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Photooxidation of Other B-Vitamins as Sensitized by Riboflavin

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ABSTRACT: Pyridoxal phosphate (PLP) was found to deactivate triplet-excited riboflavin (Rib) in aqueous solution with a deactivation constant of $3.0 \pm 0.1 \times 10^8$ L mol^{-1} s^{-1} at 25 °C. Likewise, PLP was found to quench the fluorescence emission of ^{1}Rib* with $^1k_q = 1.0 \pm 0.1 \times 10^{11}$ L mol^{-1} s^{-1} as determined by steady state fluorescence. The rather high quenching constant suggests the formation of a ground state complex, which was further confirmed by time-resolved fluorescence measurements to yield a ^{1}Rib* deactivation constant of $3.4 \pm 0.4 \times 10^{10}$ L mol^{-1} s^{-1}. Triplet quenching is assigned as one-electron transfer rather than hydrogen-atom transfer from PLP to ^{3}Rib*, as the reaction quantum yield, $\Phi = 0.82$, is hardly influenced by solvent change from water to D_2O, $\Phi = 0.78$. Neither biotin nor niacin deactivates the singlet- or triplet-excited riboflavin as it is expected from their higher oxidation potentials $E > 2$ V vs NHE.

KEYWORDS: *flavin, photooxidation, B-vitamins, photochemistry, pyridoxal, biotin, niacin*

INTRODUCTION

Food fortification and enrichment are essential tools in nutrition strategies to alleviate micronutrient deficiencies in response to the specific needs of certain population groups.[1−3] The status of B-complex vitamins is known to be marginal in populations with low consumption of food from animal sources and those that consume highly refined cereals and grain products. Milk and dairy products are, as a colloidal suspension, considered to be good vehicles for both lipid- and water-soluble vitamins. However, dairy products are rich in riboflavin, vitamin B2, which absorbs visible light and is known to be implicated in various photochemical processes, which have a crucial role in the redox stability of food and beverages.[4,5]

Recently, it was demonstrated that folic acid is efficiently photodegraded by riboflavin ($\Phi = 0.32$) in a dairy model system and that thiamin is also susceptible to light induced oxidation by riboflavin as thiamin (vitamin B1) quenches triplet-excited riboflavin with a rate constant of 6.9×10^6 L mol^{-1} s^{-1}.[5−7] Except for folic acid and thiamin, photodegradation of the other B-vitamins (pyridoxal, niacin, and biotin) as sensitized by riboflavin has not been investigated.

The present study was accordingly undertaken in order to contribute to a better understanding of the photooxidation of pyridoxal (B6), niacin (B3), and biotin (B7) as sensitized by riboflavin (B2). Such knowledge certainly will be helpful for formulation of dairy products enriched with or fortified with B-vitamins.

MATERIALS AND METHODS

Chemicals. Acetic acid, deuterium oxide, riboflavin-5′-monophosphate sodium salt hydrate (FMN), biotin, nicotinamide, nicotinic acid, pyridoxal-5′-phosphate hydrate (PLP), phenantroline, *N*,*N*-dimethylformamide (DMF), tetrabutylammonium hydroxide (TBA-OH) and potassium superoxide were purchased from Sigma-Aldrich (Steinheim, Germany) and used without further purification. Acetonitrile HPLC grade was purchased from Mallinckrodt (Phillipsburg, New Jersey). Analytical grade H_2SO_4, HCl, formic acid, and inorganic salts ($K_3[Fe(C_2O_4)_3]$, K_2HPO_4, KH_2PO_4, KOH, $NaCH_3CO_2$, and NaCl) were supplied by Merck (Darmstadt, Germany), while aqueous solutions were prepared using purified water (18 MΩ·cm) from a Milli-Q purification system (Millipore, Bedford, MA).

Potassium Ferrioxalate Actinometry.[8] The absorbance of a 0.15 mol L^{-1} solution of $K_3[Fe(C_2O_4)_3]$ was measured at 550 nm measured before and after the solution was irradiated for 20 min at 20 °C, to determine the fraction of light absorbed. The number of moles of ferrous ion produced upon light irradiation was then determined by quantitating the amount of $[Fe(phenanthroline)_3]^{2+}$ after addition of phenanthroline. This procedure was accomplished by adding 1 mL of photolyzed sample, 0.5 mL of acetate buffer (600 mL of 1 mol L^{-1} sodium acetate and 360 mL 0.5 mol L^{-1} H_2SO_4 diluted to a total volume of 1 L), and 2 mL of a 0.1% aqueous phenanthroline solution to 10 mL of water. After standing for 15 min in the dark, the amount of $[Fe(phenanthroline)_3]^{2+}$ was determined by measuring the absorbance at 510 nm ($\varepsilon = 11100$ M^{-1} cm^{-1}). The procedure was repeated with a sample of ferrioxalate kept in the dark. Five independent runs were averaged to determine the intensity of light incident on the sample. The absorbance measurements were carried out employing a Hitachi U-3501 (Hitachi-Hitech, Japan) spectrophotometer.

Laser Flash Photolysis. Laser flash photolysis experiments were carried out with an LFP-112 ns laser flash photolysis spectrometer from Luzchem (Ottawa, Canada) using the third harmonic (355 nm) of a pulsed Q-switched Nd:YAG laser (Brilliant B, Les Ulis, France) attenuated to 14 mJ·cm^{-2} as the excitation source with 8 ns resolution. A R928 photomultiplier tube from Hamamatsu Photonics (Hamamatsu City, Japan) was used to detect the transient absorption (300−800 nm). Appropriate UV cutoff filters were used to minimize the sample degradation by the monitoring light. The samples were excited in 1.0 cm × 1.0 cm fluorescence cuvettes from Hellma (Mulheim,

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Germany). Each kinetic trace was averaged 16 times, and observed rate constants were determined by fitting the data with MatLab R2008 (Mathworks Inc.). All measurements were made with fresh solutions thermostated at 25.0 ± 0.5 °C and purged with high-purity N_2 (White-Martins, Sertãozinho-SP, Brazil) for 60 min before the experiment.

Fluorescence Measurements. Fluorescence measurements were carried out using a Hitachi F-7000 fluorescence spectrometer (Hitachi High-Tech, Tokyo, Japan) at 25 °C using a thermostated cell holder. Samples were excited in 1.0 cm × 1.0 cm fluorescence cuvettes from Hellma (Mulheim, Germany), and the emission spectra were recorded for excitation at 445 nm.

Fluorescence lifetime measurements were performed with an Optical Building Blocks Corp. Fluorometer (Birmingham, U.K.), using the fluorescence time-resolved mode. The excitation and emission wavelengths were λ = 460 and 530 nm, respectively. Fluorescence decay times were fitted using a monoexponential decay function and the best fit obtained by optimized Chi-square residuals and standard deviation parameters. All solutions were previously deaerated by purging the curvette with high-purity nitrogen (White-Martins, Sertãozinho-SP, Brazil).

LC−ESI-MS/MS and Direct Infusion High-Resolution-ESI-MS. The LC−ESI-MS/MS analyses were conducted with a Shimadzu Prominence series HPLC equipped with two LC-20AD solvent delivery units for binary gradient elution, an online Shimadzu degasser DGU20A3, a manual Rheodyne model 8125 sample injector valve with sample loop of 20 μL, and a CBM-20A Shimadzu Prominence communications bus module. Samples were separated in an Agilent Extend C18 reverse phase column (2.1 mm × 150 mm x 5 μm). The mobile phase with a flow rate of 0.35 mL min^{-1} consisted of a mixture of solvents, A (water/formic acid, 99.9:0.01% v/v) and B (acetonitrile/formic acid, 99.9:0.01% v/v), using the following linear eluting gradient: 0−5 min, 0% B in A; 5−20, 15% B in A; 20−30, 90% B in A; 30−35, 0% B in A. The electrospray mass spectra were collected in the negative and positive ion modes for the identification and quantification of the target compounds using a Bruker Daltonics ion trap mass spectrometer model Esquire 4000 (Bremen, Germany). Direct infusion high-resolution accurate ESI-MS spectra of reaction products were performed on an LTQ-Orbitrap Thermo Fisher Scientific mass spectrometry system (Bremen, Germany) operating in the negative ion detection mode.

Electrochemical Studies. Cyclic voltammetry was carried out in a PAR model 264A potentiostat (Oak Ridge, TN, United States) connected to a personal computer using proprietary software for data acquisition. Electrochemical oxidations were carried out in phosphate buffer solutions with ionic strength of 0.16 mol L^{-1} (NaCl) and a three-electrode system with saturated calomel, a glassy carbon or boron doped electrode for higher potential window (up to 2 V vs NHE), and a platinum wire used as reference, work, and auxiliary electrodes, respectively.

Singlet-Excited Oxygen Deactivation by B6 Vitamin. Singlet-excited oxygen lifetime decay was recorded with a time-resolved NIR fluorometer (Edinburgh Analytical Instruments, U.K.) equipped with a Nd:YAG laser (Continuum Surelite III), λ_{exc} = 532 nm (pulse ∼30 ns). The emitted light passed through a silicon and an interference filter and a monochromator before detection with a NIR photomultiplier (Hamamatsu Co. R5509). The singlet oxygen lifetime was determined by applying first-order exponential fitting to the curve of the phosphorescence decay. Methylene blue was used as singlet-excited oxygen photosensitizer in ethanol solutions at a concentration of 1.6 × 10^{-5} mol L^{-1}.

Reactivity of B6 Vitamin (PLP) toward Superoxide Anion Radical. The reactivity of PLP toward the superoxide anion radical was probed spectrophotometrically using a Multiskan Go Thermo Fisher Scientific UV−vis spectrophotometer (Vantaa, Finland). Superoxide anion radical solutions were prepared by dissolving potassium superoxide in dry DMF. The PLP stock solution was prepared by dissolving 6 mg of PLP (ca. 2.42 × 10^{-3} mol) in 20 mL of dry DMF. Kinetic experiments were conducted using superoxide anion radical under pseudo-first-order conditions with respect to pyridoxal. Direct infusion high-resolution accurate ESI-MS spectra of reaction products were obtained on an LTQ-Orbitrap Thermo Fisher Scientific mass spectrometry system (Bremen, Germany) operating in the negative ion detection mode.

RESULTS AND DISCUSSION

Riboflavin and its derivatives (FMN and FAD) are known to become strongly oxidizing upon UV-A or blue light excitation. Light excitation of flavins results in an efficient spin-allowed transition (Φ = 0.27) yielding the short-lived singlet-excited state with a lifetime around 5 ns in water at ambient temperature with intense fluorescent emission centered at 530 nm (Figure 1).[5,9,10] Upon light UV-A or blue excitation,

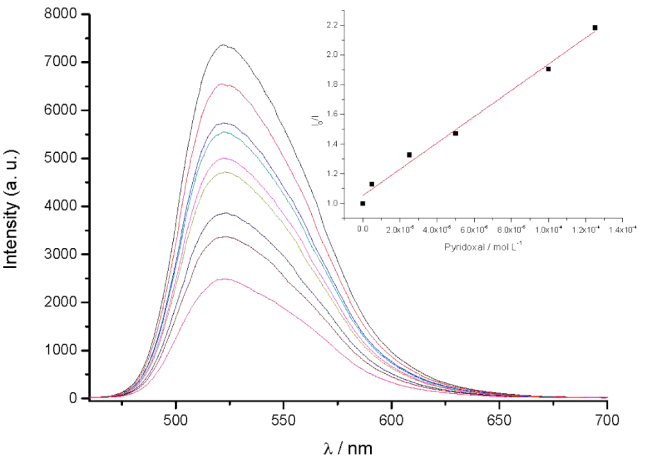

Figure 1. Fluorescence emission spectra of FMN (1.0×10^{-4} mol L^{-1}) observed at different PLP concentrations in aqueous solution at pH 6.4 at 25 °C for excitation at 440 nm (PLP concentration from 0 to 1.5×10^{-4} mol L^{-1}). Inset: Stern−Volmer analysis of the fluorescence quenching of singlet-excited FMN by PLP.

the redox potential shifts from $E°$ = −0.3 V vs NHE for the ground state to $E° \approx$ + 1.77 V vs NHE for the triplet-excited state of riboflavin.[10]

The fluorescence of the FMN was found to be partly quenched in the presence of PLP (Figure 1). Figure 1 illustrates the decrease in the steady state fluorescence intensity probed at 530 nm with excitation at 445 nm as function of increasing concentrations of PLP from 0 to 1.5×10^{-4} mol L^{-1}. In contrast biotin and niacin (both nicotinamide and nicotinic acid) were found not to quench FMN fluorescence, suggesting that these vitamins do not interact with the FMN singlet-excited state. The quenching of singlet-excited FMN by PLP was analyzed according to the Stern−Volmer equation using 530 nm band intensity for FMN (I_0) and FMN with increasing PLP concentration (I):

$$I_0/I = k_q\tau[\mathrm{PLP}] + 1$$

where τ relates to the lifetime of FMN in the absence of the quencher, 5 ns in aqueous solution,[11] and k_q is the rate constant of suppression of singlet-excited state. A correction for inner-filter effects was required due the fact of PLP absorbs a fraction of light at the FMN excitation wavelength.[12] From the linear dependence of the decrease of fluorescence intensity as function of increasing concentration of PLP, verified in the inset of Figure 1, the singlet-excited state quench rate constant could be calculated, k_q = 1.0 ± 0.1 × 10^{11} L mol^{-1} s^{-1} in aqueous solution at pH 6.4 at 25 °C. However, the rate constant for singlet-excited flavin deactivation is higher than the

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diffusion limit in water, suggesting that a ground state complex is formed. The contribution of the static quenching on the singlet-excited rate constant obtained from the static fluorescent quenching (Stern–Volmer analysis) was further calculated by probing the changes in the singlet-excited FMN decay rate constants as a function of PLP concentration (Table

Table 1. Bimolecular Second-Order Rate Constant for FMN Singlet-Excited State Quenching (^1k_q) by PLP at Varying pH and Temperature

pH	temp (°C)	^1k_q (M^{-1} s^{-1})
5.0	25	$1.2 \pm 0.3 \times 10^{11}$
6.4	25	$1.0 \pm 0.1 \times 10^{11}$
7.4	15	$2.3 \pm 0.1 \times 10^{11}$
7.4	25	$1.9 \pm 0.1 \times 10^{11}$
7.4	35	$1.8 \pm 0.1 \times 10^{11}$

Table 2. Lifetime of FMN Singlet-Excited State at Varying Concentrations of PLP in Aqueous Solution at pH 6.4

[PLP] (mM)	lifetime/ns
0	5.2 ± 0.3
0.1	4.7 ± 0.2
1.0	4.3 ± 0.2

2) as obtained by time-resolved fluorescence measurements. As can be seen in Table 2, PLP only reduces slightly the lifetime of singlet-excited FMN, thus displaying a rate constant for dynamic quenching of singlet-excited state of $^1k_q = 3.4 \pm 0.4 \times 10^{10}$ L mol^{-1} s^{-1} in aqueous solution at pH 6.4 at 25 °C. The pH changes do not systematically modify the values of the rate constants for FMN singlet-excited state quenching (Table 1). However, as expected for a quenching process occurring after formation of a ground state precursor complex, the changes in temperature slightly affect the observed quenching constant (Table 1). The increase in temperature from 15 to 35 °C reduces the observed quenching constant from $2.3 \pm 0.1 \times 10^{11}$ L mol^{-1} s^{-1} at 15 °C to $1.8 \pm 0.1 \times 10^{11}$ L mol^{-1} s^{-1} at 35 °C, suggesting formation of the ground state precursor complex to be an exothermic process.

Figure 2 shows the transient absorption spectrum for the FMN triplet-excited state in the presence of 5.0×10^{-4} mol L^{-1} PLP in N_2-saturated aqueous solution at pH 6.4 and 25 °C. The transient spectra recorded 0.8 μs after the laser pulse clearly show the triplet–triplet absorption band centered at 720 nm, which is characteristic for the triplet-excited FMN.[10,13] The spectra recorded 5.8, 15.8, and 77 μs after the laser pulse display the appearance of two new absorption bands centered at 430 and 560 nm that could be assigned to the formation of a cation radical from the 3-hydroxypyridine derivative oxidation[14] and flavin neutral radical ($FMNH^{\bullet}$; $pK_a \sim 6.8$),[15] respectively.

By monitoring the decay of the FMN triplet-excited state absorption band at 720 nm as a function of increasing concentrations of PLP (Figure 3), the second-order rate constant for triplet-excited FMN deactivation could be obtained. The decay of the triplet-excited FMN state probed at 720 nm was found to decay monoexponentially according to eq 1:

$$A_t = A_{t=0} \exp^{-k_{obs}t} \quad (1)$$

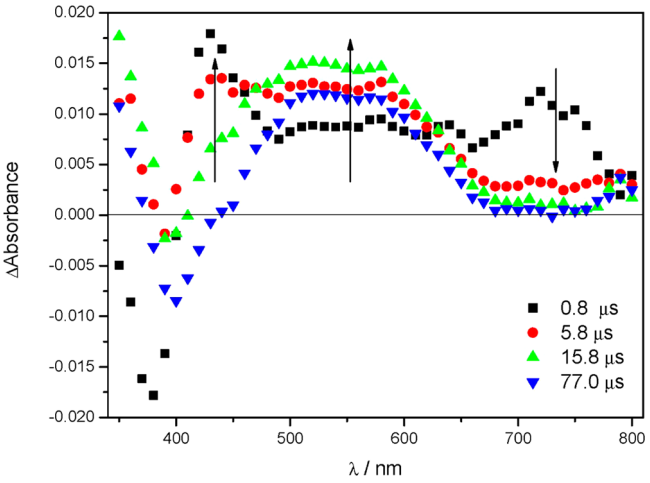

Figure 2. Transient difference absorption spectra recorded at selected delay time after laser excitation at 355 nm (14 mJ·cm^2) of a N_2-saturated aqueous solution (pH 6.4, 25 °C) containing FMN (6.0×10^{-5} mol L^{-1}) and PLP (5.0×10^{-4} mol L^{-1}).

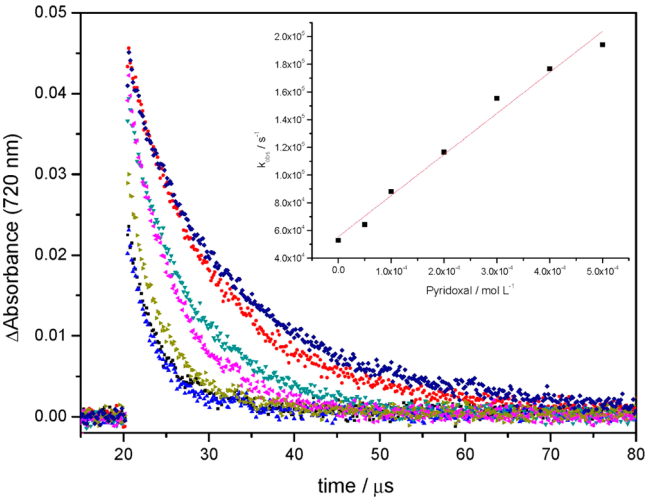

Figure 3. Kinetic traces for triplet-excited FMN decay monitored in real time at 720 nm following 8 ns laser pulses of 14 mJ cm^2 at 355 nm for increasing concentrations of PLP in N_2-saturated aqueous solution at pH 6.4 and 25 °C. Inset: Observed pseudo-first-order rate constant as a function of PLP concentration.

$A_{t=0}$ is the difference of absorbance at time zero, and k_{obs} is the observed-rate constant. As can be seen in the inset of Figure 3, the observed-rate constant for the triplet-excited FMN decay increase linearly with increasing of PLP concentration. Thus, the second-order rate constant for the bimolecular reductive quenching is calculated (eq 2):

$$k_{obs} = k_T + k_q[PLP] \quad (2)$$

where k_T is the rate constant for the triplet state natural decay and k_q is the second-order rate constant for the triplet state deactivation by the quencher. The obtained second-order rate constant for the bimolecular reductive quenching of triplet-excited FMN by PLP is $^3k_q = 3.0 \pm 0.1 \times 10^8$ L mol^{-1} s^{-1} in N_2-saturated aqueous solution pH 6.4 at 25 °C and $^3k_q = 2.2 \pm 0.2 \times 10^8$ L mol^{-1} s^{-1} in deuterium oxide solution pD 6.4 at 25 °C. The second-order constant for the bimolecular reductive quenching of triplet-excited FMN by PLP approaching the diffusion limit is accordingly competitive with the triplet-excited

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FMN deactivation by molecular oxygen ($k = 9.8 \times 10^8$ L mol^{-1} s^{-1}),[10] suggesting type I photooxidation mechanism to be preferential. A small primary kinetic isotopic effect was observed ($k_H/k_D = 1.4$) consequently suggesting the type I photooxidation operating by direct one-electron transfer (ET) or proton coupled electron transfer (PCET) from PLP to triplet-excited FMN rather than H-atom transfer as expected for phenols and pyrimidine bases.[5]

In addition to the kinetic investigation suggesting the prevailing of the type I photooxidation mechanism, the quantum yield for the photodegradation reaction was collected under anaerobic and aerobic conditions in both aqueous and deuterium oxide solutions. The reaction quantum yields may provide a more practical measurement of the efficiency in which the photodegradation occurs and may be determined as follow (eq 3):

$$\Phi = \text{molecules}_{\text{reacted}}/\text{photons}_{\text{absorbed}} = V([\text{PLP}]_{\text{dark}} - [\text{PLP}]_{\text{irradiated}})/(I_0(1 - 10^{A_{436}})) \quad (3)$$

where I_0 is the intensity of light measured by ferrioxalate chemical actinometer, A_{436} is the absorbance of the solution at the wavelength of irradiation, V is the total volume of solution, and $([\text{PLP}]_{\text{dark}} - [\text{PLP}]_{\text{irradiated}}$ is the decrease in PLP concentration upon light irradiation as determined by LC–ESI-MS/MS. The obtained quantum yields of PLP photodegradation as sensitized by FMN under different experimental conditions are collected in Table 3.

Table 3. Quantum Yield for PLP (5.0×10^{-4} M) Photodecomposition Sensitized by FMN (1.0×10^{-5} M) in the Presence and Absence of Oxygen at 25° C

ΦH_2O air-saturated	ΦD_2O air-saturated	ΦH_2O argon-saturated
0.82 ± 0.11	0.78 ± 0.12	0.33 ± 0.13

The photodegradation of PLP as sensitized by FMN is observed to be hardly influenced by solvent change from water to deuterium oxide supporting our proposal for a reductive quenching operating by one-electron transfer rather than H-atom transfer. Surprisingly, as may be seen in Table 3, oxygen is important for the efficient photodegradation of PLP sensitized by FMN, suggesting the involvement of oxygen in the photooxidation process. Aiming to evaluate the type II photooxidation process in comparison with the type I photooxidation process, the rate constant for singlet-excited oxygen quenching by PLP was determined by time-resolved singlet oxygen phosphorescence measurements. The collected rate constant of $k_q = 7.2 \times 10^6$ M^{-1} s^{-1} for singlet-excited oxygen deactivation by PLP in ethanolic solution at 25 °C is in agreement with the reported rate constant of $k_q = 3.45 \times 10^6$ L mol^{-1} s^{-1} for the pyridoxine free base.[16] By comparing the photodegradation quantum yields in aerobic and anaerobic conditions, the photodegradation in aerobic medium is 2.5 times more efficient than that found in the absence of oxygen (Table 3), suggesting the participation of singlet-excited oxygen (type II mechanism) in the photodegradation reaction. However, this observation is in opposition to the lack of influence for solvent change from water to deuterium oxide. This fact may be explained considering the participation of the superoxide anion radical generated by the oxidation of the neutral FMN radical back to FMN or by the addition of oxygen to the PLP radical avoiding the immediate back reaction following the primary oxidation of PLP by triplet-excited FMN, regenerating ground state flavin and PLP, in effect serving as physical quenching. Indeed, the relatively low value for rate constant for deactivating singlet-excited oxygen by PLP is not competitive with the reaction of singlet-excited oxygen with proteins and unsaturated lipids ($k \approx 1.0 \times 10^7$ L mol^{-1} s^{-1} and $k \approx 1.0 \times 10^5$ L mol^{-1} s^{-1}, respectively)[17] in the food matrix, and the PLP deactivation of singlet oxygen plays no role in real foods rich in proteins.

In order to support the proposed reductive quenching for triplet-excited flavins deactivation by PLP, cyclic voltammetry experiments were carried out aiming to determine the oxidation potential for the B-vitamins investigated herein. Among the investigated B-vitamins, PLP was the only vitamin that shows to be electroactive in the investigated potential window (0–2 V vs NHE). Biotin and niacin do not present oxidation potentials in the studied electrochemical window, suggesting that these vitamins have high redox potentials, i.e., $E° > 2$ V vs NHE. Figure 4 shows the cyclic voltammograms for an aqueous

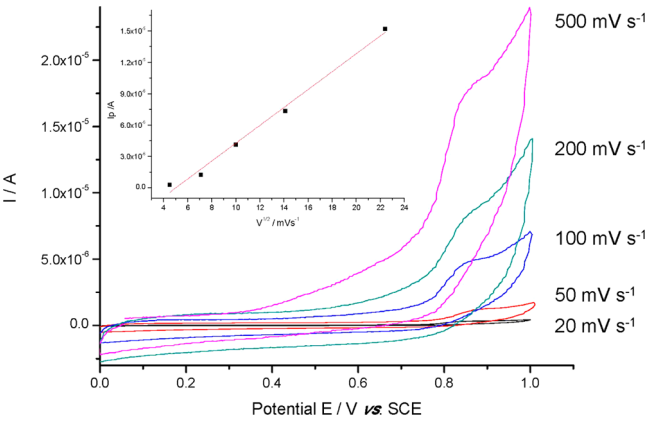

Figure 4. Cyclic voltammograms for an aqueous solution (pH 6.4) containing 1.0×10^{-4} mol L^{-1} of PLP at different scan rate. Inset: Plot of square root of scan rate versus the anodic peak current.

solution containing 1.0×10^{-4} mol L^{-1} PLP at pH 6.4 and at varying scan rate. PLP displays an irreversible one-electron anodic wave at 1.08 V vs NHE. The linear dependence of the anodic peak current on the square root of scan rate (inset Figure 4) is consistent with a diffusion process, indicating that electrochemical oxidation of PLP is kinetically controlled by the heterogeneous electron transfer from PLP to the electrode.

Pyridoxal phosphate (PLP) is a 3-pyridinol derivative which may be considered a simple phenolic compound with one nitrogen atom incorporated to the hydroxyaromatic ring. The incorporation of one or two nitrogen atoms to the hydroxyaromatic ring has been shown to reduce marginally the O–H bond dissociation energy, however, increasing substantially the ionization potential. Thus, on this ground is interesting and relevant to compare the quenching constant reported for phenolic compounds[19,20] with the rate constant of deactivation of triplet-excited flavins by PLP. Based on the Rehm–Weller equation,[18]

$$\Delta G° = E_{ox} - E_{red} - \Delta E_{0,0} - e^2/\epsilon_a$$

where e^2/ϵ_a is the Coulombic term and can be neglected in aqueous medium, $\Delta E_{0,0}$ is the energy level gap between ground state and excited singlet or triplet state (2.48 and 2.16 eV, respectively[21]), E_{red} is the one-electron reduction potential for

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triplet-excited flavin,[22] and E_{ox} is the one-electron oxidation potential for the PLP. Accordingly, the reaction free energy is calculated for the PLP quenching of singlet-excited state of FMN ($\Delta G°_{ET\text{-}S} = -107.9$ kJ mol^{-1}) and for the triplet-excited state of FMN ($\Delta G°_{ET\text{-}T} = -76.8$ kJ mol^{-1}). The driving force is high for both reductive quenching of singlet- and triplet-excited state, however, for the singlet-excited state, the deactivation is not kinetically competitive with the efficient intersystem crossing ($k_{ISC} = 2 \times 10^8$ s^{-1}).[15] In an attempt to better support the ET or PCET mechanism for triplet-excited FMN deactivation by PLP, a linear free energy relationship plot was constructed for phenols, folate, and PLP and is illustrated in Figure 5. A linear free energy relationship is clearly observed in

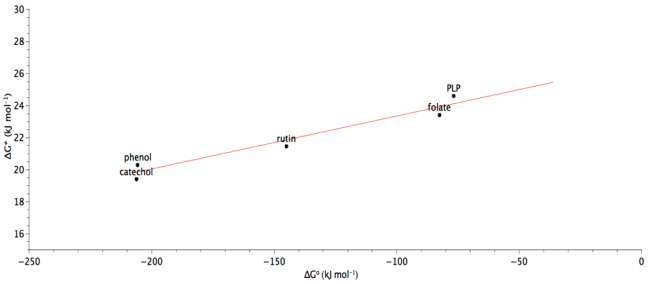

Figure 5. Free energy of activation ($\Delta G^{\ddagger}$) versus estimated free energy of reaction ($\Delta G°$) for the photoinduced electron-transfer from phenols (from refs 18 and 19) and folate to the triplet-excited state of flavins.

Figure 5, indicating a common quenching mechanism, i.e., ET or PCET as demonstrated previously for phenols, which is in accordance with the nearly diffusion-controlled rate constant, a conclusion further supported by the lack of a significant kinetic isotopic effect.[5]

In an effort to understand the role of oxygen on the quantum yield for photodegradation of PLP under aerobic conditions, the reactivity of PLP toward superoxide anion radical was investigated spectrophotometrically. The kinetic study was carried out under pseudo-first-order conditions, employing a large excess of potassium superoxide in relation to PLP in the reaction medium. Immediately upon mixing potassium superoxide and PLP solutions, a bathochromic shift from 342 to 414 nm in the PLP spectra is noticeable and may be assigned to the deprotonation of PLP by addition of the strong base (KO_2); a similar behavior is observed by adding TBA-OH base to dry DMF solutions containing PLP. A slow process is then observed corresponding to the disappearance of the band centered at 414 nm (Figure 6) with a concomitant appearance of a band centered at 306 nm.

Kinetic experiments were performed following the decay of the band centered at 414 nm versus time (inset Figure 6), which is shown to be linearly dependent on the concentration of superoxide anion radical in excess and decay accordingly to a monoexponential function: $A_t = A_{t=0} \exp^{-k_{obs}t}$. From the nonlinear fitting of the exponential function to the collected data for increasing concentrations of superoxide anion radical, k_{obs} was determined and plotted against KO_2 concentration, Figure 7, to furnish the second-order rate constant for scavenging of superoxide anion radical by PLP in dry DMF medium at 25 °C, $k_2 = 2.3 \times 10^{-2}$ L mol^{-1} s^{-1}, a value relatively low comparing to the second-order rate constant for scavenging superoxide anion radical as determined for plant phenols ranging from 10^3 to 10^7 L mol^{-1} s^{-1}.[23] The second-order rate

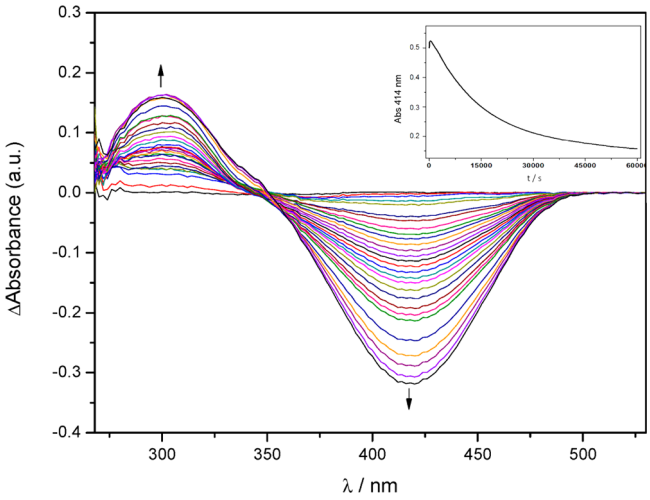

Figure 6. Differential UV−vis spectra of a reaction between 2.0 mM KO_2 and 0.1 mM PLP solutions in dry DMF at 25 °C. Inset: Decay of the band at 414 nm versus time.

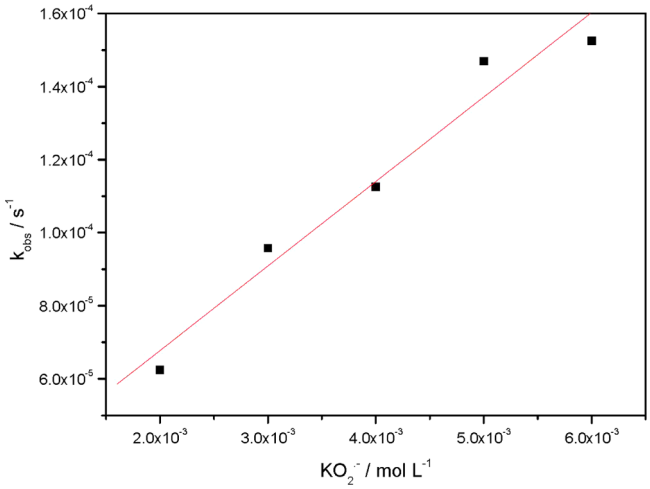

Figure 7. Observed rate constant for reaction of 0.1 mM PLP collected for increasing concentrations of KO_2.

constant for the reaction between PLP and superoxide anion radical may suggest that oxygen is involved in the photodegradation of PLP sensitized by flavin due to the generation of superoxide anion radical during the reduction of FMN neutral radical back to FMN and also contributing by reacting with PLP cation radical formed after the photoreductive quenching inhibiting the back-reaction which in effect serves as a physical quenching reflected in a lower quantum yield for photodegradation.

The structure of the photoreaction products was tentatively elucidated by direct infusion high-resolution accurate mass spectrometry (data not shown). However, it was only possible to establish the presence in the reaction mixture of PLP (m/z 246.01749, error of 0.8 ppm of the calculated value for $C_8H_9O_6NP$), FMN (m/z 457.11014, error of −3.8 ppm of the calculated value for $C_{17}H_{22}O_9N_4P$), lumicrome (m/z 241.07503, error of 12.5 ppm of the calculated value for $C_{12}H_9O_2N_4$), a PLP derivative characterized by the addition of one oxygen atom to PLP (m/z 262.01212, error of 0.8 ppm of the calculated value for $C_8H_9O_7NP$), a PLP derivative characterized by the absence of one carbon atom (m/z

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234.01750, error of 0.9 ppm of the calculated value for $C_7H_9O_6NP$), and a PLP derivative characterized by the hydrolysis of the phosphorus ester and the removal of an oxygen (m/z 182.04620, error of 1.8 ppm of the calculated value for $C_8H_8O_4N$).

In conclusion, it has been shown that flavins (vitamin B2) in food products fortified with B-vitamin may contribute to the vitamin B6 (pyridoxal) depletion upon light exposure leading to nutritional loss and deprived quality. At the same time, vitamin B6 has been shown to protect against the harmful photochemistry of vitamin B2 upon light exposure by an efficient photoreductive deactivation of the triplet-excited flavin through direct electron transfer or proton-coupled electron transfer from the hydroxy-aromatic ring to the triplet-excited flavin in an mechanism similar to that observed for phenols. Accordingly, vitamin B6 may act as a potent antioxidant in food products fortified with B-complex vitamins protecting sensitive molecules and structures against the light-induced oxidation sensitized by flavins.

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Notes

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REFERENCES

(1) Fletcher, R. J.; Bell, I. P.; Lambert, J. P. Public health aspects of food fortification: a question of balance. *Proc. Nutr. Soc.* **2004**, *63*, 605−614.

(2) Allen, L. H. B Vitamins: Proposed fortification levels for complementary foods for young children. *J. Nutr.* **2003**, *133*, 30005−30075.

(3) Lotfi, M.; Venkatesh Mannar, M. G.; Merx, R. J. H. M.; Naber-van den Heuvel,P. *Micronutrient Fortification of Foods. Current practices, research, and opportunities*; The Micronutrient Initiative (MI), International Development Research Center (IDRC)/ International Agricultural Center (IAC): Ottawa, 1996.

(4) Airado-Rodrigues, D.; Intawiwat, N.; Skaret, J.; Wold, J. P. Effect of naturally occuring tetrapyrroles on photooxidation in cow's milk. *J. Agric. Food Chem.* **2011**, *59*, 3905−3914.

(5) Cardoso, D. R.; Libardi, S. H.; Skibsted, L. H. Riboflavin as a photosensitizer. Effects on human health and food quality. *Food Funct.* **2012**, *3*, 487−502.

(6) Scurachio, R.; Skibsted, L. H.; Metzker, G.; Cardoso, D. R. Photodegradation of folate sensitized by riboflavin. *Photochem. Photobiol.* **2011**, *87*, 840−845.

(7) Natera, J.; Massad, W. A.; Garcia, N. A. Vitamin B1 as a scavenger of reactive oxygen species photogenerated by vitamin B2. *Photochem. Photobiol.* **2011**, *87*, 317−323.

(8) Hatchard, C. G.; Parker, C. A. A new sensitive chemical actinometer. II. Potassium ferrioxalato as a standard chemical actinometer. *Proc. R. Soc. London A* **1956**, *235*, 518−536.

(9) Huvaere, K.; Cardoso, D. R.; Homem-de-Mello, P.; Westermann, S.; Skibsted, L. H. Light-induced oxidation of unsatured lipids as sensitized by flavins. *J. Phys. Chem. B* **2010**, *114*, 5583−5593.

(10) Lu, C.; Lin, W.; Wang, W.; Han, Z.; Yao, S.; Lin, N. Riboflavin (VB2) photosensitized oxidation of 2′-deoxyguanosine 5′-monophosphate (dGMP) in aqueous solution: a transient intermediates study. *Phys. Chem. Chem. Phys.* **2000**, *2*, 329−334.

(11) Haggi, E.; Berolotti, S.; Garcia, N. A. Modelling the environmental degradation of water contaminants. Kinetics and mechanism of the riboflavin-sensitised-photooxidation of phenolic compounds. *Chemosphere* **2004**, *55*, 1501−1507.

(12) Gu, Q.; Kenny, J. E. Improvement of inner filter effect correction based on determination of effective geometric parameters using a conventional fluorimeter. *Anal. Chem.* **2009**, *81*, 420−426.

(13) Cardoso, D. R.; Franco, D. W.; Olsen, K.; Andersen, M. L.; Skibsted, L. H. Reactivity of bovine whey proteins, peptides and amino acids toward triplet riboflavin as studied by laser flash photolysis. *J. Agric. Food Chem.* **2004**, *52*, 6602−6606.

(14) Naik, D. B.; Hermann, R.; Mukherjee, T.; Naumov, S.; Brede, O. Ionization of mercapto- and hydroxypyridines by free electron transfer to alkyl chloride parent radical cations. *Res. Chem. Intermed.* **2005**, *31*, 113−133.

(15) Li, G.; Glusac, K. D. Light-triggered proton and electron transfer in flavin cofactors. *J. Phys. Chem. A* **2008**, *112*, 4573−4583.

(16) Bilski, P.; Li, M. Y.; Ehrenshaft, M.; Daub, M. E.; Chignell, C. F. Vitamin B6 (Pyridoxine) and Its Derivatives Are Efficient Singlet Oxygen Quenchers and Potential Fungal Antioxidants. *Photochem. Photobiol.* **2000**, *71*, 129−134.

(17) Rosein, G. E.; Miyamoto, S.; Bechara, E.; Di Mascio, P. Oxidação de proteínas por oxigênio singlete: mecanismos de dano, estratégias para detecção e implicações biológicas. *Quím. Nova* **2006**, *29*, 563−568.

(18) Rhem, D.; Weller, A. Kinetics and mechanism of electron transfer during fluorescence quenching in acetonitrile. *Ber. Bunsen-Ges. Phys. Chem.* **1969**, *73*, 8−9.

(19) Huvaere, K.; Olsen, K.; Skibsted, L. H. Quenching of triplet-excited flavins by flavonoids. Structural assessment of antioxidant activity. *J. Org. Chem.* **2009**, *74*, 7283−7293.

(20) Becker, E. M.; Cardoso, D. R.; Skibsted, L. H. Deactivation of riboflavin triplet-excited state by phenolic antioxidants: mechanism behind protective effects in photooxidation of milk-based beverages. *Eur. Food Res. Tecnhol.* **2005**, *221*, 382−386.

(21) Martin, C. B.; Walker, D.; Soniat, M. Density functional theory study of possible mechanisms of folic acid. *J. Photochem. Photobiol. A* **2009**, *208*, 1−6.

(22) Lu, C.; Bucher, G.; Sander, W. Photoinduced interactions between oxidized and reduced lipoic acid and riboflavin (vitamin B2). *Eur. J. Chem. Phys. Phys. Chem.* **2004**, *5*, 47−56.

(23) Gotoh, N.; Niki, E. Rates of interactions of superoxide with vitamin-E, vitamin-C and related-compounds as measured by chemiluminescence. *Biochim. Biophys. Acta* **1992**, *1115*, 201−207.