NRC Research Press

ARTICLE

Mutual effects between aromatic amino acids and guanosine upon vitamin B2 photosensitization in the presence of visible light

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Abstract: Considering the importance of the visible-light-induced photodynamic effect in complex bioenvironments, mutual effects between the individual aromatic amino acids (AAs) tyrosine (Tyr), tryptophan (Trp), and histidine (His) and the nucleoside guanosine (GUO) were investigated in pH 7 aqueous solution with vitamin B2 (riboflavin (Rf)) as a dye sensitizer. The quantum yields of oxygen uptake (Φ_{-O2}) for most of the AA–GUO mixtures studied, taken as a measure of overall photooxidation susceptibility, are not straightforwardly predictable from the individual behaviour of the components of the mixture. The final result depends on several connected factors, such as the respective abilities of the substrates as quenchers of the long-lived Rf triplet excited state and the generated reactive oxygen species singlet molecular oxygen ($O_2(^1\Delta_g)$) and superoxide radical anion ($O_2^{\bullet-}$). A mechanistic interpretation of the Rf-sensitized results can be roughly resumed as follows: Tyr at pH 7 exerts a protective effect on the photooxidation of the mixture Tyr–GUO due to the $O_2(^1\Delta_g)$ physical quenching by the AA. The same effect was observed for Trp–GUO and His–GUO at pH 7. In these cases, it is attributed to the quenching of $^3Rf^*$ by GUO in detriment of the Type II route. For the system Tyr–GUO at pH 9, a marked decrease in the Φ_{-O2} occurred for the mixture as compared with the respective Φ_{-O2} for the individual components. It was ascribed to the participation of a radical-mediated mechanism without oxygen consumption in a competitive pathway with the $O_2^{\bullet-}$-mediated route.

Key words: amino acids, guanosine, riboflavin, singlet molecular oxygen, superoxide radical.

Résumé : Étant donné l'importance de l'effet photodynamique induit par la lumière visible dans les milieux biologiques complexes, on a étudié les effets réciproques entre les acides aminés aromatiques (AA) tyrosine (Tyr), tryptophane (Trp) et histidine (His) individuels et le nucléoside guanosine (GUO) en solution aqueuse à pH 7 en présence de vitamine B2 (riboflavine (Rf)) comme colorant sensibilisateur. Pour la plupart des mélanges AA–GUO étudiés, le rendement quantique de la consommation d'oxygène (Φ_{-O2}), pris comme mesure de la sensibilité globale à la photooxydation, ne peut pas être prédit simplement d'après le comportement individuel des constituants du mélange. Le résultat final dépend de plusieurs facteurs reliés, dont la capacité respective des substrats à désactiver l'état excité triplet à vie longue de la Rf et les espèces réactives d'oxygène formées, à savoir l'oxygène moléculaire singulet ($O_2(^1\Delta_g)$) et l'anion radical superoxyde ($O_2^{\bullet-}$). Une interprétation mécaniste des résultats en présence de la Rf comme sensibilisateur se résume en gros comme il suit : à pH 7, la Tyr exerce un effet protecteur contre la photooxydation du mélange Tyr–GUO en raison de la désactivation physique de $O_2(^1\Delta_g)$ par l'AA. Le même effet a été observé pour Trp–GUO et His–GUO à pH 7. Dans ces cas, il est attribué à la désactivation de $^3Rf^*$ par la GUO au détriment de la voie de type II. Pour le système Tyr–GUO à pH 9, on observe une diminution marquée du Φ_{-O2} du mélange comparativement aux Φ_{-O2} respectifs des constituants individuels. Cette diminution a été attribuée à la participation du mécanisme avec médiation par un radical sans consommation d'oxygène à un chemin en compétition avec la voie ayant $O_2^{\bullet-}$ pour médiateur. [Traduit par la Rédaction]

Mots-clés : acides aminés, guanosine, riboflavine, oxygène moléculaire singulet, radical superoxide.

Introduction

Nucleic acids and proteins occupy common environments in cells and their mutual interactions in the presence oxidative agents may be the cause of a number of pathological processes.[1] The oxidative damage plays a crucial role in many diseases, and DNA–protein cross-linking is one important consequence of such damage. In particular, the photodynamic effect, mediated by endogenous photosensitizers, could lead to the formation of a wide spectrum of DNA modifications and DNA–protein interactions.[2,3,4]

Oxidative stress on DNA and RNA, including the employment of nucleotides and nucleosides as model compounds, has been recently reviewed.[3] The studies mainly include biological damage via ionizing radiation, photoirradiation, and thermal oxidation. In particular, light-induced photooxidative processes by direct and sensitized photoirradiation have been the subject of extensive research, especially within the frame of repair mechanisms. Guanosine (GUO) related compounds are by-products of DNA oxidation in cells, and these residues are susceptible to additional attack by one-electron oxidants and reactive oxygen species (ROS) such as singlet molecular oxygen $O_2(^1\Delta_g)$ and the superoxide radical anion ($O_2^{\bullet-}$).[5] In this context, the covalent photodynamic coupling of GUO and guanosine monophosphate to proteins is well known.[6]

On the other hand, peptides and proteins are recognized major targets in photodynamic processes through the degradation of the side chains of the five oxidizable amino acids histidine (His), tryptophan (Trp), tyrosine (Tyr), methionine, and cysteine.[7,8,9]

Although visible light is not highly aggressive for most biological assemblies upon direct irradiation, it is easily transmitted to dermis and basal cells. Vitamin B2 (riboflavin (Rf)), present in free

Received 28 June 2012. Accepted 17 August 2012.

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and combined forms in the human organism, constitutes one of the most important visible-light-absorbing endogenous photosensitizers. It is involved in the photooxidation of residues of DNA and proteins, despite the fact that Rf itself could be photodegraded.[10,11] When Rf is photoirradiated, especially in the presence of electron donors, ROS and reactive radicals are formed.[7,8,9] In a biological environment, closer molecules constitute the primary focus for the attack of such a reactive species. The chemical nature, local concentration of the oxidant and target molecules, and oxygen availability constitute the most important factors in determining the efficiency of the process and mechanistic aspects of the degradative events. In this context, we chose for the present study GUO as an oxidizable DNA residue and the aromatic amino acids (AAs) His, Trp, and Tyr as oxidizable protein residues in the presence of Rf as an endogenous ROS photogenerator.[3,6] These AAs were selected because they interact with the excited triplet Rf (^{3}Rf*) with rate constants quite different from each other and their mechanisms of interaction with $O_2(^1\Delta_g)$ are varied: physical plus chemical (reactive) quenching for Trp, only physical quenching for Tyr at pH 7, turning to physical plus chemical quenching at pH 9, and finally only chemical quenching for His.[7,8,9]

The assembly Rf plus AAs plus GUO can be considered a model system that acceptably mimics a natural bioscenery. The work deals with the interaction of GUO with photochemically generated ROS in the presence and in the absence of the individual AAs. Relative quantum yields for oxygen uptake (Φ_{-O2}) upon Rf photosensitization were taken as a measure of overall photooxidation susceptibility by the components of the aqueous mixtures. Kinetic information provides a meaningful insight into the feasibility of the involved mechanisms accounting for mutual interactions observed between the oxidizable substrates, the electronically excited photosensitizer, and the photogenerated ROS. This knowledge, connecting candidates to biologically significant interactions, could contribute to a deeper understanding of the potential reactivity or protection of DNA/RNA residues towards photopromoted oxidation and their proclivity to cross-linkage reactions with AAs molecules in the close surroundings.

Experimental

Materials

Rf, 99.9% deuterium oxide (D_2O), L-tryptophan, L-tyrosine, L-histidine, and superoxide dismutase (SOD) from bovine erythrocytes were purchased from Sigma Chemical Co. (St. Louis, Missouri). Rose bengal (RB) and furfuryl alcohol were from Aldrich (Milwaukee, Wisconsin). All of these chemicals were used as received. Water was triply distilled and benzene was from Sintorgan (Buenos Aires, Argentina) HPLC quality. All measurements were carried out at room temperature and with freshly prepared solutions. Buffered aqueous solutions were prepared, with 0.025 mol L^{-1} KH_2PO_4 – 0.025 mol L^{-1} Na_2HPO_4 (pH 7) and 0.01 mol L^{-1} $Na_2B_4O_7{\cdot}10H_2O$ (pH 9).[12]

Methods

All stationary photolysis was carried out at wavelengths >400 nm (cutoff filter) employing a homemade photolyzer provided with a 150 W quartz halogen lamp. Ground-state absorption spectra were registered employing a Hewlett-Packard 8452A diode array spectrophotometer. The total quenching rate constant of deactivation of the $O_2(^1\Delta_g)$ by GUO (k_t) (see reactions r6 and r7, being Q = GUO) was determined using a system previously reported.[13] Briefly, it consisted of a Nd:Yag laser (Spectron) as the excitation source. The frequency tripled output (355 nm) was used to excite Rf. The emitted ($O_2(^1\Delta_g)$) phosphorescence at 1270 nm was detected at right angles using an amplified Judson J16/8Sp Germanium detector, after having passed through the appropriate filters. The output of the detector was coupled to a digital oscilloscope and to a personal computer to carry out the signal processing. Usually, 10 shots were needed for averaging so as to achieve a good signal to noise ratio from which the decay curve was obtained. Air-equilibrated solutions were employed in all cases.

The concentration of the sensitizer (RB) was approximately 0.02 mmol L^{-1}. D_2O was used in the dynamic determinations instead of H_2O as solvent to enlarge the lifetime of $O_2(^1\Delta_g)$.[14] For the determination of k_t (reactions r6 and r7), the $O_2(^1\Delta_g)$ lifetimes were evaluated in the presence (τ) and in the absence (τ_0) of the quencher and the data were plotted according to a Stern–Volmer treatment:

$$(1)\qquad 1/\tau = 1/\tau_0 + k_t[\mathrm{GUO}]1/y$$

The reactive rate constant k_r for the reaction of GUO with $O_2(^1\Delta_g)$ (reaction r7) was determined using the method described by Scully and Hoigné[15] for which the knowledge of the reactive rate constant for the photooxidation of a reference compound R is required:

$$(2)\qquad \mathrm{Slope/slope_R} = k_r[\mathrm{GUO}]/k_{rR}[\mathrm{R}]$$

where slope and $\mathrm{slope_R}$ are the respective slopes of their first-order plots of oxygen consumption by GUO and by a reference compound under photoirradiation with visible light in the presence of dye sensitizers. Assuming that the reaction of $O_2(^1\Delta_g)$ with the quencher is the only possible pathway of oxygen consumption, the ratio of the first-order slopes of oxygen uptake by the substrate and the reference compound, each at the same concentration (slope substrate/slope reference), yields k_r/k_{rR}. Equation 2 is strictly valid when the stoichiometry substrate–$O_2(^1\Delta_g)$ is 1:1. For this reason, we call in the following the reactive rate constant k_r the *apparent* reactive rate constant (k_{rApp}), which represents an upper limit for k_r. The reference was furfuryl alcohol, with a reported pH-independent k_{rR} value of 1.2×10^8 mol L^{-1} s^{-1}.[16] RB was used as a dye sensitizer in the determination of k_r.

The quantum yields of oxygen uptake by GUO, Trp, Tyr, and His and by the respective mixtures upon Rf and RB sensitization were determined by evaluation of the initial slopes of oxygen consumption as a function of the irradiation time, employing the specific oxygen electrodes Orion 97-08 and Orion 810+.

Stationary fluorescence was measured with a Spex Fluoromax spectrofluorometer at 25 ± 1 °C in air-equilibrated solutions. For the determination of ^1k_q, accounting for the interaction of ^{1}Rf* with GUO, a classical Stern–Volmer treatment of the data was applied through eq. 3 where I and I_0 are the respective intensities of Rf fluorescence in the presence and in the absence of GUO and $^1\tau_0 = 5.2$ is the reported lifetime ^{1}Rf*:[17]

$$(3)\qquad I_0/I = 1 + K_{SV}[\mathrm{GUO}] \text{ with } K_{SV} = {}^1k_q\,{}^1\tau_0$$

Argon-saturated 0.04 mmol L^{-1} Rf aqueous solutions were photolyzed using a Laser Flash Photolysis apparatus. A Nd:YAG laser system (Spectron) at 355 nm was the excitation source, employing a 150 W Xenon lamp as the analyzing light. The detection system comprised a PTI monochromator and a red-extended photomultiplier (Hamamatsu R666). The signal, acquired and averaged by a digital oscilloscope (Hewlett-Packard 54504A), was transferred to a PC via a HPIB parallel interface where it was analyzed and stored. ^{3}Rf* was generated by a 355 nm laser pulse and the ^{3}Rf* disappearance was monitored from the first-order decay of the absorbance at 670 nm, a zone where interference from other possible species is negligible. To avoid self-quenching and triplet–triplet annihilation, the triplet decay was measured at low Rf concentration (typical 0.05 mmol L^{-1}) and at low enough laser energy.

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For the determination of the rate constant for the ^{3}Rf*–GUO interaction (reaction r4), the Stern–Volmer expression was employed:

$$(4) \qquad 1/^3\tau = (1/^3\tau_0) + {}^3k_q[\mathrm{GUO}]$$

where $^3\tau_0$ and $^3\tau$ are the experimentally determined lifetimes of ^{3}Rf* in the absence and in the presence of GUO, respectively.

Results and discussion

Main reaction scheme

The usual mechanism of action of Rf as a photosensitizer is rather complex, in many cases with the concurrent involvement of ROS.[18,19] In the presence of an interacting electron donor (Q), the mentioned mechanism could be summarized as follows:

(r1) $\mathrm{Rf} + h\nu \rightarrow {}^1\mathrm{Rf}^* \rightarrow {}^3\mathrm{Rf}^*$

(r2) ${}^3\mathrm{Rf}^* + \mathrm{O}_2({}^3\Sigma_g^-) \rightarrow \mathrm{Rf}^{\bullet+} + \mathrm{O}_2^{\bullet-}$

(r3) ${}^3\mathrm{Rf}^* + \mathrm{O}_2({}^3\Sigma_g^-) \rightarrow \mathrm{Rf} + \mathrm{O}_2({}^1\Delta_g)$

(r4) ${}^3\mathrm{Rf}^* + \mathrm{Q} \rightarrow \mathrm{Rf}^{\bullet-} + \mathrm{Q}^{\bullet+}$ rate constant ${}^3k_q$

(r5) $\mathrm{O}_2^{\bullet-} + \mathrm{Q} \rightarrow \mathrm{Products}_5$

(r6) $\mathrm{O}_2({}^1\Delta_g) + \mathrm{Q} \rightarrow \mathrm{O}_2({}^3\Sigma_g^-) + \mathrm{Q}$ rate constant k_q

(r7) $\mathrm{O}_2({}^1\Delta_g) + \mathrm{Q} \rightarrow \mathrm{Products}_7$, rate constant k_r

where $k_t = k_r + k_q$.

Upon light absorption, the Rf excited singlet state (^{1}Rf*) is generated. It can decay to the ground state or, through an intersystem crossing process, it can produce the excited triplet Rf (^{3}Rf*) (step r1). The last species can also decay to ground-state Rf or can be quenched by ground-state oxygen dissolved in the solution ($O_2(^3\Sigma_g^-)$), generating ROS. The superoxide radical anion ($O_2^{\bullet-}$) (reaction r2) and singlet molecular oxygen ($O_2(^1\Delta_g)$) (reaction r3) are produced by electron and energy transfer with quantum yields of 0.009 and 0.49, respectively.[18] Besides, the transient species ^{1}Rf* (reaction not included) and ^{3}Rf* can also interact with Q (reaction r4).

The species $O_2(^1\Delta_g)$ and $O_2^{\bullet-}$ can react with Q, producing the physical deactivation of $O_2(^1\Delta_g)$ or oxidized products through reactions r5 and r7.

Rf and RB photosensitization and oxygen uptake by GUO–AAs mixtures

The Rf-sensitized photoirradiation of the mixture His plus GUO in pH 7 water produced spectral changes that can be attributed to transformations in both substrates of the mixture (see Fig. 1). In the insets of the Fig. 1 are shown the respective spectral changes operated on His and GUO upon photoirradiation in the presence of Rf. Similar qualitative changes were observed for Trp at pH 7, for Tyr at pH 9, and for their respective mixtures with GUO.

In parallel, when pH 7 aerated aqueous solutions of 0.04 mmol L^{-1} Rf plus 0.5 mmol L^{-1} GUO in the absence and in the individual presence of 0.5 mmol L^{-1} Trp, Tyr, and His were irradiated with visible light, oxygen consumption was observed. See Fig. 2 for a representative case. The same was true when solutions of the isolated 0.5 mmol L^{-1} AAs Trp and Tyr plus 0.04 mmol L^{-1} Rf were irradiated. The respective Φ_{-O2} values are shown in Table 1. The Φ_{-O2} value for Rf alone is negligible in relative terms within typical irradiation times employed in the presence of GUO and (or) the AAs and was omitted in Table 1.

Fig. 1. Spectral evolution of a photoirradiated pH 7 aqueous solution of 0.04 mmol L^{-1} Rf in the presence of 0.03 mmol L^{-1} GUO plus 0.34 mmol L^{-1} His (main figure), 0.017 mM GUO (upper inset), and 0.17 mmol L^{-1} His (lower inset). Cutoff >400 nm. For all samples, the photoirradidtion time was 0, 390, 940, and 1580 s.

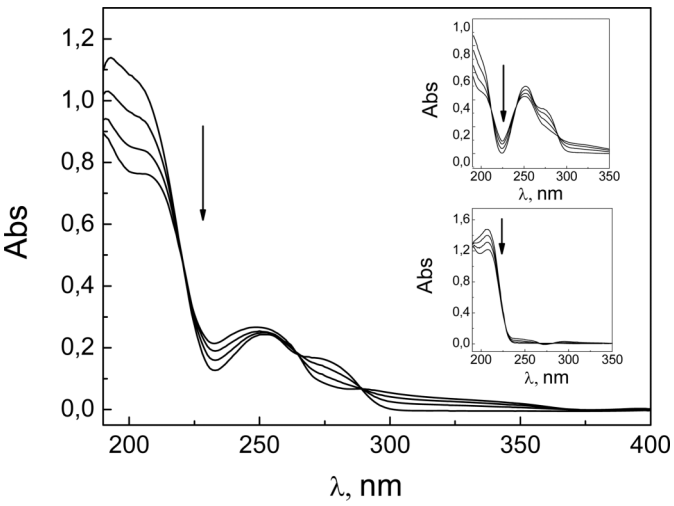

Fig. 2. Oxygen uptake as a function of photoirradiation time in a pH 7 aqueous solution of 0.04 mmol L^{-1} Rf in the presence of 0.5 mmol L^{-1} GUO (squares), 0.5 mmol L^{-1} His (triangles), and 0.5 mmol L^{-1} GUO plus 0.5 mmol L^{-1} His (circles). Cutoff >400 nm.

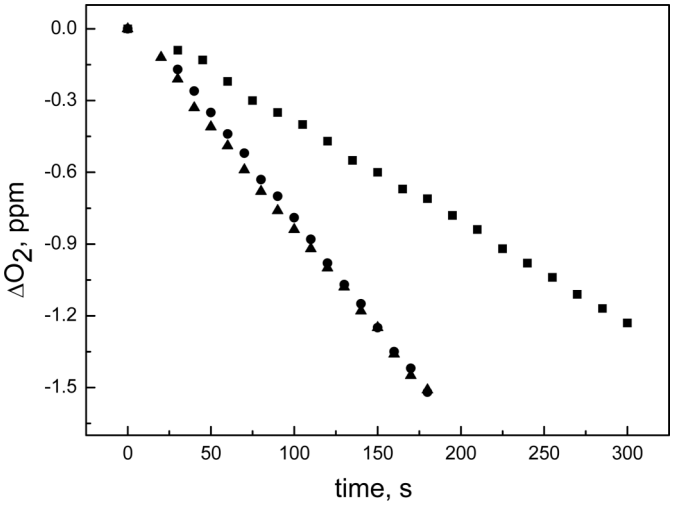

Table 1. Relative quantum yields for oxygen uptake (Φ_{-O2}) by GUO, Tyr, Trp, and His, all 0.5 mmol L^{-1}, and for the mixtures GUO plus the individual amino acids upon photoirradiation with visible light in the presence of the sensitizers riboflavin and rose bengal.

	Φ_{-O2}						
Sensitizer, pH	GUO	Tyr	GUO + Tyr	Trp	GUO + Trp	His	GUO + His
Riboflavin, pH 7	1	2.1	2.1	5	4.2	2.1	2.1
Rose bengal, pH 7	1	0.09	0.71	2.7	3.4	6.4	7.1
Riboflavin, pH 9	1	1.5	1.5				
Rose bengal, pH 9	1	2.1	2.3				

Note: All quantum yield values for each amino acid and its mixtures were normalized to the respective value of GUO.

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Due to the known dependence of the kinetics of Tyr photooxidation[20] and GUO thermal oxidation[21] on the pH of the medium, experiments of photopromoted oxygen consumption at pH 9 were also performed for Tyr, GUO and Tyr + GUO. The pK values of 10.1 and 9.2 for Tyr and GUO respectively,[9,22] indicate that a considerable fraction of their anionic species are present at pH 9. The Φ_{-O2} values are shown in Table 1.

Since RB produces $O_2(^1\Delta_g)$ in a dominant fashion under aerobic visible light irradiation,[23] similar photolysis experiments to those performed with Rf were done, for comparative purposes, replacing the vitamin by the xanthene dye sensitizer (A(549) = 0.52 for RB) and keeping constant the remaining experimental conditions.

H. Görner in 2008 reported the $O_2^{\bullet-}/H_2O_2$ generation by RB in the presence of oxygen and several electron donors including the AAs Tyr and Trp.[24] The photoinduced formation of the ROS is initiated by quenching of the triplet state of the dye (^{3}RB*) by the donor and subsequent reactions of both the dye and the donor radicals with oxygen. The rate constant reported by the author[24] for the mentioned quenching process is relatively low (2×10^7 mol L^{-1} s^{-1} for NaTyr). Hence, the generation of H_2O_2 through this pathway, in the experimental conditions of our work, would unfavourably compete with the production of $O_2(^1\Delta_g)$ by energy transfer from the triplet excited dye to dissolved oxygen. Under aerobic conditions operates a competence between $O_2(^3\Sigma_g^-)$ and Tyr towards ^{3}RB* (process r3 and process r4 of the scheme, with RB instead of Rf and Tyr instead of Q in the latter). Employing the same concentrations of Tyr and dissolved $O_2(^3\Sigma_g^-)$ (0.4–0.5 mmol L^{-1} under our work conditions) and a k_{ET} value of 7×10^8 mol L^{-1} s^{-1} in water[25] (accounting for process r3), the rate of $O_2(^1\Delta_g)$ production should be considerably higher than the corresponding one for the quenching of ^{3}RB* by Tyr.

The Φ_{-O2} values here reported represent the mean value of a set of four runs under identical conditions. All rate values of the set did not differ more than 3% from each other. Standard deviations for the individual runs were lower than 1%. Nevertheless, we assigned 3% as the error bar for the relative rates in Table 1, a more realistic estimation that assists in the interpretation of the actual magnitude of the observed effects.

It can be seen that in most of the cases, employing either Rf or RB as dye sensitizers, the Φ_{-O2} for the AAs–GUO mixtures do not constitute the simple addition of the individual values. It arises that the individual contribution of the mixture components to the overall oxidative mechanism may be affected either by the interaction of the initial by-products generated upon photoirradiation or by interactions between ROS produced by the sensitizer in the presence of the oxidizable substrates. To elucidate the mechanistic aspects of the involved processes, the first step was the evaluation of the photochemical behaviour of the individual components of the mixtures under work conditions.

The existing literature data on GUO, His, Tyr, and Trp photooxidation

The photodynamic effect on the AAs Trp, Tyr, and His employing Rf as a sensitizer is well known.[26,27,28] The AAs Tyr and Trp are oxidized by $O_2(^1\Delta_g)$ and $O_2^{\bullet-}$ produced in the photosensitization process. It is well established that Trp, Tyr, and His quench triplet flavin sensitizers with the pH-independent rate constant ^3k_q values (reaction r4 with AA instead of Q) of 2.5×10^9, 1×10^9, and 4.5×10^7 mol L^{-1} s^{-1}, respectively.[29] For Trp and Tyr, the primary products are the reduced flavin radicals (FlH$^\bullet$) and oxidized radicals of the AAs.[17] One of the last remarkable studies in the area reports on the photolysis of several flavins in air-saturated aqueous solution in the presence of electron donors, including aromatic AAs.[28] The overall reaction observed was conversion of oxygen via the hydroperoxyl/superoxide radical.

Type I (radical mediated) and especially Type II ($O_2(^1\Delta_g)$ mediated) are the main reported mechanisms responsible for Rf-photosensitized degradation of Trp and Tyr, whereas an exclusive Type II mechanism operates in the case of His.[19,30]

The interaction of His, Tyr, and Trp with $O_2(^1\Delta_g)$ has been profusely reported.[31,6,15] The imidazole-derived AA quenches the oxidative species in an exclusive chemical fashion, with a reported rate constant $k_t = k_r = 9 \times 10^7$ mol L^{-1} s^{-1}.[21] The values for the overall rate constants k_t for Tyr at pH 7 and pH 10 are 1.5×10^7 and 2×10^8 mol L^{-1} s^{-1}, respectively. The AA is practically unreactive at pH 7 ($k_r < 10^4$ mol L^{-1} s^{-1}) and reacts with a rate constant $k_r = 3.8 \times 10^7$ mol L^{-1} s^{-1} at pH 10.[9] Finally, the reported rate constant values for Trp at pH 7 are $k_t = 7.2 \times 10^7$ mol L^{-1} s^{-1} and $k_r = 4.7 \times 10^7$ mol L^{-1} s^{-1}.[9]

Regarding GUO, although a number of papers have been published on the Rf- and RB-photosensitized oxidation of GUO-related compounds, most of the investigation was done on GUO derivatives such as deoxyguanosine (dGUO), 2′-deoxyguanosine -5′-monophosphate (dGUOMP), 8-oxo-7,8-dihydroguanosine, and guanosine derivatives with improved solubility in organic solvents.[11,32,33,34,35] According to our knowledge, the only value on rate constants k_t for the interaction of GUO with $O_2(^1\Delta_g)$ in water was established to be 5.3×10^6 mol L^{-1} s^{-1}.[36,37]

Joshi and Keane published a detailed study on the Rf- and RB-sensitized photodegradation of DNA- and RNA-related purine and pyrimidine derivatives.[11] The rates of photodegradation of GUO and dGUO among other biological guanine-related compounds were compared under UVA and UVB light exposure at pH 10. Only derivatives contaninig GUO or dGUO moieties were degradable under both irradiation conditions.

The interaction of electronically excited states of Rf with GUO: generation of $O_2(^1\Delta_g)$ and $O_2^{\bullet-}$

On the basis of the data in the preceding paragraphs and to gain insight into the involved kinetic and mechanistic aspects, we carried out a study of the photopromoted interactions between GUO, Rf, and the ROS generated by the visible light irradiation of Rf and RB, in pH 7 and pH 9 aqueous solutions, as follows.

These results strongly suggest that either Rf electronically excited states, ROS produced through these states, or both mechanistic steps simultaneously operating are responsible for the photodegradation of GUO. Following, these possibilities were investigated.

The potential interaction ^{1}Rf*–GUO was evaluated by monitoring the intensity of the typical fluorescence emission band of Rf centered at 515 nm. It decreased in the presence of ≥2 mmol L^{-1} GUO but the shape of the emission spectrum did not change. The determined Stern–Volmer constant $^1K_{SV} = {}^1k_q \times {}^1\tau_o = 45.6$ allows the evaluation of the rate constant $^1k_q = 8.7 \times 10^9$ mol L^{-1} s^{-1}. These values indicate that a GUO concentration of approximately 0.5 mmol L^{-1}, similar to those employed in the Φ_{-O2} determinations, would produce a diminution in the lifetime of ^{1}Rf* lower than 2%. Hence, under work conditions, all effects derived from the interaction of GUO with electronically excited Rf could be exclusively ascribed to an interaction with ^{3}Rf*.

The decay of ^{3}Rf* was measured at a much lower Rf concentration, typically 0.02 mmol L^{-1}, and at low enough laser energy to avoid self-quenching and triplet–triplet annihilation, respectively. The disappearance of ^{3}Rf*, generated by a 355 nm laser pulse, was monitored from the first-order decay of the absorbance at 670 nm, a wavelength where interference from other possible species is negligible. The lifetime of ^{3}Rf* appreciably decreased in the presence of a GUO. The rate constant $^3k_q = 9.9 \times 10^8$ mol L^{-1} s^{-1} accounting for process r4 was obtained (Fig. 3, inset).

In the absence of a GUO, a spectrum similar to the expected one for ^{3}Rf* was observed after the laser pulse (Fig. 3),[17,38,39] while in the presence of a GUO (1.2 m, approximately 95% ^{3}Rf* quenched by the GUO), the shape of the long-lived absorption is in good agreement with that reported for the semiquinone radical RfH$^\bullet$,[9,40] formed from the radical anion Rf$^{\bullet-}$ (process r8). A similar spectrum has been published by Lu et al.[32] for the system Rf plus

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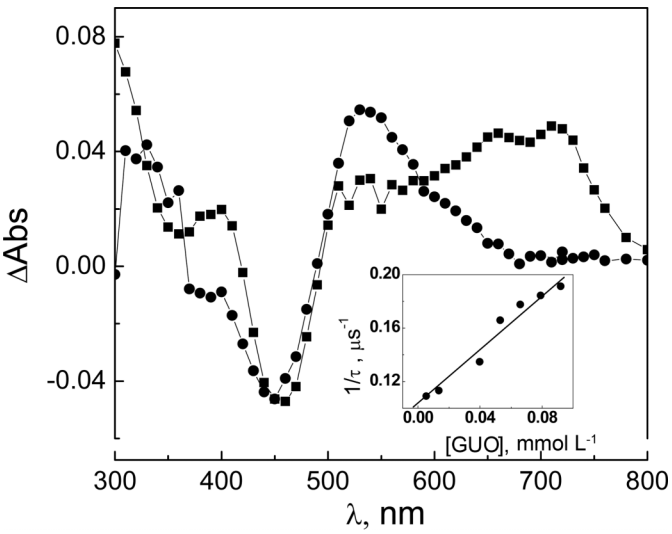
Fig. 3. Transient absorption spectra of 0.05 mmol L^{-1} Rf in an argon-saturated pH 7 aqueous solution in the absence (squares) and in the presence (circles) of 1 mmol L^{-1} GUO (Δ) (all 2 μs after the laser pulse). Inset: Stern–Volmer plot for the quenching of $^3Rf^*$ by GUO in pH 7 aqueous solution.



dGUOMP in aqueous solution, attributed to the main contribution of Rf reduced radical ($RfH^\bullet$) and oxidized dGUOMP radical to a very minor extent:

(r8) $Rf^{\bullet-} + H^+ \rightarrow RfH^\bullet$

The thermodynamic feasibility of the process can be evaluated by means of the Gibbs free energy for electron transfer, $\Delta_{ET} G_0 = E_{0(GUO/GUO^+)} - E_{0(Rf/Rf^-)} - E_{Rf^*} + C$, where $E_{0(Rf/Rf^-)}$ is the standard electrode potential of the acceptor Rf (–0.80 V), E_{Rf^*} is the $^3Rf^*$ energy (2.17 eV), C is the Coulombic energy term (–0.06 V), and $E_{0(GUO/GUO^+)}$ is the GUO standard electrode potential. We employed the literature value of 1,29 V versus NHE for $E_{0(GUO/GUO^+)}$ at pH 7.[41] The so-calculated $\Delta_{ET} G_0 = -0.14$ eV indicates that process r2 may occur and consequently that the species $RfH^\bullet$ could be spontaneously formed (process r8).

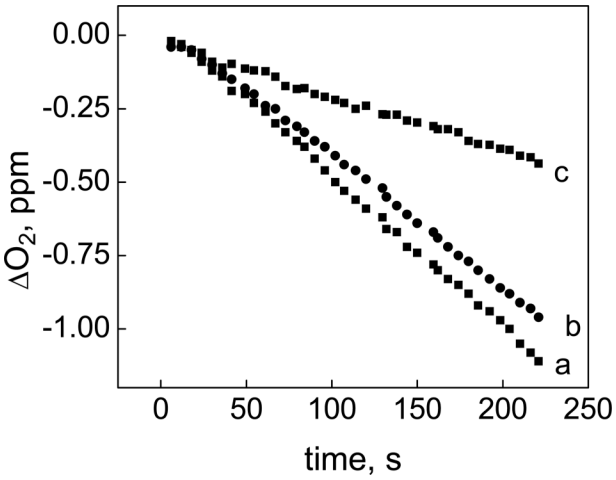
In principle, the interaction of $^3Rf^*$ with oxygen should be totally assigned to the $O_2(^1\Delta_g)$ generation pathway due to the reported extremely low quantum yield of direct $O_2^{\bullet-}$ production through reaction r7.[17] Nevertheless, as suggested by the flash photolysis results, the generation RfH^i must be included in the reaction scheme. The electron transfer processes represented by step r4 could constitute a considerable fraction of the overall process represented in the interaction between $^3Rf^*$ and GUO. In that case, although the species $O_2^{\bullet-}$ would not be straightforwardly produced from the species $Rf^{\bullet-}$, due to the rapid protonation (reaction r8), the oxygenated radical could be generated anyway through reaction r10.[42,40]

The species $RfH^\bullet$ has a different and much slower reaction path with $O_2(^3\Sigma_g^-)$ than $Rf^{\bullet-}$.[43] The bimolecular decay of $RfH^\bullet$ is known to proceed through a disproportionation reaction to yield equimolar Rf and fully reduced Rf (RfH_2) (process r9). In the presence of $O_2(^3\Sigma_g^-)$, the latter species is reoxidized[43,38] to give $O_2^{\bullet-}$ and Rf radical (process r10), which suffers a rapid deprotonation. The couple $RfH^\bullet/RfH_2^{\bullet+}$ hás a $pK_a = 2.3$.[44] Radical termination leads to Rf and RfH_2:[45]

(r9) $2RfH^\bullet \rightarrow Rf + RfH_2$

(r10) $RfH_2 + O_2(^3\Sigma_g^-) \rightarrow RfH_2^{\bullet+} + O_2^{\bullet-}$

Fig. 4. Oxygen uptake as a function of photoirradiation time in a pH 7 aqueous of 0.5 mmol L^{-1} GUO plus 0.04 mmol L^{-1} Rf in the absence (*a*) and in the presence of 1 μg mL^{-1} SOD (*b*) and 10 mmol L^{-1} NaN_3 (*c*).



The recovery of the pigment represents a crucial step in living organisms in which it is well known that $O_2^{\bullet-}$ is a key intermediate in the oxygen redox chemistry.[46]

The participation of ROS in the GUO degradation was evaluated through oxygen consumption experiments employing specific ROS quenchers. Thus, the individual presence of 10 mmol L^{-1} NaN_3 and 1 μg mL^{-1} of the enzyme SOD clearly affects the Φ_{-O2} values (Fig. 4). Similar experiments with these specific ROS quenchers have been formerly employed to confirm/discard the participation of $O_2(^1\Delta_g)$ and $O_2^{\bullet-}$, respectively, in a given oxidative event.[45,47] The salt physically quenches $O_2(^1\Delta_g)$ (process r6 with NaN_3 instead of Q) and SOD dismutates the species $O_2^{\bullet-}$ (process r11).

(r11) $2O_2^{\bullet-} + 2H^+ + SOD \rightarrow O_2(^3\Sigma_g^-) + H_2O_2$

The results confirm some degree of interaction of both ROS with GUO. Kinetic aspects of these interactions were quantified as follows.

As said, RB was chosen as a sensitizer to focalize on the potential reaction of GUO with $O_2(^1\Delta_g)$, avoiding possible interference due to interactions of the substrates with Rf electronically excited states. The decay kinetics of $O_2(^1\Delta_g)$ phosphorescence were first order and the lifetime agreed well with literature data.[29]

The interaction GUO–$O_2(^1\Delta_g)$

The addition of GUO as a quencher led to a decrease of the $O_2(^1\Delta_g)$ lifetime, unambiguously confirming the interaction of GUO with this oxidative species. The k_t values (Table 1) were graphically obtained in D_2O at pD 7 ($k_t = 1.6 \times 10^7$ mol L^{-1} s^{-1}) and pD 9 ($k_t = 4.8 \times 10^7$ mol L^{-1} s^{-1}) from TRPD measurements (Fig. 5). D_2O solutions of pD 7 and pD 9 were employed as solvents to evaluate the possible effects of pH on the rate constants of the interaction GUO–$O_2(^1\Delta_g)$.

The rate constant for process r7 was determined at pH 7 ($k_{rApp} = 1.5 \times 10^7$ mol L^{-1} s^{-1}) and pH 9 ($k_{rApp} = 3.6 \times 10^7$ mol L^{-1} s^{-1}) employing the already described actinometric method by monitoring oxygen photoconsumption (Fig. 5, inset). According to our knowledge, no reports on k_r values for GUO were previously published.

It is currently accepted that bimolecular reactions with $O_2(^1\Delta_g)$ can take place through a mechanism that involves an encounter complex.[20] Physical quenching results from the intersystem crossing to $O_2(^3\Sigma_g^-)$ within the complex. Sheu and Foote[48] studied a

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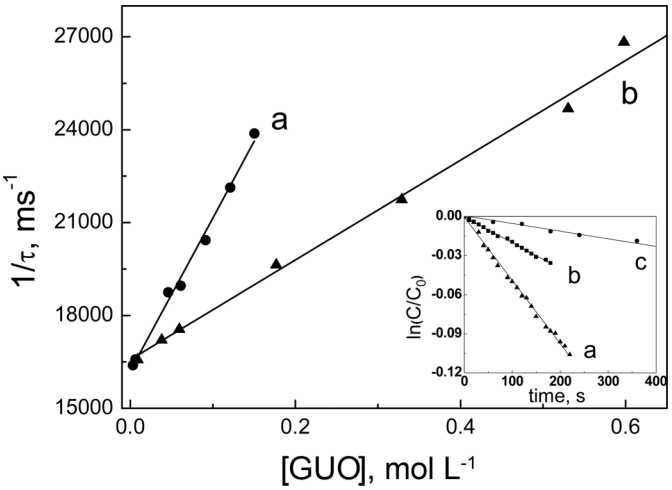
Fig. 5. Stern–Volmer plot for the quenching of $O_2(^1\Delta_g)$ by GUO in pD 9 D_2O (*a*) and in pD 7 D_2O (*b*). Inset: first-order plots of oxygen uptake upon visible light irradiation containing 0.04 mmol L^{-1} Rf plus 0.5 mmol L^{-1} furfuryl alcohol in pH 9 and pH 7 aqueous solution (*a*), 0.5 mmol L^{-1} GUO in pH 9 aqueous solution (*b*), and 0.5 mmol L^{-1} GUO in pH 7 aqueous solution (*c*).



series of synthetic 8-substituted GUO derivatives with improved solubility in organic media for which deactivation of $O_2(^1\Delta_g)$ occurs through the abovementioned general mechanism. The k_t values range 10^6–10^7 mol L^{-1} s^{-1}, depending on the presence of an electron donor or an electron-withdrawing substituent in the GUO structure. The interaction with $O_2(^1\Delta_g)$ is also highly dependent on the solvent polarity, whereas the ratio k_r/k_t in acetone-d_6 has a mean value of 0.25 for different GUO derivatives. In our case, assuming $k_r = k_{rApp}$ for aqueous GUO, the balance between physical quenching and chemical reaction, an event sensitive to spin-orbit coupling and entropy factors,[49] seems to be dominated by the reactive pathway, resulting in a practically exclusive chemical deactivation of $O_2(^1\Delta_g)$. Apparently, the high polarity of water as a solvent favours the reactive pathway within the encounter complex GUO–$O_2(^1\Delta_g)$. The pH dependency of the rate constant values k_r and k_t is also in agreement with a charge-transfer-driven encounter complex as an intermediary: the electron donor properties of GUO are highly increased in the anionic form of GUO.

The $O_2(^1\Delta_g)$-mediated photooxidation quantum efficiency Φ_r ($\Phi_r = k_r[GUO]/(k_d + k_t[GUO])$)[13] is not easy to evaluate, particularly in complex biological environments, because its determination includes the knowledge of the actual concentration of the photooxidizable substrates, represented by GUO in this case. A simpler and useful approach is the evaluation of the k_r/k_t ratio, which indicates the fraction of overall quenching of $O_2(^1\Delta_g)$ by the substrate that effectively leads to a chemical transformation. Results for GUO indicate a high contribution of the reactive channel: k_{rApp}/k_t ratios are 0.94 and 0.75 at pH 7 and pH 9 respectively.

Mechanistic aspects in the oxygen uptake by GUO–AAs mixtures

In summary, as estimated by oxygen uptake and UV absorption experiments, the photoreaction of GUO under Rf-sensitized photoirradiation should obey one or both of the following processes: oxidation due to electron transfer mechanism (reaction r9) or oxygenation through reactions r5 and (or) r7. Lee and Rodgers[33] reported an upper limit of 1×10^6 L^{-1} s^{-1} for the rate constant of reaction r5 (with dGUO instead of Q) at pH 7 and pH 10.5. This is practically coincident with the upper limit of 5×10^6 L^{-1} s^{-1} reported by Cadet and Teoule[50] for the reaction DNA + $O_2^{\bullet-}$. Our findings on the efffect of SOD in the Rf-sensitized photooxidaton of GUO is in agreement with published data by Luo et al.[34] on 8-oxo-7,8-dihydroguanosine. The authors reported a quenching effect of $O_2^{\bullet-}$ by SOD in the formation of imidazolone, the main photoproduct of GUO at pH 7, upon Rf photosensitization.

Despite the large number of competing photoprocesses in the system GUO plus AAs plus Rf that make complex the interpretation of a reaction mechanism, we will rationalize the experimental results of Rf-photosensitized oxygen uptake on the basis of a simple scheme. It includes quenching of $^3Rf^*$ by the AAs and GUO, production of $O_2(^1\Delta_g)$ and $O_2^{\bullet-}$, and interaction of the generated ROS with the oxidizable substrates.

GUO–Tyr mixture, pH 7

In spite that in the RB photosensitization there mainly operates a $O_2(^1\Delta_g)$-mediated process, results in Fig. 2 show that the overall Φ_{-O2} for the mixture GUO plus Tyr at pH 7 is lower than that expected for the simple addition of individual rates. This apparent discrepancy could be due to the fact that the phenolic AA physically quenches $O_2(^1\Delta_g)$ with a rate constant value ($k_t \sim k_q$ in this case) similar to that for the reactive rate constant k_{rApp} of GUO. The former represent a process that does not contribute to oxygen consumption. As a result, the lifetime of $O_2(^1\Delta_g)$ in the mixture solution, and concomitantly the rate of oxygen uptake, should be reduced due to the presence of Tyr as compared with the case of isolated GUO.

In the Rf sensitization, Tyr competes with GUO as a quencher of $^3Rf^*$. Both compounds possess similar ^3k_q values. Nevertheless, the Φ_{-O2} for isolated Tyr is practically twice that for the corresponding one for the nucleotide, reflecting a much more effective oxidative reaction of the AA with photogenerated ROS. The Φ_{-O2} for the mixture is the same as that for isolated Tyr, attributable again to the effect of physical quenching of $O_2(^1\Delta_g)$ exerted by Tyr, protecting the nucleotide against this oxidative species.

GUO–Trp mixture, pH 7

In the RB-sensitized process, the contribution of the GUO–Trp mixture to the overall Φ_{-O2} is close to the simple addition of the individual rates of the nucleoside and the AA (Fig. 1, inset). Both components of the mixture exhibit high k_r/k_t ratios, with chemical quenching of $O_2(^1\Delta_g)$ as a dominant source of oxygen uptake.

In the case of Rf sensitization, the Φ_{-O2} for GUO remains as a low fraction of the overall contribution by the mixture, but the rate for isolated Trp is slightly higher than that for the mixture. This effect could be due to the quenching of $^3Rf^*$ by GUO starting a Type I process. Whereas the Type II route is the dominant mechanism in the Rf-sensitized photooxidation of Trp,[51] the quenching of $^3Rf^*$ by GUO decreases the stationary concentration of $O_2(^1\Delta_g)$ with the concomitant reduction of the overall Φ_{-O2} by the mixture.

GUO–His mixture, pH 7

The contribution from the $O_2(^1\Delta_g)$-mediated step to overall oxygen uptake by the GUO–His mixture should be high in relative terms, as indicated by the value of approximately 1 for the ratio k_{rapp}/k_t and 1 for k_r/k_t for the GUO and the AA, respectively. The Φ_{-O2} for the RB–GUO–His mixture practically represents the addition of the corresponding Φ_{-O2} for the individual runs (Table 1).

The possible operation of a $O_2^{\bullet-}$-mediated mechanism in the Rf–His system, initiated by an electron transfer quenching from the AA to $^3Rf^*$, must be disregarded due to the relatively low rate constant for the quenching of the excited flavin by the AA.[26] The rate constant for the competitive quenching by oxygen) is approximately 2 orders of magnitude higher (reaction r4 versus reaction r3, with His instead of Q).

The value for Φ_{-O2} for the mixture in the Rf-sensitized system is somewhat lower than the expected one from the simple addition of the individual rates of GUO, and His could obey a decrease in the efficiency of $O_2(^1\Delta_g)$ generation due to relatively fast competitive quenching of $^3Rf^*$ by GUO, lowering the Φ_{-O2} by His.

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GUO–Tyr mixture, pH 9

Regarding the case of RB sensitization at pH 9, it can be seen that the rate for oxygen consumption by the mixture GUO plus Tyr is much lower than the addition of the respective individual rates. It is known that Tyr, in the alkaline pH range, is efficiently oxidized by $O_2(^1\Delta_g)$, producing unstable endoperoxides via [1,4]-cycloaddition.[52] The endoperoxides could generate radical intermediates, strong reactants that could favourably interact with GUO, without additional oxygen consumption, in a competitive pathway with the $O_2^{\bullet-}$ route (process r5). This argument for the additional radical mechanism has already been employed to explain a similar situation in the photooxidation of ascorbic acid in the presence of AAs.[52]

In the case of Rf sensitization, the situation is qualitatively similar to that described above. The rate constant values for the quenching of ^{3}Rf* by GUO and Tyr are practically the same, as are the respective rate constant values for the reactive pathway with $O_2(^1\Delta_g)$. On this basis, the marked decrease in Φ_{-O2} observed for the mixture should be attributed to the effect of the endoperoxide/radical mechanism already mentioned in the case of RB sensitization.

Conclusions

The observed parallelism in the behaviour of the overall rates of oxygen uptake by photoirradiated aqueous mixtures of GUO plus AAs upon RB and Rf sensitization strongly suggests that the mechanism seems to be highly dominated by the $O_2(^1\Delta_g)$ pathway. Nevertheless, this rate is not straightforwardly predictable from the individual behaviour of the isolated substrates. The final result is highly dependent on a number of connected factors, such as the respective abilities of the substrates as quenchers of both the long-lived Rf triplet excited state and the generated ROS. These findings could be of interest within the frame of photochemically induced interactions between protein and nucleic acids, which is the case of covalent addition of membrane AA residues to RNA/DNA molecules in a proteinaceous environment. The photochemically induced interactions of oxidizable residues of nucleic acids with oxidizable AAs must be individually described from the behaviour of the respective mixtures of both compounds.

Acknowledgements

Financial support from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Secretaría de Ciencia y Técnica of the Universidad Nacional de Río Cuarto (SECyT UNRC), and Secretaría de Ciencia y Técnica of the Universidad Nacional de San Luis (SECyT UNSL), all in Argentine, is grateful acknowledged.

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