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 (O2(1Δg)), 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
DAAsA : 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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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$_2$O$_2$) 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 (NaN₃), 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 (D₂O, 99.9% D) (from Aldrich), and triply distilled H₂O. Phosphate buffer was used to regulate pH = 7 or pD = 7. D₂O was employed in the time-resolved determinations of O₂(1Δg) 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 °C 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.

RESULTS AND DISCUSSION

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 (pKₐ = 4.17) [22]. An air-equilibrated pH 7 aqueous solution of Rf (A₄₄₀ = 0.4) plus 0.5 mM AsA was stable when stored under dark conditions. The absorption spectrum of the described solution changes upon photoirradiation (λₘₚ > 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
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

\[ \text{Rf} + \text{hv} \rightarrow ^1\text{Rf}^* \rightarrow ^3\text{Rf}^* \] (1)

\[ ^1\text{Rf}^* + \text{Q} \rightarrow \text{Rf} + \text{Q} \text{ or } \text{P}(2) \]

\[ ^3\text{Rf}^* + \text{O}_2(3\Sigma_g^-) \rightarrow \text{Rf}^{*+} + \text{O}_2^- \] (3)

\[ ^3\text{Rf}^* + \text{Q} \rightarrow \text{Rf}^{*+} + \text{Q}^- \] (4) rate constant \( ^3k_q \)

\[ \text{Rf}^{*+} + \text{H}^- \rightarrow \text{RfH}^+ \] (5)

\[ 2\text{RfH}^+ \rightarrow \text{Rf} + \text{RfH}_2 \] (6)

\[ \text{RfH}_2 + \text{O}_2(3\Sigma_g^-) \rightarrow \text{RfH}_2^{*+} + \text{O}_2^- \] (7)

\[ \text{RfH}_2^{*+} + \text{O}_2^- \rightarrow \text{Rf} + \text{H}_2\text{O}_2 \] (8)

\[ \text{O}_2^- + \text{Q} \rightarrow \text{P}(9) \] rate constant \( k_0 \)

\[ \text{H}_2\text{O}_2 + \text{Q} \rightarrow \text{P}(10) \]

\[ \text{H}_2\text{O}_2 + \text{O}_2^- \rightarrow \text{OH}^- + \text{OH}^- \] (11)

\[ \text{OH}^- + \text{Q} \rightarrow \text{P}(12) \]

\[ ^3\text{Rf}^* + \text{O}_2(3\Sigma_g^-) \rightarrow \text{Rf} + \text{O}_2(1\Delta_g^-) \] (13) rate constant \( k_{ET} \)

\[ \text{O}_2(1\Delta_g^-) \rightarrow \text{O}_2(3\Sigma_g^-) \] (14)

\[ \text{O}_2(1\Delta_g^-) + \text{Q} \rightarrow \text{O}_2(3\Sigma_g^-) + \text{Q} \] (15) rate constant \( k_q \)

\[ \text{O}_2(1\Delta_g^-) + \text{Q} \rightarrow \text{P}(16) \] (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.

**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 (I₀) and in the presence (I) of different AsA concentrations (Fig. 2), the classical Stern-Volmer treatment (I/I₀ = 1 + kq[AsA]), with kq = kSV/τo, allows the determination of the Stern-Volmer constant (kSV = 12.5 M⁻¹), kq being the rate constant for process (2) and τo = 5.2 ns, the reported value for the fluorescence lifetime of 1Rf* [24]. Results are shown in Fig. 2, from which a kq value of 7.2 x 10⁹ 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 1Rf*, in the range of 5%. Hence, the participation of 1Rf* in the photodegradation of AsA must be disregarded under work conditions.

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

Neither 1Rf* nor 3Rf* 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 NaN₃, 1 μg/ml CAT, 1 μg/ml SOD and 10 mM Mann in air-equilibrated pH 7 aqueous solution of Rf (A₄46 = 0.4) and AsA 0.5 mM decreases the OUR upon photoirradiation (λirr > 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 O₂ (Δg), H₂O₂, O₂• and OH• in a given oxidative event [28, 29, 30]. The enzyme SOD dismutates the species O₂• (reaction (17)), whereas CAT decomposes H₂O₂ (reaction (18)),

![Fig. 2. Stern-Volmer plots for the quenching of riboflavin stationary fluorescence by AsA in pH 7 aqueous solution (●) and for the quenching of O2(Δg) phosphorescence by AsA in pH 7 D2O solution (●). I₀ and I f represent the stationary fluorescence intensities of riboflavin in the absence and in the presence of AsA and τ represents the O2(Δg) phosphorescence lifetime in the presence of different AsA concentrations.](image-url)
indicates, in principle, the participation of $O_2(1\Delta g)$, $H_2O_2$, $O_2^•$ and $OH^•$ 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, $^3Rf^•$ is efficiently quenched by AsA [11]. The species $RfH^•$ 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 $RfH^•$ and $O_2^•^−$, and, eventually, $Rf$ and $H_2O_2$ (process (8)) [31, 32]. Finally, the species $OH^•$ 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]),

Table 1. Rate constants for overall quenching ($k_i$) and reactive quenching ($k_{app}$ for AsA and $k_r$ for trp) of $O_2(1\Delta g)$ phosphorescence; $k_i/k_i$ ratios and rate constants for the quenching of $^3Rf^•$ ($k_{q1}$) and $^3Rf^•$ ($k_{q3}$).

<table>
<thead>
<tr>
<th>Compound</th>
<th>pH/pD</th>
<th>$k_i/10^8$ (M⁻¹s⁻¹)</th>
<th>$k_r/10^8$ (M⁻¹s⁻¹)</th>
<th>$k_{q1}/10^9$ (M⁻¹s⁻¹)</th>
<th>$k_{q3}/10^9$ (M⁻¹s⁻¹)</th>
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</thead>
<tbody>
<tr>
<td>AsA</td>
<td>pH 7</td>
<td>1.42 (a)</td>
<td>1.11 (c)</td>
<td>0.78</td>
<td>7.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.60 (b)</td>
<td></td>
<td></td>
<td>10 (d)</td>
</tr>
<tr>
<td>Trp</td>
<td>pH 7</td>
<td>0.72 (e)</td>
<td>0.47(e)</td>
<td>0.65</td>
<td>2.5 (f)</td>
</tr>
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(a) pD 7; (b) from ref. [25]; (c) $k_{reff}$ instead of $k_i$; (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 photoradiation: 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 NaN₃ 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.

NaN₃ deactivate $O_2(1\Delta g)$ with NaN₃ instead of AsA in reaction (15), and Mann deactivates the species $OH^•$ (reaction (19)).

$2O_2^•^− + 2H^+ + SOD \rightarrow O_2(3\Sigma_g^−) + H_2O_2$ (17)

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

$OH^• + Mann \rightarrow$ deactivation (19)

The observed delay in the rates of oxygen uptake in the presence of the selective ROS interceptors indicates, in principle, the participation of $O_2(1\Delta g)$, $H_2O_2$, $O_2^•$ and $OH^•$ 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.

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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 O2^- 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 O2(^1Δg) generation (process (13)). Considering the k_{ET} value of process (13) in H2O (1.2×10^9 M^-1 s^-1, i.e. 1/9 of the diffusional value) [36], and the reported value of 3{k_o} (process (4)) 1×10^{10} M^-1 s^-1 (Table 1), it can be deduced that, for the same concentrations of AsA and dissolved O2(^1Σ^-), the rate for the generation of the initial O2^- precursor RhH^* (via RF^*, process (8)) is ca. ten times higher than the corresponding one for O2(^1Δg) generation (process (5)). This is a very interesting finding since, as already mentioned, the respective reaction steps for the generation of H2O2 and OH^* depend on the initial generation of the species O2^-.

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 O2^- 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_o (step (9) in Scheme 1) was reported at pH 7.4, employing O2^- generated by the xanthine-xanthine oxidase system [38]. Regarding H2O2, 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 H2O2 to the same species, threonic acid [6].

In reference to the interaction AsA-OH^*, several works describe the in vivo-suppression of OH^* 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 O2(^1Δg) by AsA**

When a solution of the well-known exclusive O2(^1Δg) generators PN (A400 = 0.3) or RB (A530 = 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 O2(^1Δg)-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 O2(^1Δg), avoiding potential interferences due to interactions of the substrates with RF electronically excited states and eventually with other ROS different from O2(^1Δg).

In the TRPD experiments, the decay kinetics of O2(^1Δg) 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 O2(^1Δg) lifetime, unambiguously confirming the interaction of the vitamin with the oxidative species. The k_i value, graphically obtained, as shown in Fig. 2, was 1.42 x 10^8 M^-1 s^-1 at pH 7, employing RB as a dye-sensitizer. Rougée et al. [25] reported a k_i value of 1.6 x 10^8 M^-1 s^-1 at pH 7 which is very close to the value determined here at pH 7. The expression 1/τ_o = 1/τ_o + k_i [AsA] was employed, where τ_o and τ_o are the O2(^1Δg) 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 (A530 = 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 photolrradiation of a solution of RB (A530 = 0.4) + AsA 0.5 mM in an
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 $O_2(1\Delta_g)$, $O_2^-$, H$_2$O$_2$ and $O_2^-$. 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 $k_q$ of $2.5 \times 10^9$ M$^{-1}$ s$^{-1}$ [27].

Oxidized radicals of the AAs and the reduced flavin radicals (FlH •) 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].

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The interaction Trp with $O_2(1\Delta_g)$ has been profusely studied [26, 44, 6, 15]. Rate constant values $k_t = 7.2 \times 10^7$ M$^{-1}$s$^{-1}$ and $k_r = 4.7 \times 10^7$ 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 mechanically 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 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 $4.5 \times 10^8$ 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 $k_{reff} = 1.11 \times 10^8$ M$^{-1}$s$^{-1}$ (reaction 16, with $k_{reff}$ instead of $k_t$).

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.

![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).](image-url)
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 parallel, oxygen consumption was observed in pH 7 aerated aqueous solutions of 0.04 mM Rf (A_{445} = 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.
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 $k_e/k_t$ 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 occurs 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 photodegradation 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 $^3$Rf 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.

REFERENCES