavior of the ascorbate ion-glutathione mixture in the visible-light-mediated oxidation of tryptophan

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ABSTRACT

Within the framework of the frequently invoked correlation between antioxidant contents and potential antioxidative protection, the stability of the amino acid (AA), tryptophan (trp), taken as a prototype of relevant photooxidizable biological target, was evaluated in the presence of the known antioxidant ascorbate ion (AsA). The peptide glutathione (GSH) was also included in order to eliminate thermal decomposition of AsA. Riboflavin (Rf, vitamin B2), endogenous in most biological environments, was employed as a photosensitizer. A systematic kinetic and mechanistic study was conducted under aerobic conditions in pH 7 aqueous medium. The evaluation of oxygen uptake rates (OUR) in the photoirradiated mixtures, taken as an overall indicator of the total oxidation degree of the mixture components, constitutes an interesting and useful tool in this case. In the presence of Rf, the isolated AA and AsA undergo photodegradation under conditions currently found in natural media. The photodegradations occur in a non-simple reaction, mediated by reactive oxygen species (ROS) such as singlet molecular oxygen ($O_2(^1\Delta_g)$), superoxide radical anion, hydrogen peroxide and hydroxyl radical. All ROS are photogenerated from triplet excited Rf after interaction with the ground state of AsA and trp. The photoirradiation also triggers an already described parallel thermally-driven and dark reaction on AsA which is stopped by the addition of GSH. The global outcome can be interpreted as a sort of conjunctive antioxidant protection exerted on the system AsA + trp + GSH upon Rf-photosensitization, a result that cannot be predicted from the individual behavior of the mixture components.

KEYWORDS: ascorbate ion, glutathione, photodegradation, photooxidation, riboflavin, ROS, tryptophan

ABBREVIATIONS

AA	:	amino acid
AAs	:	aminoacids
AsA	:	ascorbate ion
CAT	:	catalase
DAsA	:	dehydroascorbic acid
FFA	:	furfuryl alcohol
GSH	:	glutathione
Mann	:	D-mannitol
OUR	:	oxygen uptake rates
PN	:	perinaphthenone
Q	:	an eventual electron-donating and oxidizable substrate

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RB : rose bengal
Rf : riboflavin
ROS : reactive oxygen species
SOD : superoxide dismutase
trp : tryptophan

INTRODUCTION

Dietary intake of natural antioxidants in humans contributes to the defensive mechanism against carcinogens and mutagens, mainly due to the scavenging effect of these compounds on oxidative species, including free radicals and reactive oxygen species (ROS) [1]. For this reason, the establishment of a direct correlation in biological environments between antioxidant contents and potential antioxidative protection is a common practice. Nevertheless, an initial and essential requirement to ensure the effectiveness of a given antioxidant is its own stability/persistence in the presence of the oxidative species. Ascorbate ion (AsA), currently known as vitamin C, has many well-known beneficial biological properties, including antioxidant activity [2, 3]. However, AsA decomposes in a relatively easy fashion upon exposure to ultraviolet photoirradiation and heat among other external factors which also include reactions with metals and oxidants [4, 5, 6]. Although AsA only absorbs in the ultraviolet range, the photoirradiation with visible light in the presence of adequate absorbers is an important route for the *in vivo* degradation of the vitamin. This is due to the so-called process of photosensitization. In this context riboflavin (Rf, vitamin B2)-photosensitized reactions of AsA possibly are the most studied degradation modes [7, 8, 9, 10, 11, 12, 13, 14, 15]. These studies focused on the kinetic and mechanistic aspects of the flavin mononucleotide-sensitized oxidation of AsA [7]; the effect of the light dose and Rf content on the decomposition of AsA in the presence of amino acids (AAs) [8], the antioxidative protection exerted by AsA on a parenteral nutrition solution upon flavin-sensitized photoirradiation [8], the generation of hydrogen peroxide (H_2O_2) in the anaerobic Rf-photosensitized AsA degradation [10], electron transfer reactions between flavines and several electron donors, including AsA [11, 12] and tryptophan (trp) degradation upon UVA photoirradiation in the presence of Rf and glycation end products, in relation to the problem of lens-cataract development. In the last years, two papers dealt with the protective effect of AsA on the Rf-sensitized oxidation of aromatic amino acids, including trp [14], and on the degradation of Rf in milk [15]. In the first of the above-mentioned papers, after an interesting study, the authors conclude that Type I mechanism was mainly responsible for the degradation of the AAs. The quenching of $O_2(^1\Delta_g)$ by AsA exerts a protection of the sensitizer, favoring the photodegradation of the amino acids.

On the basis of this knowledge, other literature reports and our own results, we present a comprehensive interpretation of the role of AsA as a protective antioxidant of trp under Rf-photosensitization. It was done upon visible-light irradiated Rf + AsA + trp mixtures, in the absence and in the presence of the peptide, glutathione (GSH), employing oxygen consumption measurements. The amino acid (AA) trp was taken as a prototype of photooxidizable biological target and the peptide has been reported as a stabilizer for the thermal AsA-degradation [16]. We made a complete and systematic kinetic study, taking into account the presence of a series of different parallel competitive reactions. Besides, we present a mechanistic evaluation of all the possible individual reactions initiated by the interaction between photoexcited Rf, and ground state AsA, all under a unique experimental pattern. This is an important condition that makes the reaction steps absolutely comparable. The work includes a kinetic analysis of the Rf-photopromoted and dark processes, and provides a quantification of the dark degradative reaction of AsA in comparison to the photochemical one and its consequence in the photoprotection of trp.

Riboflavin is one of the endogenous visible-light absorbers which has been postulated as a possible sensitizer for the *in vivo* photooxidative degradation of several biologically relevant substrates [17, 18]. The combination Rf-AsA-GSH-trp reasonably mimics a natural biological scenery, in which an endogenous sensitizer, a biologically relevant oxidizable target and the antioxidant photoprotector system are simultaneously present in a given environment, illuminated by daylight.

MATERIALS AND METHODS

Chemicals

Riboflavin (Rf), rose bengal (RB), ascorbic acid (AsA), perinaphthenone (PN), sodium azide (NaN_3), catalase (CAT) from bovine liver, glutathione (GSH), superoxide dismutase (SOD) from bovine erythrocytes and D-mannitol (Mann) and L-tryptophan (trp) were purchased from Sigma Chem. Co. Furfuryl alcohol (FFA) was from Riedel de Haën. The solvents employed were deuterated water (D_2O, 99.9% D) (from Aldrich), and triply distilled H_2O. Phosphate buffer was used to regulate pH = 7 or pD = 7. D_2O was employed in the time-resolved determinations of $O_2(^1\Delta_g)$ phosphorescence emission, in order to enlarge the lifetime of this species [19].

Instrumentation and methods

Stationary Rf fluorescence experiments were carried out in a RF 5301-PC Shimadzu spectrofluorimeter at 25 ± 1 ºC in air equilibrated solutions. Excitation and emission wavelengths for Rf were 445 and 515 nm, respectively. Ground state absorption spectra were registered in a Hewlett Packard 8452A diode array spectrophotometer. Stationary aerobic photolysis of aqueous solutions containing AsA 0.1-0.5 mM and Rf *ca*. 0.04 mM were carried out in a PTI unit (150 W Xe lamp) with a high pass monochromator, irradiating with 440 ± 10 nm, or in a home-made photolyzer for non-monochromatic irradiation (150 W quartz-halogen lamp), using a cut-off filter of 400 nm in order to ensure that the light was only absorbed by Rf. Rf- or RB-sensitized photooxygenation rates of the substrates were determined from the initial slopes of the plots oxygen consumption *vs*. irradiation time, employing a specific oxygen electrode (Orion 97-08). The oxygen uptake rates (OUR) we are reporting in this work represent the mean value of a group of four runs taken under identical experimental conditions. All OUR values of these sets did not differ more than 3-4% each other. Although standard deviations for the individual determinations resulted lower than 1%, we assigned 3% as the error bar for the relative OUR. This constitutes a more realistic estimation in our opinion, and assists in the interpretation of the actual magnitude of the observed effects.

The reactive rate constant, k_r, for the reaction of $O_2(^1\Delta_g)$ with AsA (process (16) of Scheme 1, see below) was determined as described in the literature [20] using the expression $slope/slope_R = k_r\,[AsA]/k_{rR}\,[R]$, for which the knowledge of the reactive rate constant for the photooxidation of a reference compound, R, at similar concentration, is required, and where slope and $slope_R$ are the respective slopes of the first-order plots of AsA and R consumption, or oxygen consumption by the same compounds, under sensitized irradiation.

Time-resolved phosphorescence detection (TRPD) of $O_2(^1\Delta_g)$ was carried out with a laser-kinetic spectrophotometer previously described [21], using pD 7 aqueous solutions of the sensitizer RB with absorbance close to 0.4 at 532 nm. The decay kinetics was first order in all cases.

RESULTS AND DISCUSSION

The self-defined reaction steps included in the following mechanistic scheme have been already employed for the interpretation and discussion of reactions between photoexcited Rf and different substrates [7, 9, 11, 18]. Q represents an electron-donor and photooxidizable substrate.

As already mentioned, the main objective of the present work was a detailed elucidation of the kinetic and mechanistic aspects in the photoprotection exerted by AsA towards the Rf-sensitized degradation of trp. In doing this, we initially undertake a detailed characterization of the photochemical behavior of isolated AsA and trp on the basis of our own results obtained in the present and previous works and results from other authors, all in pH 7 aqueous solution, as follows:

Ascorbate ion photodegradation under stationary photolysis

In neutral aqueous solution ascorbic acid is present as ascorbate ion ($pK_a = 4.17$) [22]. An air-equilibrated pH 7 aqueous solution of Rf (A_{446} = 0.4) plus 0.5 mM AsA was stable when stored under dark conditions. The absorption spectrum of the described solution changes upon photoirradiation ($\lambda_{irr} > 400$ nm), exhibiting transformations in both the AsA and Rf components, the latter to a minor extent. When the light was suppressed, after an initial irradiation period, the absorption spectrum

$Rf + h\nu \rightarrow {}^1Rf^* \rightarrow {}^3Rf^*$ (1)

${}^1Rf^* + Q \rightarrow Rf + Q$ or P(2) (2), rate constant ${}^1k_q$

${}^3Rf^* + O_2({}^3\Sigma_g^-) \rightarrow Rf^{\bullet+} + O_2^{\bullet-}$ (3)

${}^3Rf^* + Q \rightarrow Rf^{\bullet-} + Q^{\bullet+}$ (4) rate constant ${}^3k_q$

$Rf^{\bullet-} + H^+ \rightarrow RfH^\bullet$ (5)

$2RfH^\bullet \rightarrow Rf + RfH_2$ (6)

$RfH_2 + O_2({}^3\Sigma_g^-) \rightarrow RfH_2^{\bullet+} + O_2^{\bullet-}$ (7)

$RfH_2^{\bullet+} + O_2^{\bullet-} \rightarrow Rf + H_2O_2$ (8)

$O_2^{\bullet-} + Q \rightarrow P(9)$ (9) rate constant k_9

$H_2O_2 + Q \rightarrow P(10)$ (10)

$H_2O_2 + O_2^{\bullet-} \rightarrow OH^\bullet + OH^-$ (11)

$OH^\bullet + Q \rightarrow P(12)$ (12)

${}^3Rf^* + O_2({}^3\Sigma_g^-) \rightarrow Rf + O_2({}^1\Delta_g)$ (13) rate constant k_{ET}

$O_2({}^1\Delta_g) \rightarrow O_2({}^3\Sigma_g^-)$ (14)

$O_2({}^1\Delta_g) + Q \rightarrow O_2({}^3\Sigma_g^-) + Q$ (15) rate constant k_q

$O_2({}^1\Delta_g) + Q \rightarrow P(16)$ (16) rate constant k_r

Being $k_t = k_r + k_q$

Scheme 1. Possible reaction steps in the Rf-sensitized photooxidation of a hypothetical substrate Q.

of the solution was still changing. The decrease in the 265 nm absorption maxima of AsA as a function of irradiation time, representing the rate of AsA degradation, is neatly lowered in the absence of light, and constitutes *ca*. 12% of the rate value obtained under photoirradiation (data not shown). The thermal degradation component of AsA consumption is totally suppressed when the experiment was done in the presence of 0.5 mM of the peptide GSH. Results are shown in Fig. 1.

From parallel experiments on similar photoirradiated solutions, oxygen uptake was observed. Likewise, oxygen consumption was still occurring, although at a lower OUR, after suppression of the irradiation source. This effect is shown in Fig. 1. The solutions did not consume any oxygen before photoirradiation.

The reported first step in the oxidation of AsA is the formation of dehydroascorbic acid (DAsA), with loss of H in the carbons C2 and C3 [6]. The dark reaction described in the present work, starts upon Rf-sensitized photoirradiation of pH 7 aqueous solutions containing 0.5 mM AsA. Experimental evidence strongly suggests that DAsA is formed. Apparently this is the case of the dark reaction we found in the photochemically

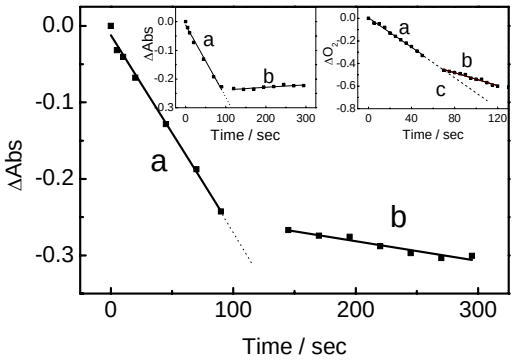

Fig. 1. Absorbance changes at 265 nm in a solution of 0.1 mM AsA as a function of photoirradiation time in the presence of 0.04 mM riboflavin, up to 90 sec (a). The same in the absence of light after the initial 90 sec photoirradiation (b). Left inset: Absorbance changes at 265 nm in a solution of 0.1 mM AsA as a function of photoirradiation time in the presence of 0.04 mM riboflavin and 0.5 mM glutathione, up to 90 sec (a). The same in the absence of light after the initial 90 sec photoirradiation (b) Right inset: Oxygen uptake by a 0.1 mM AsA solution as a function of photoirradiation time in the presence of 0.04 mM riboflavin, up to 50 sec (a). The same in the absence of light after the initial 50 sec photoirradiation (b). Extrapolation of the photochemically- mediated trace (c). All in pH 7 aqueous solutions.

initiated photodegradation of AsA. The thermal component is absolutely suppressed in the presence of GSH. Besides, and according to oxygen uptake experiments shown in Fig. 1, the rate of the overall component (thermal plus photochemical reactions) is *ca.* 8-fold faster than the thermally driven reaction, under work conditions. This fact ensures a slow but continuous degradation of the vitamin even under dark conditions.

This collection of preliminary experimental evidence indicates that under visible light irradiation the overall interaction Rf-AsA includes the participation of electronically excited states of the pigment and, according to the oxygen uptake experiments, also includes the participation of dissolved ground state oxygen and/or reactive oxygenated species formed in the medium. On this basis, we carried out a systematic kinetic study in order to evaluate and characterize the nature, mechanism and extent of the possible processes involved in the Rf-sensitized degradation of AsA, including the detected thermally-driven AsA-consumer and oxygen-consumer reactions, induced by the initial photoirradiation of the sensitizer.

Quenching of Rf electronically excited states

The fluorescence properties of Rf in water are well known. Rf exhibits an intense emission band at 515 nm with a fluorescence quantum yield of 0.25 [23]. The presence of AsA ≥10 mM produces a detectable quenching of the Rf fluorescence, as determined by stationary fluorescence measurements. On monitoring the fluorescence intensity of Rf in the absence (I_o) and in the presence (I) of different AsA concentrations (Fig. 2), the classical Stern-Volmer treatment ($I_o/I = 1 + {}^1K_{SV}$ [AsA], with ${}^1K_{SV} = {}^1k_q \, . \, {}^1\tau_o$) allows the determination of the Stern-Volmer constant (${}^1K_{SV} = 12.5\ M^{-1}$), ${}^1k_q$ being the rate constant for process (2) and τ_o = 5.2 ns, the reported value for the fluorescence lifetime of ^{1}Rf* [24]. Results are shown in Fig. 2, from which a ${}^1k_q$ value of $7.2 \times 10^9\ M^{-1} s^{-1}$ is deduced (Table 1).

According to the fluorescence quenching data, an AsA concentration of 0.5 mM -ten times higher than the concentrations employed in the Rf-sensitized experiments- would produce a negligible decrease in the lifetime of ^{1}Rf*, in the range of 5%. Hence, the participation of ^{1}Rf* in the photodegradation of AsA must be disregarded under work conditions.

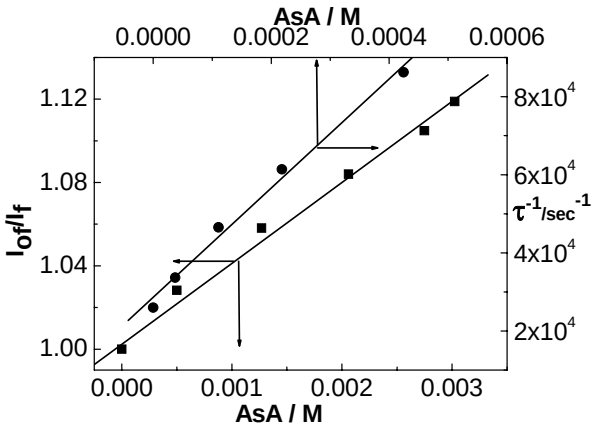

Fig. 2. Stern-Volmer plots for the quenching of riboflavin stationary fluorescence by AsA in pH 7 aqueous solution (■) and for the quenching of $O_2({}^1\Delta_g)$ phosphorescence by AsA in pD 7 D_2O solution (●). I_{of} and I_f represent the stationary fluorescence intensities of riboflavin in the absence and in the presence of AsA and τ^{-1} represents the $O_2({}^1\Delta_g)$ phosphorescence lifetime in the presence of different AsA concentrations.

Recently, the work by H. Görner [11] experimentally demonstrated that AsA quenches ^{3}Rf* in water. The published value of the rate constant ${}^3k_q$ for process (4) is included in Table 1.

Neither ^{1}Rf* nor ^{3}Rf* lifetimes were affected by the presence of GSH up to concentration 0.5 mM, according to stationary-fluorescence and laser flash photolysis experiments, similar to those described above for AsA.

Interaction of AsA with photogenerated ROS

The potential participation of Rf photogenerated ROS was evaluated through oxygen consumption experiments, employing specific ROS interceptors.

The individual presence of 10 mM NaN_3, 1 μg/ml CAT, 1 μg/ml SOD and 10 mM Mann in air-equilibrated pH 7 aqueous solution of Rf (A_{446} = 0.4) and AsA 0.5 mM decreases the OUR upon photoirradiation ($\lambda_{irr} > 400$ nm), as shown in Fig. 3. The traces are the mean values from three runs not differing more than 5% each other. Similar experiments with ROS-interceptors have been formerly employed to confirm/discard the participation of $O_2({}^1\Delta_g)$, H_2O_2, $O_2^{\bullet-}$ and $OH^{\bullet}$ in a given oxidative event [28, 29, 30]. The enzyme SOD dismutates the species $O_2^{\bullet-}$ (reaction (17)), whereas CAT decomposes H_2O_2 (reaction (18)),

Table 1. Rate constants for overall quenching (k_t) and reactive quenching (k_{rapp} for AsA and k_r for trp) of $O_2(^1\Delta_g)$ phosphorescence; k_r/k_t ratios and rate constants for the quenching of ^{1}Rf* (k_{q1}) and ^{3}Rf* (k_{q3}).

Compound pH/pD	$k_t/10^8$ ($M^{-1}s^{-1}$)	$k_r/10^8$ ($M^{-1}s^{-1}$)	k_r/k_t	$k_{q1}/10^9$ ($M^{-1}s^{-1}$)	$k_{q3}/10^9$ ($M^{-1}s^{-1}$)
AsA pH 7	1.42 (a) 1.60 (b)	1.11 (c)	0.78	7.2	10 (d)
Trp pH 7	0.72 (e)	0.47(e)	0.65		2.5 (f)

(a) pD 7; (b) from ref. [25]; (c) k_{reff} instead of k_r; (d) from ref. [11]; (e) from ref. [26]; (f) from ref. [27].

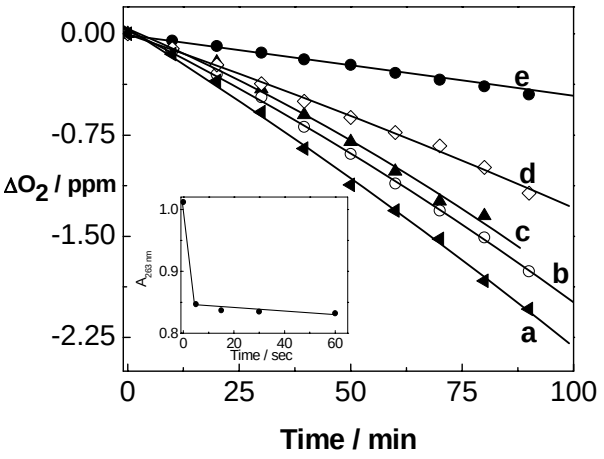

Fig. 3. Oxygen consumption by the following solutions in the presence of 0.05 mM Rf upon photoirradiation: 0.5 mM AsA (a); 0.5 mM AsA plus 10 mM Mann (b); 0.5 mM AsA plus 1 μg/ml CAT (c); 0.5 mM AsA plus 1 μg/ml SOD (d) and 0.5 mM AsA plus NaN_3 10 mM (e), all in pH 7 aqueous solution. Inset: temporal absorbance changes of a 0.5 mM solution of AsA after the addition of 0.05 mM H_2O_2 observed at the wavelength of the maximum of the absorption band at pH 7.

NaN_3 deactivate $O_2(^1\Delta_g)$ with NaN_3 instead of AsA in reaction (15), and Mann deactivates the species $OH^\bullet$ (reaction (19)).

$$2O_2^{\bullet-} + 2H^+ + SOD \rightarrow O_2(^3\Sigma_g^-) + H_2O_2 \quad (17)$$

$$2H_2O_2 + CAT \rightarrow 2H_2O + O_2(^3\Sigma_g^-) \quad (18)$$

$$OH^\bullet + Mann \rightarrow deactivation \quad (19)$$

The observed delay in the rates of oxygen uptake in the presence of the selective ROS interceptors indicates, in principle, the participation of $O_2(^1\Delta_g)$, H_2O_2, $O_2^{\bullet-}$ and $OH^\bullet$ in the overall AsA oxidative event.

The viability of the reaction between AsA and H_2O_2 (reaction (10)), was independently checked by a simple test. Fig. 3, inset, shows the time monitoring of the absorbance maximum in the AsA absorption spectrum after the addition of 0.05 mM of H_2O_2. Neat spectral changes indicate the oxidation of AsA.

Oxygen uptake results, spectroscopic evidence and auxiliary specific experiments in the Rf-sensitized photoprocesses strongly support the effective participation of ROS in the degradation of AsA through a non-simple mechanism.

As recently demonstrated by Görner, ^{3}Rf* is efficiently quenched by AsA [11]. The species $RfH^\bullet$ is generated in this interaction. The author demonstrates that the bimolecular decay of the neutral radical is known to proceed through a disproportionation reaction yielding Rf and fully reduced Rf (RfH_2) (process (6)). In the presence of $O_2(^3\Sigma_g^-)$, RfH_2 is reoxidized, giving rise to $RfH_2^{\bullet+}$ and $O_2^{\bullet-}$, and, eventually, Rf and H_2O_2 (process (8)) [31, 32]. Finally, the species $OH^\bullet$ can be formed through reaction (11).

The thermodynamic feasibility of the electron transfer process (4) for the case of Rf-AsA can be evaluated by means of the Gibbs free energy for electron transfer [33], using the expression $\Delta G^0 = E_{(AsA/AsA+)} - E_{(Rf/Rf-)} - E_{Rf*} + C$, where $E_{(AsA/AsA+)}$ is the oxidation potential of AsA (0.058 V [34]), $E_{(Rf/Rf-)}$ is the redox potential of Rf (–0.80 V [35]),

E_{Rf*} is the ^{3}Rf* energy (2.17 eV [35]), and C is the coulombic energy term (-0.06 V [35]). The so-calculated ΔG^0 value (-1.39 eV) indicates that process (4) may be operative and, consequently, that the species $O_2^{\bullet-}$ could be formed after electron transfer from Rf (process (4)), a possibility that is in total agreement with the experimental findings by Görner [11].The effective operation of this pathway in aerated medium will depend on whether it is kinetically competitive or not with the $O_2(^1\Delta_g)$ generation (process (13)). Considering the k_{ET} value of process (13) in H_2O (1.2×10^9 $M^{-1}s^{-1}$, i.e. 1/9 of the difussional value) [36], and the reported value of ^3k_q (process (4)) 1×10^{10} $M^{-1}s^{-1}$ (Table 1), it can be deduced that, for the same concentrations of AsA and dissolved $O_2(^3\Sigma_g^-)$, the rate for the generation of the initial $O_2^{\bullet-}$ precursor $RfH^{\bullet}$ (vía $Rf^{\bullet-}$, process (8)) is *ca*. ten times higher than the corresponding one for $O_2(^1\Delta_g)$ generation (process (5)). This is a very interesting finding since, as already mentioned, the respective reaction steps for the generation of H_2O_2 and $OH^{\bullet}$ depend on the initial generation of the species $O_2^{\bullet-}$. Our experimental evidence for the scavenging effect of AsA on the mentioned Rf-photogenerated ROS, includes and confirms previously reported data. It was established [37] that $O_2^{\bullet-}$ takes part in the oxidation of AsA in aqueous medium through a complex reaction mechanism, with participation of ascorbate anion and ascorbate radical, the final products being dehydroascorbic acid and its decomposition products. A rate constant value of 2.7×10^5 M^{-1} sec^{-1} for k_9 (step (9) in Scheme 1) was reported at pH 7.4, employing $O_2^{\bullet-}$ generated by the xanthine-xanthine oxidase system [38]. Regarding H_2O_2, it is well known that the oxidative species is generated by Rf-photosensitization in the presence of AsA [10, 11]. It has also been reported that AsA plus its primary oxidation product, DAsA, react with the peroxide [39, 40]. AsA and DAsA are ultimately oxidized by H_2O_2 to the same species, threonic acid [6].

In reference to the interaction AsA-$OH^{\bullet}$, several works describe the *in vivo*-suppression of $OH^{\bullet}$ production in the presence of AsA [41] and the oxidation of the vitamin by the radical [42], forming different oxidation products in basic or in acidic solutions.

Quenching of $O_2(^1\Delta_g)$ by AsA

When a solution of the well-known exclusive $O_2(^1\Delta_g)$ generators PN (A_{400} = 0.3) or RB (A_{530} = 0.4) [43] was irradiated in pH 7 aqueous solution with visible light in the presence of AsA, both modifications in the AsA spectral component and oxygen consumption were observed. Any spectral change in the absorption band of the sensitizer was not detected. In similitude with the Rf-sensitized runs, the mentioned spectral changes and oxygen consumption were still occurring even when the light was suppressed after an initial photoirradiation time (not shown). These results strongly suggest both, some degree of interaction $O_2(^1\Delta_g)$-AsA, and the presence of a dark reaction. RB and PN were chosen as sensitizers in order to focalize on the potential reaction of AsA with $O_2(^1\Delta_g)$, avoiding potential interferences due to interactions of the substrates with Rf electronically excited states and eventually with other ROS different from $O_2(^1\Delta_g)$.

In the TRPD experiments, the decay kinetics of $O_2(^1\Delta_g)$ phosphorescence was first order, and the lifetime agreed well with literature data [19]. The addition of a AsA as a quencher leads to a decrease of the $O_2(^1\Delta_g)$ lifetime, unambiguously confirming the interaction of the vitamin with the oxidative species. The k_t value, graphically obtained, as shown in Fig. 2, was 1.42 x 10^8 $M^{-1}s^{-1}$ at pD 7, employing RB as a dye-sensitizer. Rougée *et al*. [25] reported a k_t value of 1.6 x 10^8 $M^{-1}s^{-1}$ at pH 7 which is very close to the value determined here at pD 7. The expression $1/\tau_\Delta = 1/\tau_{\Delta o} + k_t$ [AsA] was employed, where τ_Δ and $\tau_{\Delta o}$ are the $O_2(^1\Delta_g)$ lifetimes in the presence and in the absence of AsA, respectively.

The rate constant for reactive interaction of the AsA, (k_r, process (16)), was obtained by the already mentioned actinometric method, by monitoring oxygen photoconsumption, employing FFA as a reference compound and RB (A_{530} = 0.4) as a dye sensitizer. A typical first order plot employed for the determination of k_r is shown in Fig. 4 for FFA. The trace corresponding to AsA clearly exhibits a curvature, indicating an increase of the OUR with time.

The OUR upon sensitized photoirradiation of a solution of RB (A_{530} = 0.4) + AsA 0.5 mM in an

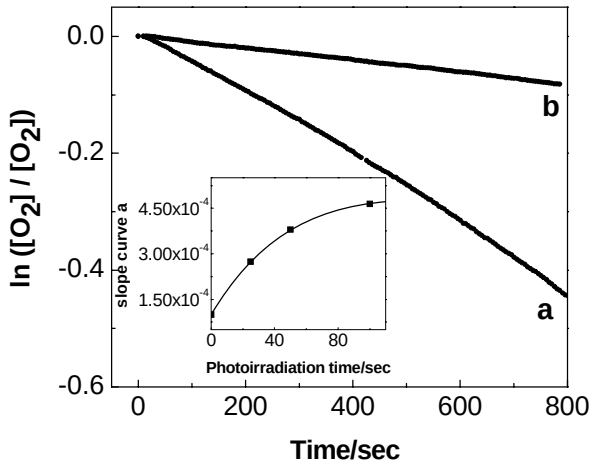

Fig. 4. First order plot of oxygen uptake in the rose bengal-sensitized photooxidation of furfuryl alcohol 0.5 mM (b) and AsA 0.5 mM (a). Slope of the first order plot (curve (a) of the main figure) as a function of photoirradiation time. All in pH 7 aqueous solution.

air saturated pH 7 aqueous solution was decreased by 80% in the presence of NaN_3 50 mM. In this concentration, the salt, possessing a k_q value of 4.5 x 10^8 $M^{-1}s^{-1}$ for the physical deactivation of $O_2(^1\Delta_g)$, reduce the lifetime of the excited species from 4 to 0.07 μs. On the basis that RB is an exclusive $O_2(^1\Delta_g)$-generator, no oxygen uptake at all should be expected in the presence of NaN_3 50 mM. The observed remaining oxygen consumption constitutes an additional evidence for the occurrence of an independent reaction, parallel to the $O_2(^1\Delta_g)$-mediated one. In this context, we interpreted the curvature in the first order plot for AsA (Fig. 4), absolutely absent in the FFA trace, as due to the contribution of the mentioned parallel dark reaction. Considering that the dark reaction is started by the photochemical step, the extrapolation to photoirradiation time = 0 of the slopes in the first order plots for oxygen uptake by AsA (Fig. 4, inset) enables the calculation of an effective reactive rate constant k_{reff} = 1.11 x 10^8 $M^{-1}s^{-1}$ (reaction 16, with k_{reff} instead of k_r).

The rate constant values for the interaction AsA-$O_2(^1\Delta_g)$ and the k_{reff}/k_t ratio are included in Table 1. The latter indicates the fraction of overall quenching of $O_2(^1\Delta_g)$ by the substrate that leads to a chemical transformation.

In synthesis, at this point of the work, results clearly indicate that AsA, in aerated aqueous medium and in the presence of Rf and visible light, participates in the generation and deactivates the ROS $O_2(^1\Delta_g)$, $O_2^{\bullet-}$, H_2O_2 and $O_2^{\bullet-}$. The photoirradiation produces the degradation of AsA, that behaves as a typical sacrificial scavenger, and starts a parallel dark reaction that consumes oxygen at a rate *ca*. 1/9 of the rate for the overall (photochemical + thermal) reaction. The dark degradation is totally suppressed by the presence of 0.05 mM GSH.

The existing information on the photopromoted degradation of trp sensitized by Rf and RB

Mechanistic aspects of the Rf-sensitized photodegradation of trp are well known. Triplet flavin is quenched by the amino acid (reaction 4 with AA instead of Q) with the pH-independent rate constant value ^3k_q of 2.5 x 10^9 M^{-1} s^{-1} [27]. Oxidized radicals of the AAs and the reduced flavin radicals ($FlH^{\bullet}$) are the primary products [17]. One of the more recent studies in this area reports on the photolysis of several flavins in air-saturated aqueous solution in the presence of electron donors, including aromatic AAs [31]. The overall reaction observed was conversion of oxygen via the hydroperoxyl/superoxide radical.

Type II ($O_2(^1\Delta_g)$-mediated) and Type I (radical-mediated) were the reported mechanisms responsible for the photosensitized degradation of trp [19].

The interaction Trp with $O_2(^1\Delta_g)$ has been profusely studied [26, 44, 6, 15]. Rate constant values k_t = 7.2 x 10^7 $M^{-1}s^{-1}$ and k_r = 4.7 x 10^7 $M^{-1}s^{-1}$ were reported by ourselves and are included in Table 1 for the purpose of comparison [26].

Rf- and RB-photosensitization and oxygen uptake by trp-AsA-GSH mixtures

In the preceding part of this study, we have kinetically and mechanistically characterized the Rf-photopromoted AsA decomposition through a systematic work, all done under identical experimental conditions. In the following we focus our interest on the evaluation of AsA in the presence of GSH as an eventual photoprotector system in the Rf-sensitized oxidation of trp. The Rf (A_{445} = 0.36)-sensitized photoirradiation of pH 7 solutions containing 0.074 mM trp, 0.078 mM

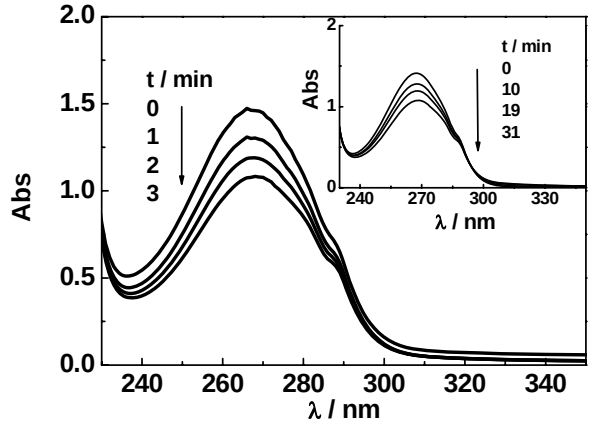

Fig. 5. Evolution of the absorption spectra of the mixture Rf (A_{445} = 0.35) + 0.074 Mm trp + 0.078 mM AsA + 0.044 mM GSH upon photoirradiation in pH 7 aqueous solution. Inset: The same but replacing Rf by RB (A_{546} = 0.46). Cut-off 400 nm.

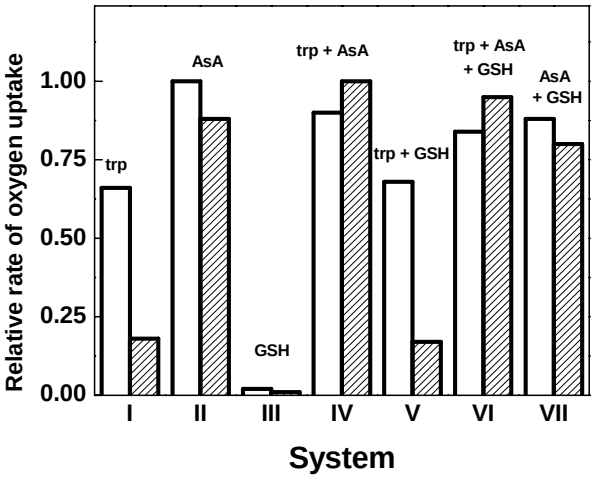

Fig. 6. Diagram of the relative rates of oxygen uptake upon photoirradiation of different mixtures, described at the top of the columns, in the presence of Rf (A_{445} = 0.36) (white columns) or RB (A_{546} = 0.44) (dashed columns). Experiments for each sensitizer were performed under identical experimental conditions, in pH 7 aqueous solutions. In all cases the concentration of the respective components of the mixtures, except the described sensitizers, was 0.5 mM. Cut-off 400 nm.

AsA and 0.044 mM GSH produced spectral changes that can be mainly attributed to transformations in the substrates, as shown in Fig 1. Since RB produces $O_2(^1\Delta_g)$ in a dominant fashion under aerobic visible-light irradiation [45] similar photolysis experiments to those performed with Rf were made, for comparative purposes. The vitamin was replaced by the xanthene dye sensitizer (A_{546} = 0.46 for RB), and the remaining experimental conditions were kept constant. Results are shown in the inset of Fig. 5.

In both photosensitization runs, GSH is the only non reactive component of the mixture, within typical photoirradiation times employed (data not shown). The absolute rates of substrate consumption appreciated from the evolution of the respective spectra in Fig. 5 should not be directly compared because the number of photons absorbed by each sensitizer was different.

In parallel, oxygen consumption was observed in pH 7 aerated aqueous solutions of 0.04 mM Rf (A_{446} = 0.36) or RB (A_{546} = 0.46) plus the already mentioned individual substrates all in concentration 0.5 mM or their respective mixtures namely: trp; AsA; GSH; trp + AsA; trp + GSH; AsA + GHS and trp + AsA + GSH. The obtained results, expressed as relative OUR are shown in Fig. 6. The respective OUR for the sensitizers alone were negligible in relative terms, and hence omitted in the Figure.

The individual contribution of the mixture components to the overall oxidative mechanism may be affected by: (a) the interaction of the initial by-products generated upon photoirradiation or (b) by interactions between ROS produced by the sensitizer in the presence of the oxidizable substrates. In order to rationalize mechanistic aspects of the involved processes, we drive the discussion through the described behavior of the individual components of the mixtures under work conditions.

Despite the large number of competing photoprocesses in the system Rf +AsA + GSH + trp, that make the interpretation of a reaction mechanism complex, we rationalize the experimental results of Rf-photosensitized oxygen uptake on the basis of a simple scheme. It includes quenching of $^3Rf^*$ by AsA and trp, production of ROS and interaction of the generated ROS with the oxidizable substrates.

In the RB-sensitized process -dashed columns in Fig. 6- the relative OUR value for trp is close to 1/4 of the corresponding one for isolated AsA. A similar situation can be observed for the runs

performed in the presence of GSH. The contribution of the mixture AsA-trp to the overall OUR practically corresponds to the simple addition of the individual rates of the components of the mixture. The same is true when these experiments in the presence of GSH are compared. The only observable difference is that the respective OUR are slightly decreased as compared with those in the absence of the peptide, possibly due to inhibition of the parallel thermal oxygen-consumer reaction by AsA, photochemically initiated. AsA and trp exhibit high k_r/k_t ratios (Table 1), with chemical quenching of $O_2(^1\Delta_g)$ (process (16)) as a dominant source of oxygen consumption. All experimental evidence on OUR strongly suggests a simple reaction scheme. Both substrates, in the absence and in the presence of GSH behave as individual oxidizable targets, exhibiting additive OUR in an exclusively $O_2(^1\Delta_g)$-mediated process.

From the observation of the Rf-sensitized set of results in Fig. 6 represented by the white columns, it turns out that ratio OUR_{trp}/OUR_{AsA} is *ca*. 0.66. It is drastically increased as compared to the same situation upon RB-sensitization. This ocurrs both in the absence and in the presence of GSH. But the most remarkable result is that the OUR for the mixture AsA-trp is significantly lower than the simple addition of the respective rates for the individual components. Again parallel results were obtained in the presence of the GSH.

Whereas Type I and Type II routes have been the independently proposed mechanisms for the Rf-sensitized photooxidation of trp [14, 46], our present results indicate that the main photodegradation route for AsA operates through a $O_2(^1\Delta_g)$-mediated process. In other words, Type II mechanism constitutes the common oxidative pathway for the mixture. This fact explains why the competitive quenching of $^3Rf^*$ by trp + AsA, that decreases the $O_2(^1\Delta_g)$ stationary concentration, produces the concomitant reduction of the overall OUR by the mixture.

The overall outcome can be interpreted as a sort of conjunctive antioxidant protection exerted on the system AsA + trp + GSH upon Rf-photosensitization, a result that cannot be predicted from the individual behavior of the mixture components.

CONCLUSION

The simultaneous presence of AsA + GSH + trp exerts a photoprotective effect against ROS-mediated oxidation of the mixture as compared with the extent of oxidation of the individual components, when exposed to visible light irradiation in the presence of Rf.

The kind of evaluation employed in the present contribution constitutes an interesting tool that allows the observation of the oxidizable system as a whole if the OUR, taken in relative terms, are considered as an overall oxidation tendency indicator in a given environment.

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CONFLICT OF INTEREST STATEMENT

There are no conflicts of interest.

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