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Vitamin B1 as a Scavenger of Reactive Oxygen Species Photogenerated by Vitamin B2

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ABSTRACT

Kinetics and mechanism of photoprocesses generated by visible light-irradiation of the system riboflavin (Rf, vitamin B2) plus Thiamine (Th) and Thiamine pyrophosphate (ThDP), representing vitamin B1, was studied in pH 7 water. A weak dark complex vitamin B2–vitamin B1, with a mean value of 4 ± 0.4 M^{-1} is formed. An intricate mechanism of competitive reactions operates upon photoirradiation, being the light only absorbed by Rf. Th and ThDP quench excited singlet and triplet states of Rf, with rate constants in the order of 10^9 and 10^6 M^{-1} s^{-1}, respectively. With Vitamin B1 in a concentration similar to that of dissolved molecular oxygen in water, the quenching of triplet excited Rf by the latter is highly prevalent, resulting in the generation of $O_2(^1\Delta_g)$. Superoxide radical anion was not detected under work conditions. A relatively slow $O_2(^1\Delta_g)$-mediated photodegradation of Th and ThDP was observed. Nevertheless, Th and especially ThDP behave as efficient physical deactivators of $O_2(^1\Delta_g)$. The thiazol structure in vitamin B1 appears as a good scavenger of this reactive oxygen species. This characteristic, that presents at vitamin B1 as a potential photoprotector of biological entities against $O_2(^1\Delta_g)$ attack, was been experimentally confirmed employing the protein lisozime as a photo-oxidizable target.

INTRODUCTION

Vitamins constitute a particular group of relevant molecules in living organisms, essential for sustaining normal physiological activity and healthy life quality. Some vitamins are relatively labile and susceptible to certain chemical changes that may affect their specific biological roles (1,2). These changes are generated by different factors such as temperature, extreme pH values, reactions with native or externally added molecules and light exposure, among others (1,2).

Vitamins B1 and B2 are part of the vitamin-B complex for which thiamine (Th) and riboflavin (Rf) constitute the respective chromophoric moieties (see chemical structures in Scheme 1). As both vitamins are collectively concerned in biological interactions and occupy common microenvironments (3,4), may be mutually affected by potential photopromoted interactions when exposed to visible light. It is well known that vitamins B2, B6 and vitamin B1 derivatives produce reactive oxygen species (ROS), especially singlet molecular oxygen ($O_2(^1\Delta_g)$), upon adequate photoirradiation (5–7). Vitamin B1, as most of the biologically relevant molecules is transparent to visible light, whereas Rf is one of the most important endogenous visible-light absorbers (5). The latter has been postulated as a sensitizer for the *in vivo* photopromoted reactions, that can produce physiological changes in the surrounding molecules (5,8–10).

When Rf is photoirradiated, ROS and reactive radicals are formed (5,8). In a biological environment, closer molecules constitute the primary focus for the attack of such a species, being the chemical nature and concentration of these target molecules, oxygen availability and pH of the medium, the most important factors in determining the efficiency and mechanism governing the degradative photoprocess (11,12).

There is a sustained interest on photochemical reactions of biologically relevant compounds, due to the possible involvement of these events in naturally occurring and biomedical processes (13–16). Within this frame, the main aim of our study was the elucidation of possible effects on the lability of vitamins B1 and B2 upon photoirradiation with environmental light, typically visible light. This knowledge may contribute to the substantial understanding of unexpected biochemical transformations that could take place due to photochemical instability either *in vivo* or under current laboratory conditions.

In synthesis, in the present work we discuss results of a systematic kinetic and mechanistic study on the interaction of Rf-photogenerated ROS and radicals with vitamin B1, represented by thiamine (Th) and thiamine pyrophosphate (ThDP), under visible-light irradiation in aqueous medium. The photodegradability of vitamins B1 and B2, and the ability of the former to act as a natural photoprotector in biological environments were evaluated. The exclusive ($O_2(^1\Delta_g)$)-generator perinaphthenone (PN) (17) was employed as an auxiliary photosensitizer.

MATERIALS AND METHODS

Materials. Thiamine hydrochloride (Th), thiamine pyrophosphate chloride (ThDP), Rf, PN, superoxide dismutase (SOD) from bovine erythrocytes and lisozime (Liso), were purchased from Sigma Chem. Co. Deuterium oxide (D_2O; 99.9 atom % D) was from Aldrich. Sodium azide (NaN_3) was from Merck. All these compounds were used as received. Water was triply distilled and buffered solutions (KH_2PO_4, and $K_2HPO_4.3H_2O$, both from Aldrich) were employed to prepare pH/pD 7 aqueous solutions (18).

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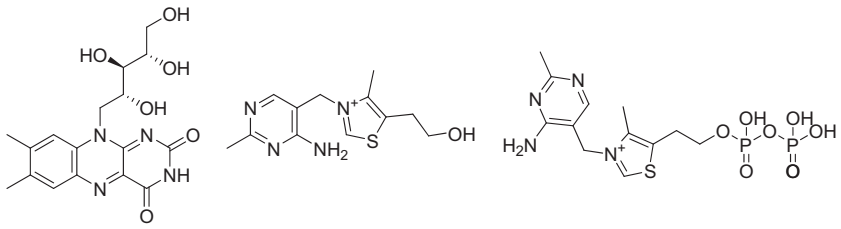

Vitamin B2 — Riboflavin (Rf)

Vitamin B1 — Thiamine (Th)

Vitamin B1 — Thiamine pyrophosphate (ThDP)

Scheme 1. Chemical structures of vitamin B2 (riboflavin, Rf) and vitamin B1 (thiamine, Th and thiamine pyrophosphate, ThDP).

Absorption and fluorescence measurements. Ground state absorption spectra were registered in a UV–Vis Shimadzu UV-2401PC spectrophotometer. Steady-state fluorescence was measured with a Spex Fluoromax spectrofluorimeter at 25 ± 1°C in air-equilibrated solutions. Excitation and emission wavelengths for Rf were 445 and 515 nm, respectively. Fluorescence lifetimes were determined with a time-correlated single photon counting (SPC) technique on an Edinburgh FL-9000CD instrument equipped with a blue LED (PicoQuant PLS-8-2-208). Excitation and emission wavelengths for Rf were 450 and 515 nm, respectively.

Continuous photolysis. Continuous aerobic photolysis of aqueous solutions containing Rf plus Ths, was performed a home-made photolyser with a 150-W quartz-halogen lamp provided with a filter (cutoff >400 nm).

The reactive rate constant, k_r, for the reaction of $O_2(^1\Delta_g)$ with each Th (process [13] in Scheme 2) was determined as previously described (19), using the expression $slope/slope_R = k_r\,[Ths]/k_{rR}\,[R]$, for which the knowledge of the reactive rate constant for the photo-oxidation of a reference compound, R, at similar concentration, is required, and where slope and $slope_R$ are the respective slopes of the first-order plots of Ths and R consumption, or oxygen consumption by the same compounds, under sensitized irradiation. Oxygen uptake in water was monitored with a 97-08 Orion electrode. Employing PN as a sensitizer, it was assumed that the reaction of $O_2(^1\Delta_g)$ with each Th is the only way of oxygen consumption. The reference R was FFA, with a reported pH-independent k_{rR} value in water of 1.2×10^8 M^{-1} s^{-1} (20).

Laser flash photolysis experiments. Argon-saturated aqueous solutions of Rf 0.04 mM were irradiated with a flash photolysis apparatus. A ns Nd:YAG laser system (Spectron) at 355 nm was used for excitation, employing a 150-W Xenon lamp as a source for 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 via a HPIB parallel interface to a PC where it was analyzed and stored. The disappearance of ^{3}Rf*, a species generated by the 355 nm pulse, was monitored from the first-order decay of the absorbance at 670 nm, a zone where the interference from other possible species is negligible. The decay was measured at low Rf concentration (typically 0.04 mM) and at low enough laser energy to avoid self-quenching and triplet–triplet annihilation. The rate constant for the interaction ^{3}Rf*–Ths (process [5]) was determined by the Stern-Volmer expression $1/^3\tau = (1/^3\tau_0) + k_{q3}$ [Ths], where $^3\tau$ and $^3\tau_0$ are the experimentally determined lifetimes of ^{3}Rf* in the presence and in the absence of Ths, respectively.

Time-resolved detection (TRPD) of $O_2(^1\Delta_g)$ phosphorescence. The overall quenching rate constant of deactivation of $O_2(^1\Delta_g)$ by each Th (k_t, the sum of k_q plus k_r, processes (13) and (14), respectively, Scheme 2) was determined using a previously reported system (21). Briefly, a Nd:YAG laser (Spectron) was used for the excitation (355 nm) of the sensitizer PN ($A_{355} = 0.3$), and the emitted radiation ($O_2(^1\Delta_g)$ phosphorescence at 1270 nm) was detected at right angles using an amplified Judson J16/8Sp germanium detector, after passing through two Wratten filters. The output of the detector was coupled to a digital oscilloscope and to a personal computer for the signal processing. Usually, 16 shots were needed for averaging, so as to achieve a good signal to noise ratio, from which the decay curve was obtained. Air-saturated solutions were employed in all the cases. In the dynamic determinations, D_2O instead of H_2O was used as a solvent in order to enlarge the lifetime of $O_2(^1\Delta_g)$ (20). The $O_2(^1\Delta_g)$ lifetimes were evaluated in the presence (τ_Δ) and in the absence ($\tau_{\Delta 0}$) of the quencher, and the data were plotted as a function of concentration, according to a simple Stern-Volmer treatment, $1/\tau_\Delta = 1/\tau_{\Delta 0} + k_t$ [Ths].

$Rf + Ths \rightleftharpoons [Rf\text{-}Ths]$	[1] Association constant K_{ass}
$Rf + h\nu \longrightarrow {}^1Rf^* \longrightarrow {}^3Rf^*$	[2]
${}^1Rf^* + Ths \longrightarrow Rf + Ths$ *or* $Rf^{\bullet-} + Ths^{\bullet+}$ *or* P_3	[3] rate constant k_{q1}
${}^3Rf^* + O_2({}^3\Sigma_g^-) \longrightarrow Rf^{\bullet+} + O_2^{\bullet-}$	[4]
${}^3Rf^* + Ths \longrightarrow Rf + Ths$ *or* $Rf^{\bullet-} + Ths^{\bullet+}$ *or* P_5	[5] rate constant k_{q3}
$Rf^{\bullet-} + H^+ \longrightarrow RfH^\bullet$	[6]
$2\,RfH^\bullet \longrightarrow Rf + RfH_2$	[7]
$RfH_2 + O_2({}^3\Sigma_g^-) \longrightarrow RfH_2^{\bullet+} + O_2^{\bullet-}$	[8]
$O_2^{\bullet-} + Ths$ or $Rf \longrightarrow P_9$	[9]
${}^3Rf^* + O_2({}^3\Sigma_g^-) \longrightarrow Rf + O_2({}^1\Delta_g)$	[10] rate constant k_{ET}
$O_2({}^1\Delta_g) \longrightarrow O_2({}^3\Sigma_g^-)$	[11] rate constant k_d
$O_2({}^1\Delta_g) + Ths \longrightarrow O_2({}^3\Sigma_g^-) + Ths$	[12] rate constant k_q
$O_2({}^1\Delta_g) + Ths \longrightarrow P_{13}$	[13] rate constant k_r
Being $k_t = k_r + k_q$	

Scheme 2. Possible reaction steps upon photoirradiation of vitamin B2 in the presence of a substrate transparent to the incident light.

RESULTS

Proposed mechanistic steps

The main photoinduced processes that can take place when a solution containing a potentially oxidizable substrate, such as vitamin B1 in this case, and a dye sensitizer, represented by Rf is irradiated with visible light in the presence of oxygen, are shown in self-defined Scheme 2 (22,23). These processes include dark and photoinduced reactions, both in the presence and in the absence of Ths, the prevalence of which usually depends on the experimental conditions and on the chemical nature of the involved compounds, Ths and the sensitizer. A similar Scheme has been previously discussed in regard with other biological relevant substrates (24). Two oxidative species are formed from ground state oxygen ($O_2({}^3\Sigma_g^-)$), $O_2^{\bullet-}$ (reaction [4]) and $O_2(^1\Delta_g)$ (reaction [10]), with quantum yields of

0.009 and 0.49–0.68, respectively (6,7,25). The discrepancy in the latter might be due to the employment of different experimental methodologies and different solvents.

Dark association Ths–Rf and quenching of $^1Rf^*$

Dark association between Rf and heteroaromatics has been reported (24,26). Nevertheless, no spectral perturbations owing to this process could be detected in the difference absorption spectra of Rf plus either of the Th or ThDP herein studied, within the concentration ranges employed in the stationary photoirradiation experiments, *i.e.* Rf *ca* 0.05 mM and Ths *ca* 0.5 mM. Even so, Rf fluorescence measurements in the presence and in the absence of Ths strongly suggest the occurrence of a dark association between vitamins B1 and B2. The fluorescence quenching method (27) was employed for evaluation of the apparent association constant (K_{ass}, step [1], Scheme 2) for the systems Rf–Th and Rf–ThDP, in aqueous solution. Rf presents an intense fluorescence emission band, centered at 515 nm, with a reported emission quantum yield (Φ_F) of 0.25 (33). In the presence of Ths, the fluorescence quenching of excited singlet Rf (^{1}Rf*, Scheme 2) produces a decrease in the stationary emission intensity, but the shape of the fluorescence spectrum does not change. The fluorescence decay of Rf in absence and in the presence of Ths, as determined by the SPC technique, was monoexponential. Figure 1 shows the respective Stern-Volmer plots obtained from stationary and time-resolved methods for the case of Th as a representative example.

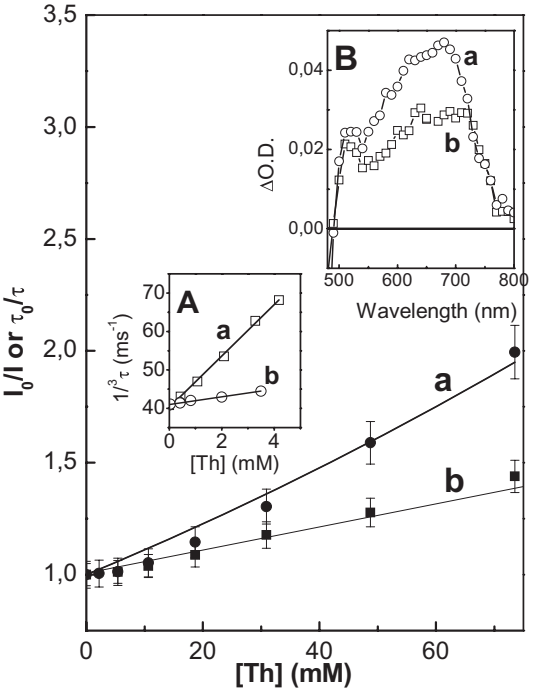

Figure 1. Stern-Volmer plots for the stationary (a) and time-resolved (b) fluorescence quenching of $^1Rf^*$ by Th. Inset (A): Stern-Volmer plot for the $^3Rf^*$ quenching by thiamine (Th) (a) and thiamine pyrophosphate (ThDP) (b). Inset (B). Transient absorption spectra of Rf (Abs 0.30 at 355 nm) in argon-saturated aqueous solution, in the absence (a) and in the presence (b) of 5 mM ThDP, taken 10 μs after the laser pulse. All in pH 7 aqueous solution.

The first one presents a neat positive curvature, whereas the plot for the time-resolved data is linear. This behavior corresponds to the typical case in which a fluorescent probe is simultaneously quenched by dark association with its ground state and by collisional interaction with its excited singlet state. It is also well known that these systems can be treated using the modified Stern-Volmer equation (28,29):

$$I_0/I = (1 + K_D[\text{Ths}])(1 + K_{ass}[\text{Ths}]) \qquad [14]$$

where I_0 and I represent the stationary fluorescence intensities in the absence and in the presence of Ths, respectively. The constant K_D, being $K_D = k_{q1} \times {}^1\tau_0$, accounts for the dynamic component of the fluorescence quenching and was determined independently of the time-resolved measurements. From Fig. 1, the respective K_D values of 5.28 and 33.6 M^{-1} were found for Th and ThDP. Employing the obtained $^1\tau_0$ value of 4.8 ns, which is in excellent agreement with literature reports (8), k_{q1} values of 1.1 10^9 M^{-1} s^{-1} for Th and 7.0 10^9 M^{-1} s^{-1} for ThDP were calculated. Hence, the respective values of 5 ± 0.5 and 3 ± 0.2 M^{-1} were obtained for K_{ass} by nonlinear least square fitting employing the previously mentioned values.

Continuous photolysis

The continuous photoirradiation of an air-equilibrated pH 7 aqueous solution of individual *ca* 0.06 mM Th and ThDP, sensitized by 0.04 mM Rf produces different changes in the whole absorption spectrum of the mixture, which reflect the addition of chemical changes in both, the substrates and the sensitizer, as shown in Fig. 2 for Th as a representative example. The corresponding absorption spectrum of Rf in pH 7 aqueous solution is also shown in Fig. 2, for comparative purposes. In parallel, the photoirradiation of the mixtures Rf–Ths gave rise to oxygen consumption.

The pieces of evidence herein shown strongly suggest that the incidence of visible light on solutions containing the system Rf–Ths starts a series of processes that could include reactions of Rf electronically excited states and ROS with Ths ground state and also with ground state of the very Rf. Following, we systematically investigated these possibilities.

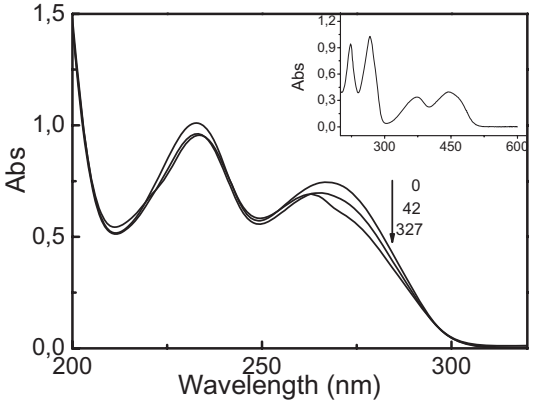

Figure 2. Absorption spectra of 0.05 mM riboflavin (Rf) plus 0.06 mM thiamine (Th) *vs* 0.05 mM Rf in pH 7 aqueous solution, upon visible light photoirradiation. Numbers on the spectra represent photoirradiation time, in seconds. Inset: Absorption spectrum of Rf 0.03 mM in pH 7 aqueous solution.

Table 1. Rate constant values for the quenching of ^{1}Rf* (k_{q1}) and ^{3}Rf* (k_{q3}), overall (k_t) and reactive (k_r) quenching of $O_2(^1\Delta_g)$ and by thiamine (Th) and thiamine pyrophosphate (ThDP), and relative rates of oxygen consumption ($\Delta O_2/\Delta t$) upon perinaphthenone (PN) and riboflavin (Rf) sensitization in pH 7 air-equilibrated solutions. The determination of k_t was made in pD 7 aqueous solution.

Compound	k_{q1} ($\times 10^9$)	k_{q3} ($\times 10^6$)	k_t ($\times 10^8$)	k_r ($\times 10^7$)	k_r/k_t	Relative $\Delta O_2/\Delta t$ PN	Rf
Thiamine	1.1 ± 0.05	5.1 ± 0.3	4.0 ± 0.1	3.3 ± 0.1	0.083	1	1
Thiamine pyrophosphate	7.0 ± 0.06	2.1 ± 0.5	1.4 ± 0.1	0.55 ± 0.1	0.039	0.16	0.58

The interaction Ths–^{3}Rf*

By monitoring as described in the experimental section, it was observed that the disappearance of Rf triplet state (^{3}Rf*) only decreased in the presence of Ths concentrations at least one order of magnitude superior to those employed in the stationary photolysis experiments. A Stern-Volmer treatment of the triplet quenching (Fig. 1, inset A), yielded the values for the bimolecular rate constants k_{q3} (reaction [5]) (Table 1).

The transient absorption spectrum of the Rf solution 2 μs after the laser pulse (Fig. 1, inset B) is similar to that reported for the Rf neutral triplet state in water (23). The corresponding trace in Fig. 1, inset B, recorded at 40 μs after the laser pulse, in the presence of 2 mM Th, under identical experimental conditions, does not show any modification that could suggest the generation of a new transitory species, different from ^{3}Rf*. The same qualitative result was observed for ThDP.

Involvement of ROS

In Fig. 3 are shown the results of oxygen uptake experiments obtained upon photoirradiation of 0.05 mM Rf and of the mixtures 0.05 mM Rf plus 0.5 mM Th and ThDP. The relative values for the rate of oxygen consumption are collected in Table 1. The participation of ROS was evaluated through oxygen consumption experiments in the presence of specific ROS quenchers (Fig. 3). Thus, the presence of NaN_3 (10 mM) practically suppressed oxygen consumption, whereas the presence of the enzyme SOD (1 μg mL^{-1}) did not affect this rate, all for both Ths. The salt NaN_3 and the enzyme SOD are selective quenchers for the species $O_2(^1\Delta_g)$ and $O_2^{\bullet-}$, respectively, and concentrations similar to those herein employed have been formerly used in order to confirm or discard the participation of any of said species in a given reaction mechanism (23,30,31). Some of these results, shown in Fig. 3, strongly suggest the involvement of the oxidative species $O_2(^1\Delta_g)$.

Determination of the rate constants k_t and k_r

The extent of Ths as $O_2(^1\Delta_g)$ quenchers was evaluated through the rate constants k_t and k_r (being $k_t = k_q + k_r$ (reactions [12] and [13]). They were determined in pD 7 aqueous solution, as described in the experimental section. PN was employed as a dye sensitizer instead of Rf in order to avoid possible interferences produced by photodecomposition of vitamin B2 and eventual interactions between its electronically excited states and Ths. PN is an exclusive $O_2(^1\Delta_g)$-generator extremely photostable upon photoirradiation, with a reported quantum yield *ca* 1 for photoproduction of the oxidative species (17).

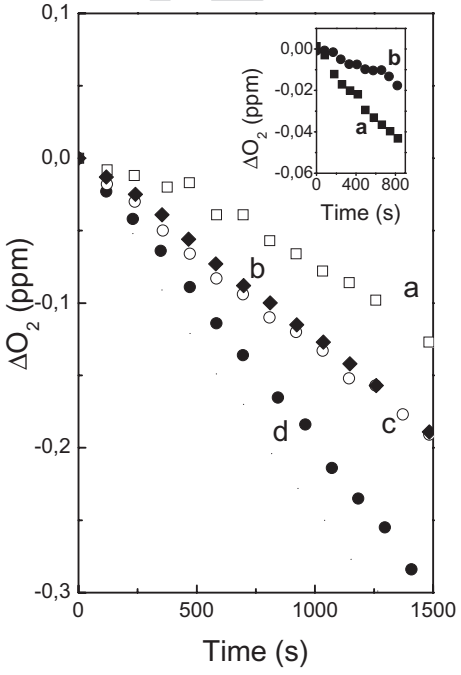

Figure 3. Rate of oxygen consumption in photoirradiated solutions of: 0.05 mM riboflavin (Rf) (a); 0.05 mM Rf plus 0.5 mM thiamine pyrophosphate (ThDP) (b); 0.05 mM Rf plus 0.5 mM ThDP plus 1 μg mL^{-1} superoxide dismutase (c); 0.05 mM Rf plus 0.5 mM Th. Inset: Rate of oxygen consumption in photoirradiated solutions of: PN A_{364} = 0.4 plus 1.5 μg mL^{-1} Liso (a) and PN A_{364} = 0.4 plus 1.5 μg mL^{-1} Liso plus 0.5 mM ThDP (b).

The $O_2(^1\Delta_g)$-phosphorescence was quenched by Th and ThDP in the sub-mM concentration range, as shown in Fig. 4. Through a simple Stern-Volmer treatment, the overall quenching constant k_t was determined. This experiment unambiguously demonstrates an interaction between $O_2(^1\Delta_g)$ and Ths, which may be merely physical in nature (process [12]), purely reactive (process [13]) or a simultaneous composition of both mechanisms.

The rate constant values k_r (reaction [13]), collected in Table 1, were determined for both Ths through oxygen uptake experiments, using the method described by Scully and Hoigné (19) and again employing PN as a dye sensitizer, through the first order plots shown in Fig. 4, inset. Typically, PN (A_{364} = 0.4), 0.5 mM Ths and 0.5 mM FFA, the reference, were employed in these experiments, in buffered pH 7 aqueous solutions.

The $O_2(^1\Delta_g)$-mediated photo-oxidation quantum efficiency Φ_r ($\Phi_r = k_r$ [Ths]/($k_d + k_t$ [Ths])) is not easy to evaluate,

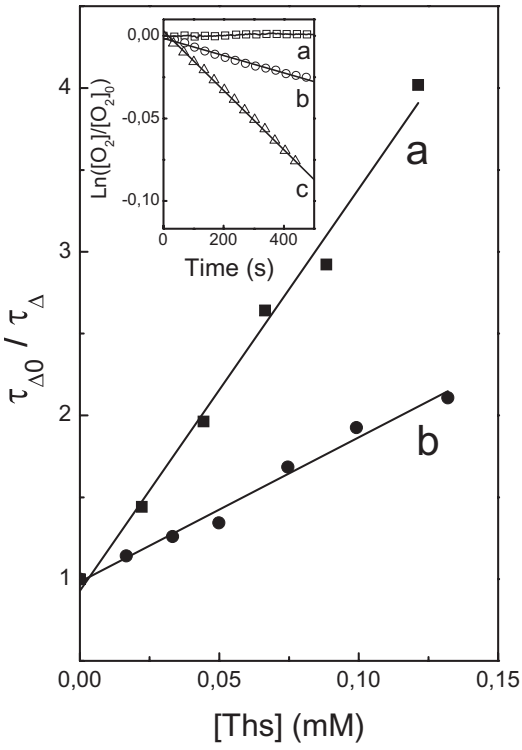

Figure 4. Stern-Volmer plot for the quenching of O_2 ($^1\Delta_g$) phosphorescense by thiamine (Th) (a) and thiamine pyrophosphate (b) in pD7 D_2O. Inset: First-order plots for oxygen uptake upon visible-light irradiation in aqueous solutions containing: PN A_{364} (a); PN A_{364} plus 0.5 mM Th (b) and PN A_{364} plus 0.5 mM FFA (c).

particularly in biological environments (32), because its determination includes the knowledge of the actual concentration of the photo-oxidizable substrates, represented by the Ths in this case. A simpler and useful approach is the evaluation of the k_r/k_t ratio (Table 1), which indicates the fraction of overall quenching of $O_2(^1\Delta_g)$ by the substrate that effectively leads to a chemical transformation.

Photoprotective effect of Ths towards protein photo-oxidation

In order to evaluate the potential photoprotective effect of Ths towards photo-oxidizable biological targets, the rate of PN-sensitized photo-oxidation of the protein lisozime, in a concentration 1.5 μg mL^{-1} in pH 7 water was evaluated, in the presence and in the absence of 5 mM ThDP, a biologically active form of vitamin B1 (2). It is known that the PN-sensitized photo-oxidative degradation of Liso in aqueous solution operates through a $O_2(^1\Delta_g)$-mediated mechanisms (33,34). Results in Fig. 3, inset, show that the photo-oxidation rate of Liso, as estimated by the rate of oxygen consumption upon PN-photosensitization, suffers a delay of *ca* 30% in the presence of ThDP, for which the proportion of physical scavenging of $O_2(^1\Delta_g)$ is highly prevalent, reaching a k_r/k_t value of 0.039 (Table 1).

DISCUSSION

According to our knowledge the only study devoted to the photochemistry of mixtures of vitamin B1, represented by Th and Rf has been published by Vaid in 1997 (35). Most effort of the work was directed to the elucidation of photoproducts. As a result, UV photoirradiation of the vitamins mixture gave rise to the oxidation products 2,7-dimethyl-5*H*-thiachromine-8-ethanol (thiochrome) and 2-methyl-4-amino-5-aminomethyl-pyrimidine,α-aceto-γ-mercaptopropanol. Thiochrome seems to be the habitual oxidative product of vitamin B1, independently of the reaction involved. It has also been described by Petrov (36) as a metabolic oxidation product of Th, and it was more recently reported as generated during the electrochemical oxidation of the vitamin (37).

Comparative experiments on the velocity of Th photodegradation upon direct photoirradiation in different mixtures with Rf are described in the work by Vaid (35). The main results indicate that the presence of Rf inhibits the photodegradation of Th, being the decomposition of vitamin B1 faster in the acidic pH range.

Turning to our work, kinetic and mechanistic aspects arising from the experimental results obtained on the dark and photoinduced interactions Rf–Ths can be analyzed as follows:

Rf–Ths dark association

The very low values determined for K_{ass} (expression [1]) denote a weak dark-interaction between Rf and Ths and indicate that the respective fractions of complexed vitamins can be ignored under the experimental conditions employed in our work, *i.e.* Rf *ca* 0.02 mM and Ths in the sub-mM concentration range.

Interaction of Ths with Rf electronically excited states and ROS generation

The oxygen photoconsumption observed in the systems Rf–Ths and PN–Ths may be due to the action of ROS.

The species $O_2^{\bullet-}$ and $O_2(^1\Delta_g)$ are usually present in aerobic Rf-photosensitized processes in aqueous solutions (25). Superoxide radical anion is straightforwardly formed through the interaction of ^{3}Rf* with dissolved oxygen, according to reaction [4]. Nevertheless, the quantum yield for this process is quite low, close to 0.009 (25), and the stationary concentration of $O_2^{\bullet-}$ produced by this pathway must be disregarded. Even so, it has been profusely reported (21–23) the generation of $O_2^{\bullet-}$ starting with the quenching of electronically singlet and/or triplet excited states of Rf by electron donor substrates, a situation typically represented by reactions [3] and [5]. The species $Rf^{\bullet-}$ is protonated (reported pK_a = 8.3 for the deprotonation of RfH$^\bullet$, reaction [6]) (38) and subsequently, through reactions, [7] and [8] $O_2^{\bullet-}$ is generated. It may react with Ths by step [9], constituting a possible oxygen consumer pathway.

According to our experimental results, both singlet and triplet electronically excited states of Rf are quenched by Ths in aqueous solutions, in processes represented by the respective equations [2] and [5] of Scheme 2. The respective rate constant reach values close to the diffusional limit in the case of k_{q1} and the opposite, extremely low values in the case of k_{q3}. A simple calculation through a Stern-Volmer treatment employing the mean values for both Ths of 4×10^9 M^{-1} s^{-1} for k_{q1}, and 3.5×10^6 M^{-1} s^{-1} for k_{q3}, indicates that the lifetime of ^{1}Rf* and

$^3Rf^*$ under stationary photoirradiation conditions—0.5 mM Ths—only decreases around 1%, and 2.5%, respectively. This fact indicates that the fraction of electronically excited singlet and triplet Rf intercepted by Ths is not enough to generate significant concentrations of any new transitory species, in particular $Rf^{\bullet}$. This argument is also supported by two additional experimental findings. First the absence of modifications in the oxygen consumption rates by the photoirradiated system Rf–Ths when run in the presence of the enzyme SOD, a specific $O_2^{\bullet -}$ scavenger. Second, the constancy in the spectral shape of the transient species detected in the laser flash photolysis experiments, indicating that only $^3Rf^*$ was present, even at relatively long times after excitation and at relatively high Ths concentrations.

Quenching of $O_2(^1\Delta_g)$ by Ths and Rf, and photoprotective effect exerted by Ths

Regarding $O_2(^1\Delta_g)$, its presence was unambiguously demonstrated by the TRPD experiments whereas its participation in the oxygen-consuming runs was indirectly supported by the suppression of oxygen uptake in the photoirradiated systems Rf–Ths and PN–Ths both in the presence of NaN_3.

The overall rate constants for the interaction of Ths with $O_2(^1\Delta_g)$, in the range of 10^7–10^8 M^{-1} s^{-1} (Table I), indicate that Th and ThDP are fairly good quenchers of this ROS.

Both k_t and k_r values are moderately higher for Th than for the pyrophosphate derivative. At least two main reasons could account for these differences. One lies on possible oxidation potential differences (unfortunately no oxidation potential values for vitamin B1 and related compounds are available in the literature) and the second one can be due to an eventual protective effect exerted by the phosphate groups towards the effective attack of the species $O_2(^1\Delta_g)$ in solution.

Vitamin B1 can be structurally described as formed by the composition of an amino-methyl-pyrimidine and a thiazole moiety. We think that the thiazole component is responsible for $O_2(^1\Delta_g)$ quenching since, according to previous results from our laboratory (39), amino-pyrimidine and methyl-pyrimidine in D_2O did not show neither chemical nor physical interaction with the oxidative species.

A positive result that arises in the context of oxidative interactions between biomolecules, in this case represented by the couple vitamin B1–vitamin B2, is the dominant physical scavenging of $O_2(^1\Delta_g)$, the oxidative B2-photogenerated species. The considerably low values for the quotient k_r/k_t exhibited by Ths (Table 1), is a desirable quality. The final result is the elimination of $O_2(^1\Delta_g)$ without appreciable loss of the scavenger. This action, in a biological environment, represents a protection for proteins, DNA and other cell matrix components, which due to their known reactivity and high local concentration, constitute the primary targets for the attack of ROS or other reactive species generated upon Rf-photosensitisation (40,41). This photoprotective effect exerted by vitamin B1 was evident as observed by the clear decrease in the rate of oxygen uptake of the system Liso + ThDP as compared with that of the protein alone, upon PN-sensitization. Liso, with $k_r = 2.5\ 10^8$ M^{-1} s^{-1} and $k_r/k_t = 0.19$ in D_2O (17), is frequently employed to model photodynamic effect in proteins (34,35).

Turning to the photodegradation of Ths, their relative rates of oxygen uptake at pH 7 employing Rf or PN as sensitizers (Table 1) show a similar trend (oxidation rate Th > ThDP) suggesting, in principle the operation of a common oxidative mechanism. Nevertheless, the kinetic behavior is not totally parallel. In other words, the ratio between the mentioned relative rates of both Ths is not the same with both sensitizers. We think that this fact simply arises from the additive reactive quenching exerted by Rf, affecting more markedly the oxygen uptake rate of ThDP, the substrate that exhibits lower rate constants for reactive $O_2(^1\Delta_g)$ scavenging. A rate constant $k_r = 6 \times 10^7$ M^{-1} s^{-1} has been reported for Rf in MeOH (25). This possibility is absent in PN, which is unreactive towards $O_2(^1\Delta_g)$ (42).

CONCLUSIONS

The visible-light irradiation of a mixture of Rf and vitamin B1 yields mainly the ROS $O_2(^1\Delta_g)$ which is formed by energy transfer from triplet excited Rf to ground state oxygen. Although Ths interact with electronically excited Rf, the fraction of excited states intercepted, with vitamin B1 in the sub-mM range, is not enough to generate detectable amounts of any new transitory species, in particular $Rf^{\bullet}$ and $O_2^{\bullet -}$ in an ulterior step. At physiological pH values, Ths comply the requirements for potentially efficient photoprotectors of biologically active targets, exhibiting relatively moderate to high values for the overall rate constants of $O_2(^1\Delta_g)$-scavenging and a low proportion of reactive quenching.

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