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PAPER

The role of vitamin B6 as an antioxidant in the presence of vitamin B2-photogenerated reactive oxygen species. A kinetic and mechanistic study†

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We report on the photostability of a mixture of vitamins B6 and B2 (riboflavin, Rf) upon visible light irradiation and on the possible role of the vitamin B6 family (B6D) as deactivators of reactive oxygen species (ROS). The work is a systematic kinetic and mechanistic study under conditions in which only Rf absorbs photoirradiation. Pyridoxine, pyridoxal hydrochloride, pyridoxal phosphate and pyridoxamine dihydrochloride were studied as representative members of the vitamin B6 family. The visible light irradiation of dissolved Rf and B6D in pH 7.4 aqueous medium under aerobic conditions induces photoprocesses that mainly produce B6D degradation. The overall oxidative mechanism involves the participation of ROS. Photogenerated ^{3}Rf* is quenched either by oxygen, giving rise to $O_2(^1\Delta_g)$ by electronic energy transfer to dissolved ground state oxygen, or by B6D yielding, through an electron transfer process, the neutral radical RfH$^{\cdot}$, and $O_2^{\cdot-}$ in an subsequent step. B6D act as quenchers of $O_2(^1\Delta_g)$ and $O_2^{\cdot-}$, the former in a totally reactive event that also inhibits Rf photoconsumption. The common chromophoric moiety of B6D represented by 3-hydroxypyridine, constitutes an excellent model that mimics the kinetic behavior of the vitamin as an antioxidant towards Rf-generated ROS. The protein lysozyme, taken as an $O_2(^1\Delta_g)$-mediated oxidizable biological target, is photoprotected by B6D from Rf-sensitized photodegradation through the quenching of electronically excited triplet state of the pigment, in a process that competes with $O_2(^1\Delta_g)$ generation.

1. Introduction

Vitamins constitute a particular group of relevant molecules in living organisms, essential for sustaining normal physiological activity and healthy life quality. Pyridoxine and derivatives (B6D), a group of structurally related compounds known as vitamin B6, participate in many enzymatic reactions, and in recent years have become a focus of interest in the evaluation of reactions of amino acid metabolism.[1] The vitamin exists in six interconvertible forms: pyridoxine, pyridoxal, and pyridoxamine and their respective phosphate forms (see Scheme 1). All of them have multiple functions in the human metabolism, basically taking part in the vitamin B6-dependent enzymes on amino acids transformations, including transfer of the amino group, decarboxylation, racemization, and beta- or gamma-elimination or replacement.[1] Most vitamins, including the B6 family, are relatively labile and susceptible to certain chemical changes that may affect their specific roles.[2] Numerous reports have been published on the biological disorders promoted by a non adequate biological availability of pyridoxine. For instance, its deficiency was detected in both type 1 and type 2 diabetic patients.[3,4] In this context, the stability of vitamin B6, and particularly its degradation by different biological and artificial mechanisms, has been the subject of intense research work in recent decades. These mechanisms include ionizing irradiation,[5] enzymatic conversion, thermal degradation in the presence of an oxidant,[6] coupling of catalytic wet air oxidation and biological treatment,[7] and oxidation mediated by reactive oxygen species (ROS).[8–11]

Vitamins B2 and B6 are part of the vitamin B complex for which 3-hydroxypyridine and isoalloxazine (Rf) constitute the respective chromophoric moieties (Scheme 1). Both vitamins may occupy common microenvironments in biological media[12,13] and are affected by mutual interactions. Among the vitamin B6 family, only pyridoxal 5-phosphate (pyridoxal PH) (Scheme 1) absorbs light at wavelengths slightly higher than 350 nm. The rest of B6D, as most of the biologically-relevant molecules, are practically transparent to visible light, whereas Rf is one of the most important endogenous visible-light absorbers. The latter has been postulated as a sensitizer for *in vivo* photo-promoted reactions that can produce physiological changes in the surrounding molecules.[14–16]

It is well known that vitamin B2 and derivatives generate the ROS singlet molecular oxygen ($O_2(^1\Delta_g)$) and superoxide radical anion ($O_2^{\cdot-}$) with quantum yields of 0.49 and 0.009 respectively, upon adequate photoirradiation.[17–19]

The interaction of B6D with ROS has been studied under different selected experimental conditions.[8–11] Nevertheless,

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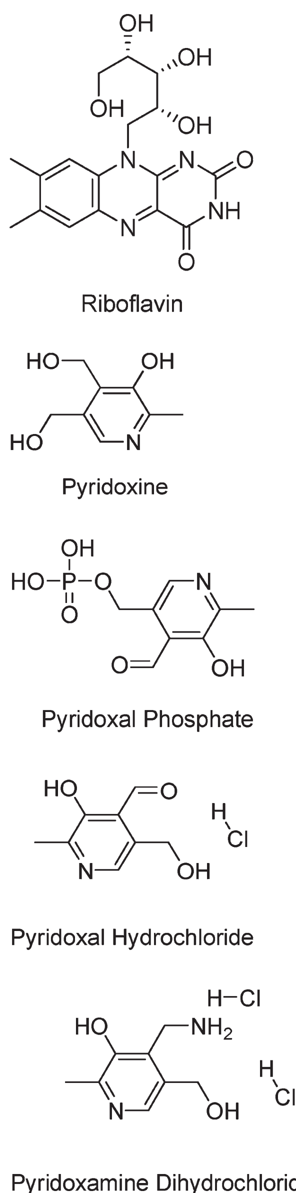


Scheme 1 Chemical structures of riboflavin, pyridoxine, pyridoxal hydrochloride, pyridoxal phosphate and pyridoxamine dihydrochloride.

according to our knowledge the combination of aqueous Rf-B6D mixture and visible light, adequate to mimic a potential scenery in living organisms, has never been explored. The results may contribute to the substantial understanding of unexpected biochemical transformations that could take place due to photochemical instability of the vitamins either *in vivo* or under current laboratory conditions. The main aim of our present study was the elucidation of the degree of lability of vitamins B6 and B2 upon photoirradiation and the possible role of the B6 family as deactivator of ROS in natural bioenvironments. We carried out a systematic kinetic and mechanistic study on the interaction of Rf-photogenerated ROS and radicals with pyridoxine, pyridoxal hydrochloride (pyridoxal HC), pyridoxal PH and pyridoxamine dihydrochloride (pyridoxamine DHC) as representative members of the vitamin B6 family.

2. Materials and methods

Materials

Riboflavin (Rf), pyridoxine, pyridoxal 5-phosphate, pyridoxal hydrochloride and pyridoxamine dyhydrochloride, deuterium oxide 99.9% (D_2O), superoxide dismutase (SOD) from boverythrocytes sodium azide (NaN_3) and lysozime were purchased from Sigma Chemical Co. Rose Bengal (RB), and furfuryl acetate (FFAc) were from Aldrich. D_2O was employed in the time-resolved determinations of $O_2(^1\Delta_g)$ in order to increase the lifetime of this species. Water was triply distilled.

The pH/pD of the final aqueous solutions for all photochemical experiments was in the range 7.4 ± 0.2, employing buffered aqueous solutions prepared, with 0.025 M KH_2PO_4/0.025 M Na_2HPO_4.[20] The presence of the salts in the mentioned concentrations did not affect neither the lifetimes nor the profiles of the optical spectra of Rf electronically excited states, as compared to those obtained in pure water.

Absorption and fluorescence experiments

Ground state absorption spectra were registered employing a Hewlett Packard 8453 diode array spectrophotometer. Fluorescence lifetimes were determined with a time-correlated single photon counting technique (SPC) on an Edinburgh FL-9000CD instrument. Excitation and emission wavelengths for Rf were 445 and 515 nm, respectively. A classical Stern–Volmer treatment of the data was applied through eqn (1), where $^1\tau$ and $^1\tau_0$ are the respective fluorescence lifetimes of Rf in the presence and in the absence of B6D, and ^1k_q is the rate constant of the quenching of excited singlet Rf (^{1}Rf*) by B6D.

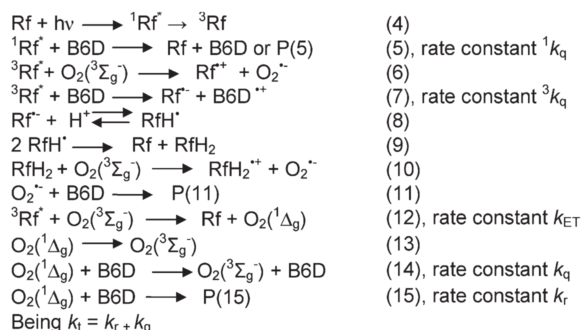
$$^1\tau_0/^1\tau = 1 + ^1k_q\,^1\tau_0[\mathrm{B6D}] \quad (1)$$

Laser flash photolysis experiments

Argon-saturated 0.04 mM Rf aqueous solutions were photolysed using a flash photolysis apparatus. A ns Nd:Yag laser system (Spectron) at 355 nm was the excitation source, employing a 150 W xenon lamp as 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 Hewlett-Packard Interface Bus (HPIB), where it was analyzed and stored.

The decay of the triplet Rf (^{3}Rf*) was generated by the laser pulse, and its disappearance was monitored at 670 nm, a spectral region where the interference from other possible species is negligible. The triplet decay was measured at low Rf concentration (typically 0.05 mM) and at low enough laser energy to avoid self-quenching and triplet–triplet annihilation. The rate constant for the interaction of ^{3}Rf* and B6D (^3k_q, reaction (7) in Scheme 2) was determined from a Stern–Volmer treatment (eqn (2)):

$$1/^3\tau = 1/^3\tau_0 + ^3k_q[\mathrm{B6D}] \quad (2)$$



Scheme 2 Possible reaction pathways for the riboflavin-sensitized photooxidation of vitamin B6 derivatives.

where $^3\tau$ and $^3\tau_0$ are the experimentally determined lifetimes of ^{3}Rf* in the presence and in the absence of a B6D, respectively.

Time-resolved $O_2(^1\Delta_g)$ phosphorescence detection (TRPD)

The total quenching rate constant (k_t, see Scheme 2) for $O_2(^1\Delta_g)$ deactivation by B6D was determined by near-IR time resolved phosphorescence, employing eqn (3), with τ and τ_0 being the respective $O_2(^1\Delta_g)$ lifetimes in the presence and in the absence of B6D.

$$1/\tau = 1/\tau_0 + k_t[\text{B6D}] \tag{3}$$

The 355 nm output from a Nd:Yag laser (Spectron) was used as the excitation source. The emitted ($O_2(^1\Delta_g)$) phosphorescence at 1270 nm was detected at right angles using a Edinburgh EI-P Germanium detector, after having passed through 1270 nm interference and two Wratten filters. The output of the detector was coupled to a 400 MHz digital oscilloscope (HP 54504A) and to a personal computer for signal processing. Usually, 10 shots were averaged in order to achieve a signal with a good signal to noise ratio, from which the decay curve was obtained. Air-equilibrated solutions were employed in all cases.

Stationary photolysis and oxygen uptake experiments

Stationary aerobic photolysis of aqueous solutions containing typically 0.2–0.5 mM B6D plus either 0.04 mM Rf or RB (A_{550} = 0.5) were carried out in a PTI unit, provided with a high pass monochromator and 150 W Xe lamp, irradiating with 445 ± 10 nm, or in a home-made photolyser for non-monochromatic irradiation (150 W quartz-halogen lamp). In this case cut-off filters (400 nm) ensured that the light was only absorbed by the sensitizer.

The Rf- or RB-sensitised photooxygenation rates of 0.5 mM B6D were determined by evaluation of the initial slopes of oxygen consumption *vs.* irradiation time, employing a specific oxygen electrode (Orion 97–08).

Reactive (chemical) rate constants for the interaction B6D–$O_2(^1\Delta_g)$ (k_r, see Scheme 2) were obtained as described by Tratniek *et al.*,[21] from the ratio of the first order slopes of B6D and reference consumption, each at the same concentration, yielding k_r/k_{rR}. The reference was FFAc, with a $k_{rR} = 5.5 \times 10^7$ $M^{-1}\ s^{-1}$ determined by ourselves.[22]

Rates of B6D consumption and oxygen consumption were obtained from the initial slopes of the plots of absorbance decrease in the respective B6D absorption maxima and oxygen uptake respectively, as a function of photoirradiation time.

3. Results

Scheme 2 has been employed for the interpretation of the results. P(n) represent different photoproducts.

Stationary photolysis and photoprotection of the protein lysozyme

Air-equilibrated pH 7,4 aqueous solutions of Rf (*ca.* 0.05 mM) plus each B6D (*ca.* 0.5 mM) were stable when stored under dark conditions. They were spectrophotometrically controlled upon dark storage by several hours. Fig. 1 shows the changes in the difference absorption spectrum (0.05 mM Rf plus 0.1 mM pyridoxal HC *vs.* 0.05 mM Rf) after irradiation (λ_{irr} > 450 nm) of such a solution. Upon prolonged irradiation also the sensitizer exhibits slight spectral changes. The same qualitative behavior was observed for the rest of B6D studied.

From parallel experiments on similar photoirradiated solutions oxygen consumption was observed. Relative rates of oxygen uptake for all B6D studied are shown in Fig. 2. The solutions did not consume any oxygen before photoirradiation.

In order to make a preliminary evaluation of the potential photoprotective effect of B6D on the oxidative degradation of biologically relevant substrates, the oxygen consumption rates by a 0.04 mM aqueous solution of Rf plus 0.1 mM lysozyme (pH 7.4), in the absence and in the presence of pyridoxal PH were compared. The protein, for which the oxidative degradation upon Rf-sensitization is well known,[23] was taken as a typical oxidizable biological target. Results in the inset of Fig. 2 show a decrease in the overall rate of oxygen uptake in the presence of the B6D indicating, in principle, a photoprotection exerted by pyridoxal PH. In parallel, a solution containing 0.04 mM of the sensitizer alone practically does not show oxygen consumption within the photoirradiation times employed.

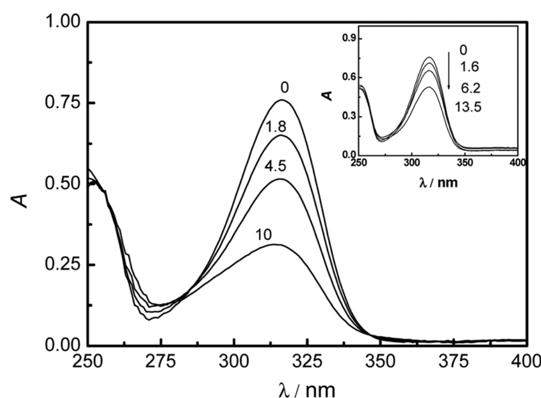


Fig. 1 Spectral evolution of pyridoxal HC 0.1 mM plus Riboflavin 0.04 mM (main figure) and of pyridoxal 0.1 mM plus Rose Bengal (A_{560} = 0.5) (inset) in pH 7.4 aqueous solution. In all cases cut-off > 400 nm. Inserted numbers indicate photoirradiation time in min.

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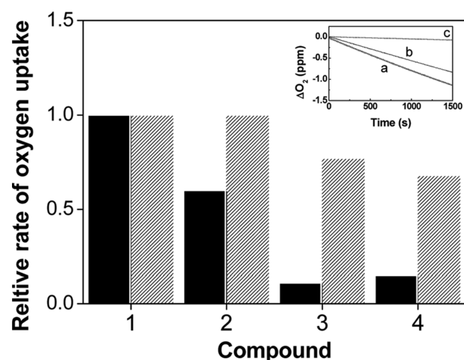

Fig. 2 Relative rates of oxygen uptake by vitamin B6 derivatives in pH 7.4 buffer upon visible light photoirradiation, (■) in the presence of 0.05 mM riboflavin and (▨) in the presence of Rose Bengal (A_{560} = 0.5), . with the following compounds all in concentration 0.5 mM: (1) pyridoxal phosphate; (2) pyridoxamine dihydrochloride; (3) pyridoxine; (4) pyridoxal hydrochloride. The respective higher rate values of oxygen uptake upon riboflavin-photosensitization and Rose Bengal-photosensitization were arbitrarily normalized to one and are not straightforwardly comparable in absolute terms. Inset: Oxygen consumption as a function of photoirradiation time (cut-off > 400 nm) of pH 7.4 aqueous solutions of (a) 0.04 mM Riboflavin + 1 μg ml^{-1} lysozime; (b) 0.04 mM riboflavin + 1 μg ml^{-1} lysozime + 0.1 mM pyridoxal HC and (c) 0.04 mM riboflavin.

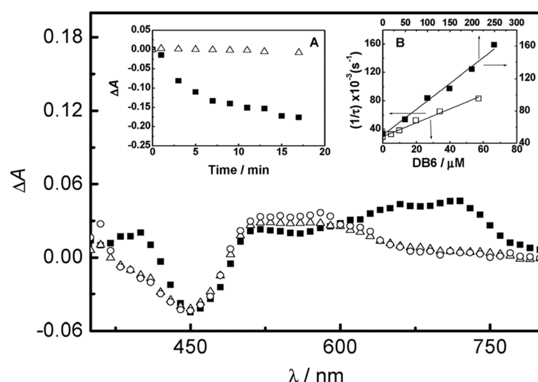

Fig. 3 Transient absorption spectra of Rf (0.05 mM) in argon-saturated pH 7.4 aqueous solution (■) in the absence and (△) in the presence of 1 mM pyridoxamine dihydrochloride; (○) in the presence of 1 mM pyridoxine (all 2 μs after the laser pulse). Inset A: Absorbance change of 0.04 mM riboflavin at 445 nm upon photoirradiation (cut-off > 400 nm) (■) in the absence and (△) in the presence of 0.5 mM pyridoxal HC. Inset B: Stern–Volmer plots for the quenching of ^{3}Rf* (b) by pyridoxal phosphate and pyridoxamine DHC in pH 7.4 aqueous solution.

The presence of any of the B6D studied, in the sub-mM concentration range, in deoxygenated solutions, produced a marked decrease in the rate of Rf consumption. It was evaluated by monitoring the rates of absorbance decrease of the 445 nm absorption band of Rf, in the absence and in the presence of B6D (see Fig. 3, inset A) for the representative case of pyridoxal HC. Photodegradation of Rf is known to proceed from its electronically excited states.[24] Hence, the modification in the photodegradation rate of Rf strongly suggests a process of quenching of the excited states of the pigment by B6D.

This collection of preliminary experimental evidence indicates that under visible light irradiation the overall interaction Rf-B6D includes the participation of electronically excited states of the pigment and dissolved ground state oxygen and/or reactive oxygenated species formed in the medium, and suggests a possible photoprotection from B6D on the Rf-sensitized photodegradation of relevant biological substrates. On this basis, we carried out a systematic kinetic study in order to evaluate and characterize the nature, mechanism and extent of the processes started with the visible-light photoirradiation of Rf plus B6D.

Table 1 Rate constants for the quenching of electronically excited singlet state (k_{q1}, M^{-1} s^{-1}) and electronically excited triplet state (k_{q3}, M^{-1} s^{-1}) of riboflavin by vitamin B6-derivatives; overall rate constants (k_t) for the quenching of $O_2(^1\Delta_g)$ by vitamin B6-derivatives and ratios of the reactive and overall rate constants for the quenching of $O_2(^1\Delta_g)$ by vitamin B6-derivatives (k_r/k_t ratios)

Compound	$k_{q1}/10^9$	$k_{q3}/10^9$	$k_t/10^8$	k_r/k_t ± 0.1
Pyridoxine	3.7 ± 0.2	1.7 ± 0.08	1.2 ± 0.1	1
Pyridoxal phosphate	ND[a]	0.9 ± 0.04	0.5 ± 0.05	1
Pyridoxal hydrochloride	0.4 ± 0.05	2.2 ± 0.08	1.3 ± 0.1	1
Pyridoxamine dihydrochloride	4.0 ± 0.4	0.4 ± 0.02	1.3 ± 0.1	1
3-Hydroxypyridine	5.9[b]	0.9[b]	0.3[c], 2.6[d]	0.66[c], 1[d]
Trolox	6.2[e]	4.7[e]	3.5[f]	0.63[f]
Ascorbic Acid	7.2	10[g]	1.6	0.70

[a] Not determined. [b] Ref. 39. [c] pH ~6, ref. 38. [d] pH 11, ref. 38. [e] Ref. 35. [f] Ref. 34. [g] Ref. 48.

Quenching of Rf electronically excited states

Fluorescence experiments and the interaction ^{1}Rf*–B6D. Rf presents an intense fluorescence emission band, centered at 515 nm, with a reported fluorescence quantum yield of 0.25.[24] In the presence of B6D in the mM concentration range, the fluorescence quenching of excited singlet Rf (^{1}Rf*, Scheme 2) produces a decrease in the stationary emission intensity, but the shape of the emission spectrum does not change. In parallel, the fluorescence decay of Rf in the absence and in the presence of B6D, as determined by the SPC technique, was monoexponential. The respective k_{q1} values for pyridoxine, pyridoxal HC and pyridoxamine DHC were determined from the Stern–Volmer plots (Table 1). The experiment could not be done for pyridoxal PH due to its low solubility in aqueous solution of pH 7.4 (solubility limit in aqueous solution *ca.* 1 mM).

Laser flash photolysis experiments and the interaction ^{3}Rf*–B6D. The ^{3}Rf* lifetime in N_2-saturated aqueous solution appreciably decreases in the presence of B6D, suggesting the occurrence of an interaction between B6D and the mentioned electronically excited state of Rf (reaction (7)). As before, a Stern–Volmer treatment of the triplet quenching (eqn (2)) yielded the bimolecular rate constants k_{q3} (Fig. 3, inset B and Table 1).

The transient absorption spectrum of Rf, obtained 2 μs after the laser pulse (Fig. 3, main), is similar to that reported for ^{3}Rf* in water.[25] Under identical conditions but in the presence of

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Fig. 4 Absorbance decrease of 1 mM pyridoxine at 324 nm + riboflavin (A_{445} = 0.5) as a function of photoirradiation time (cut-off 400 nm), in pH 7.4 aqueous solution, (c) in the absence and (a) in the presence of 10 mM NaN_3; (b) in the presence of 1µg ml^{-1} SOD. Inset: Oxygen consumption as a function of photoirradiation time (cut-off 400 nm) by riboflavin (A_{445}) = 0.5 (a) in the absence and (b) in the presence of 0.5 mM pyridoxal hydrochloride; (c) in the presence of 0.5 mM trolox; (d) in the presence of 0.5 mM ascorbic acid. All in pH 7.4 aqueous solution.

1 mM of any of the B6D (between 86% and 97% ^{3}Rf* quenched by B6D), the shape of the long-lived absorption spectrum is in good agreement with that reported for the neutral Rf radical (RfH˙).[26,27]

The Interaction of B6D with Rf-photogenerated ROS

The potential participation of Rf photogenerated ROS was evaluated through B6D consumption, employing specific ROS interceptors.

The rate of 0.2 mM B6D consumption upon sensitized photoirradiation of a solution of Rf (A_{446} = 0.4) in an air saturated pH 7.4 aqueous solution decreased in the presence of NaN_3 10 mM. A representative result is shown for pyridoxal HC in Fig. 4. The observed decrease in the rate of pyridoxal HC consumption is *ca.* 50%. The reported rate constant for the physical deactivation of $O_2(^1\Delta_g)$ by the salt[28] (reaction 14 with NaN_3 instead of B6D) is $k_q = 4.5 \times 10^8\ M^{-1}\ s^{-1}$. Employing a simple Stern–Volmer calculation, a decrease of 95% in the degradation rate should be observed for B6D in the presence of NaN_3 10 mM. The remaining oxygen consumption strongly suggests the occurrence of at least a second independent reaction, parallel to the $O_2(^1\Delta_g)$-mediated one. Hence, the possible participation of $O_2^{\cdot -}$ in the Rf-sensitized degradation of B6D was tested, by running the same described photolysis experiments but replacing NaN_3 by 1 µg ml^{-1} SOD. Results clearly show a decrease of the rate of BD6 consumption. Similar experiments with ROS-interceptors have been formerly employed to confirm/discard the participation of $O_2(^1\Delta_g)$, and $O_2^{\cdot -}$ in a given oxidative event.[28,29] The enzyme SOD dismutates the species $O_2^{\cdot -}$ (reaction 16), whereas NaN_3, as said, physically deactivates $O_2(^1\Delta_g)$.

$$2O_2^{\cdot -} + 2H^+ + SOD \rightarrow O_2(^3\Sigma_g^-) + H_2O_2 \qquad (16)$$

These experimental results indicate the participation of $O_2(^1\Delta_g)$ and $O_2^{\cdot -}$ in the overall Rf-photopromoted oxidative event of B6D.

Quenching of $O_2(^1\Delta_g)$ by B6D

On the basis that Rf generates $O_2(^1\Delta_g)$ and other ROS in the presence of electron-donating substrates as the vitamin B6 family, the well-known $O_2(^1\Delta_g)$ generator RB (A_{530} = 0.4)[30] was employed, in order to exclusively study the $O_2(^1\Delta_g)$-interaction. Visible light-irradiated aqueous RB solutions (pH 7.4) in the presence of B6D produced oxygen consumption and modifications in the spectral component of B6D (Fig. 1, inset). No spectral change was detected for the sensitizer. The relative rates for oxygen uptake for all B6D tested are shown in Fig. 2.

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.[28] The addition of a B6D as a quencher led 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 values, graphically obtained through a Stern–Volmer treatment, eqn (3), are shown in Table 1. They are in agreement with those already reported by Bilski *et al.*[11] Although the authors noted a significant contribution to total quenching by chemical reaction (step (15)), the respective k_r values were not evaluated.

In biologically relevant $O_2(^1\Delta_g)$-mediated photoreactions, the photooxidation quantum efficiency Φ_r of a given substrate Q, ($\Phi_r = k_r[Q]/(k_d + k_t[Q])$) constitutes highly valuable information in order to realistically estimate the extent of oxidative photodamage.[31] Nevertheless this value is not easy to determine, particularly in natural environments, because its evaluation includes the knowledge of the actual concentration of Q. A simpler and useful approach is the calculation 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.

The respective rate constants for reactive interaction of B6D, were obtained by the already mentioned actinometric method,[21] by monitoring substrate consumption, employing FFAc as a reference compound and RB (A_{560} = 0.4) as a dye sensitizer (experiments not shown).

The ability of biologically relevant compounds such as ROS quenchers, and their own photodegradation under oxidative stress, constitute meaningful information on both the photoprotection of living environments and on the fading of these compounds, followed by the concomitant loss of the potential photoprotective effect. In this way it is important to compare the ROS quenching ability of B6D with those of recognized natural and artificial antioxidant photoprotectors. In doing this, the rates of oxygen uptake for B6D, ascorbic acid (AsA) and Trolox (Tx), all 0.5 mM, were comparatively determined employing Rf as a photosensitizer. The representative case for pyridoxal PH is shown in the inset of Fig. 4. AsA is a well-known natural antioxidant and the artificial molecule Tx is a recognized water-soluble analogue of the natural antioxidant α-tocopherol.[32–35] It is also known that Tx and AsA interact with Rf-generated $O_2^{\cdot -}$ and $O_2(^1\Delta_g)$. The reported rate constants for the reaction of Tx and AsA with $O_2^{\cdot -}$ (reaction (11) with Tx and AsA instead of B6D) are $1.7 \times 10^4\ M^{-1}\ s^{-1}$ and $2.7 \times 10^5\ M^{-1}\ s^{-1}$, respectively.[36]

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The k_t and k_r values for both antioxidants are also included in Table 1. The overall result show that Tx and Asa interact with Rf-generated ROS with a higher rate of oxygen uptake than those exhibited by B6D. Again, Fig. 4 also shows that Rf, the sensitizer (upper trace), practically does not consume any oxygen upon photoirradiation, within typical times employed for Tx, AsSA and pyridoxal PH.

4. Discussion

Oxygen uptake results, spectroscopic evidence and auxiliary specific experiments in the Rf-sensitized photoprocesses strongly support the effective interaction B6D-ROS through a non-simple mechanism.

The non-parallel behavior exhibited by the two families of rates of oxygen uptake for B6D, employing Rf or RB as sensitizers (Fig. 2), clearly indicates differences in their respective reaction mechanisms.

The kinetic behavior of the B6D studied are all very similar, with relatively high rate constants for the overall interaction with the oxidative species and a value of *ca.* 1 for the quotient k_r/k_t. As said, a set of similar k_t values was already reported by Bilski *et al.*[11] Although the authors did not specifically evaluate the respective k_r values, they observed a high efficiency in the reactive pathway (step 15). Bilski *et al.*[11] also reported an increase in the quenching rate constants of the oxidative species by B6D by increasing solvent polarity and pH. In this context, we think that the mechanistic behavior of B6D could be described through the initial formation of an encounter complex [$O_2(^1\Delta_g)$-substrate] with partial charge-transfer character, from which an irreversible electron transfer process would yield the photooxidation products. The formation of this complex, typical for phenols and amines,[37] depends on the electron-donating ability of the substrate, and is favored in polar solvents. In phenols, the corresponding OH-ionized forms are the most photooxidizable species.[31]

In all B6D cases, the molecular moiety responsible for the interaction with $O_2(^1\Delta_g)$ seems to be the 3-hydroxypyridine (3OHP) fraction. We included in Table 1, for comparative purposes, our already reported data on $O_2(^1\Delta_g)$-mediated 3OHP photooxidation.[38,39] Its k_t value at pH 6 is in the same range than those of B6D at pH 7.4, with a relatively high k_r/k_t value, a quotient that reaches the value of 1 in at pH 10, a condition in which the hydroxy-group of 3OHP is totally ionized (pK_a = 8.75).[40]

Apparently the high reactivity exhibited by B6D in neutral medium, superior to that of 3OHP, could be ascribed to the increase of the electron-donating capacity due to the inductive effect exerted by the methyl group in the nuclear position 2 of the B6D molecular structure, absent in 3OHP. This fact also supports the above mentioned encounter-complex mechanistic model.

BD6 interacts with Rf electronically excited states, ^{1}Rf* and ^{3}Rf*. Nevertheless, according to the fluorescence quenching data, a B6D 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*, lower than 1%. Hence, the participation of ^{1}Rf* in the photodegradation of B6D must be disregarded under our experimental conditions.

^{3}Rf* is efficiently quenched by B6D. The species RfH$^\cdot$ is generated in this interaction. It is well established that the bimolecular decay of the neutral radical proceeds through a disproportionation reaction[27] yielding Rf and fully reduced Rf (RfH_2) (step 9). In the presence of $O_2(^3\Sigma_g^-)$, RfH_2 is reoxidized, giving rise to $RfH_2^{\cdot+}$ and $O_2^{\cdot-}$(reaction 10).

The thermodynamic feasibility of the electron transfer process[41] (reaction 7) for the case of Rf–B6D can be evaluated by means of the Gibbs energy of photoinduced electron transfer, using the following expression[42] that includes the abbreviations for the reaction partners employed in this work:

$$\Delta_{ET}G_0 = N_A e[E^\circ(\mathrm{B6D/B6D^{\cdot+}}) - E^\circ(\mathrm{Rf/Rf^{-\cdot}})] + w(\mathrm{B6D^{\cdot+}\ Rf^{-\cdot}}) - w(\mathrm{B6D\ Rf}) - \Delta E_{0,0},$$

where e is the elementary charge, N_A is the Avogradro constant, $E^\circ(\mathrm{B6D/B6D^{\cdot+}})$ is the standard electrode potential of the donor (the only available data in the literature[43,44] is 0.72 V and 0.80 V for pyridoxine), $E^\circ(\mathrm{Rf/Rf^{-\cdot}})$ is the standard electrode potential for the acceptor (−0.80 V),[45] the difference $w(\mathrm{B6D^{\cdot+}\ Rf^{-\cdot}}) - w(\mathrm{B6D\ Rf})$ is the coulombic energy term (−0.06 V)[45] and $\Delta E_{0,0}$ is the vibrational zero energy of the excited partner (2.17 eV)[45] The so-calculated $\Delta_{ET}G_0$ values,−0.71 eV and −0.63 eV for pyridoxine, indicate that reaction (7) may be operative and consequently that the species $O_2^{\cdot-}$ could be formed by electron transfer from RfH_2 (reaction (10)). The effective operation of this pathway in aerated medium will depend on whether reaction 7 is kinetically competitive or not with the $O_2(^1\Delta_g)$ generation (reaction (12)). The k_{ET} value of reaction (12) in H_2O is *ca.* 1/9 of the diffusional value,[46] *i.e.* 7×10^8 M^{-1} s^{-1}, and the obtained values of ^3k_q for the B6D are between 0.4×10^9 M^{-1} s^{-1} for pyridoxamine DHC and 2.2×10^9 M^{-1} s^{-1} for pyridoxal HC (Table 1). Hence, it can be deduced that, for the same concentrations of B6D and dissolved $O_2(^3\Sigma_g^-)$, the generation rate of the initial $O_2^{\cdot-}$ precursor, *via* $Rf^{\cdot-}$ (reaction (7)), is between 3 times higher for pyridoxal PH and 0.6 times lower for pyridoxamine DHC than the corresponding one for $O_2(^1\Delta_g)$ generation (step 12). In other words, the rate values for $O_2(^1\Delta_g)$-generation and for the precursory pathway for $O_2^{\cdot-}$ production are relatively close and may compete. This means that photoirradiated Rf, in the presence of B6D, generates $O_2(^1\Delta_g)$ and $O_2^{\cdot-}$.

Experimental evidence on the ability of B6D as quenchers of $O_2^{\cdot-}$ in the context of biological environments has been published. Sushil and Lim[10] demonstrated that pyridoxine and pyridoxamine DHC inhibit $O_2^{\cdot-}$, preventing lipid peroxidation, whereas Chumnantana *et al.*[9] reported that either of the B6D herein studied increase the viability of yeast cells under oxidative stress produced by the presence of menadione, a $O_2^{\cdot-}$ generator.

The interaction of 3OHP, the chromophoric moiety of B6D, with photoirradiated Rf has been already studied by ourselves.[39] Again the results, as in the case of the RB-photosensitization,[38] indicate a clear qualitative parallelism with those we are reporting for the vitamin B6 family. In photoirradiated solutions with *ca.* 0.01 mM Rf and 0.4 mM 3-OHP, the dominant pathways are the generation of the species $O_2(^1\Delta_g)$ and the concomitant photooxygenation of both 3-OHP and the very Rf, confirming the importance of the hydroxy-pyridine structure as a ROS scavenger [39]

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The observed decrease in the overall rate of oxygen consumption by the mixture Rf + lysozyme + pyridoxal PH as compared to that of Rf + lysozyme (Fig. 2, inset), shows in principle a photoprotective effect by B6D on the degradation of the protein. Lysozyme quenches $O_2(^1\Delta_g)$ with a reported rate constant k_t = 2.5×10^8 M^{-1} s^{-1}.[28] On this basis and considering that the interaction of pyridoxal PH with $O_2(^1\Delta_g)$ is a totally reactive (chemical) event, the expected rate of oxygen uptake by the mixture should contain, at least, the individual contributions from B6D and the protein. In other words, the rate of oxygen consumption by the mixture should be higher than that obtained for the protein alone. An explanation for the observed results is a selective quenching of the species $O_2^{\cdot-}$ by B6D. Nevertheless, in a recent paper Li *et al.*[23] observed an antioxidant effect on lysozyme, exerted by salicylic acid, due to the quenching of $^*Rf^3$ by the aspirin metabolite. The reported rate constant for reaction (7), with salicylic acid instead of B6D, is k_{q3} = 2.25×10^9 M^{-1} s^{-1}, with k_t = 2×10^7 M^{-1} s^{-1} for salicylic acid,[28] both in the same range as the respective rate constant values for B6D (Table 1). Similarly, the quenching of $^*Rf^3$ by pyridoxal PH could be the mechanistic pathway responsible for the inhibition of lysozyme oxidation.

Attempting to standardize the antioxidant ability of B6D in the presence of Rf-photogenerated ROS, its stability was compared with those of the recognized antioxidants, such as the naturally-occurring AsA and the synthetic Tx (Table 1). The overall rate constants for $O_2(^1\Delta_g)$ quenching are higher for B6D than for Tx and AsA. The balance between rate constants for overall and chemical $O_2(^1\Delta_g)$ deactivation indicates that all compounds, *i.e.* Tx, ASA, and most especially the B6D family, act as typical sacrificial scavengers. Practically 100% of the collisions with $O_2(^1\Delta_g)$ cause B6D degradation, whereas this value reaches around 80% for AsA and 60% for Tx. Nevertheless, according to our results (Fig. 4) Tx and AsA are better oxygen-consumers than B6D upon Rf-sensitization, through a combination of $O_2(^1\Delta_g)$-oxidation and radical-mediated mechanisms. This result would suggest an important contribution of the $O_2^{\cdot-}$-mechanism in the overall oxygen consumption process. It seems that the difference of AsA and Tx with B6D lies in the much lower $^3Rf^*$ quenching rate constant of the latter, decreasing, in relative terms, the $O_2^{\cdot-}$ production route.

A remarkable point is the fate of Rf under photoirradiation in the presence of B6D. Although Rf also participates as a potential reactant, the sensitizer is almost not degraded. This vitamin is reduced by process (7), and also may react with $O_2(^1\Delta_g)$, (step (15), with Rf instead of B6D) with a rate constant $k_{rRf} = 6 \times 10^7$ M^{-1} s^{-1}.[47] Nevertheless, process (9) constitutes a source of Rf regeneration. Under working conditions, 10[Rf] ∼ [B6D] and the rate constant for $O_2(^1\Delta_g)$ quenching by Rf is similar or lower than the corresponding ones for B6D (Table 1), the sensitizer will be not decomposed by $O_2(^1\Delta_g)$ in the presence of B6D to a considerable extent.

5. Conclusions

The visible light irradiation of Rf and B6D, in aqueous medium under aerobic conditions at pH 7.4, induces photoprocesses that mainly produce B6D degradation. The overall oxidative mechanism involves the participation of at least the ROS $O_2(^1\Delta_g)$ and $O_2^{\cdot-}$ and includes intermediary radical species. B6D act as efficient quenchers of $O_2(^1\Delta_g)$ in a totally reactive event that also highly inhibits Rf photoconsumption. 3OHP constitutes an excellent model for the kinetic behavior of B6D as quenchers of Rf-generated ROS.

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