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Vitamin B₆ (Pyridoxine) and Its Derivatives Are Efficient Singlet Oxygen Quenchers and Potential Fungal Antioxidants

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ABSTRACT

Vitamin B₆ (pyridoxine, 1) and its derivatives: pyridoxal (2), pyridoxal 5-phosphate (3) and pyridoxamine (4) are important natural compounds involved in numerous biological functions. Pyridoxine appears to play a role in the resistance of the filamentous fungus *Cercospora nicotianae* to its own abundantly produced strong photosensitizer of singlet molecular oxygen (¹O₂), cercosporin. We measured the rate constants (k_q) for the quenching of ¹O₂ phosphorescence by 1–4 in D₂O. The respective total (physical and chemical quenching) k_q values are: $5.5 \times 10^7 M^{-1} s^{-1}$ for 1; $7.5 \times 10^7 M^{-1} s^{-1}$ for 2, $6.2 \times 10^7 M^{-1} s^{-1}$ for 3 and $7.5 \times 10^7 M^{-1} s^{-1}$ for 4, all measured at pH 6.2. The quenching efficacy increased up to five times in alkaline solutions and decreased ~10 times in ethanol. Significant contribution to total quenching by chemical reaction(s) is suggested by the degradation of all the vitamin derivatives by ¹O₂, which was observed as declining absorption of the pyridoxine moiety upon aerobic irradiation of RB used to photosensitize ¹O₂. This photodegradation was completely stopped by azide, a known physical quencher of ¹O₂. The pyridoxine moiety can also function as a redox quencher for excited cercosporin by forming the cercosporin radical anion, as observed by electron paramagnetic resonance. All B₆ vitamins fluoresce upon UV excitation. Compounds 1 and 4 emit fluorescence at 400 nm, compound 2 at 450 nm and compound 3 at 550 nm. The fluorescence intensity of 3 increased ~10 times in organic solvents such as ethanol and 1,2-propanediol compared to aqueous solutions, suggesting that fluorescence may be used to image the distribution of 1–4 in *Cercospora* to understand better the interactions of pyridoxine and ¹O₂ in the living fungus.

INTRODUCTION

The term vitamin B₆ is used to describe all biologically interconvertible forms of pyridoxine that include pyridoxine, pyridoxal, pyridoxal 5-phosphate and pyridoxamine. Vitamin B₆ is an essential co-factor in numerous enzymatic reactions involved primarily in amino acid metabolism. All living organisms require vitamin B₆ and they must either synthesize it or, like humans, derive it from nutrients. In addition to its role as a vital co-factor, our recent findings (1) suggest that vitamin B₆ may function as an antioxidant by interacting with singlet molecular oxygen during (photo)oxidative stress in the filamentous fungus *Cercospora nicotianae*. The wild-type strain of this fungus produces and is highly resistant to the potent photosensitizer cercosporin and is also highly resistant to other singlet oxygen photosensitizers of diverse structure and solubility.

Production of *C. nicotianae* mutant strains (2,3) sensitive to both cercosporin and other photosensitizers led to the identification of a gene apparently required for the resistance exhibited by the wild-type strain (4,5). Further characterization of this gene (1) revealed that it encoded a component necessary for pyridoxine synthesis in *C. nicotianae* as well as in other organisms and that pyridoxine and its derivatives are capable of quenching singlet oxygen *in vitro*. Other vitamins such as vitamin C and vitamin E are powerful antioxidants and scavengers of active oxygen species including singlet oxygen, but the ability of vitamin B₆ to quench singlet oxygen had not previously been reported.

Singlet oxygen (¹O₂) is a strong oxidizer and a potent initiator of radical oxidation in biological systems. It is best detected by its infrared phosphorescence at 1270 nm, which, although difficult to measure, is very specific for ¹O₂ and is usually free of spectral interference from other emissions. The phosphorescence is quenched by antioxidants, and the rate constants for this quenching may be a direct and convenient measure of an antioxidant potential. We measured the rate constants for ¹O₂ quenching by pyridoxine and its derivatives, and found that they are strong quenchers and good substrates for ¹O₂, especially in an aqueous environment. We also characterized pyridoxine fluorescence that may help to determine the distribution of pyridoxine in the fungus by fluorescence microscopy.

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MATERIALS AND METHODS

Chemicals. Ethanol, 1,4-dioxane, 1,2-propanediol (all spectrophotometric grade), Triton X-100, rose bengal (RB) † and 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) were purchased from Aldrich Chemical Co. (Milwaukee, WI). The DMPO was vacuum distilled and stored at -20°C . Deuterium oxide was from Cambridge Isotope Laboratories (Andover, MA). The pyridoxine vitamers and surfactant benzalconium chloride were purchased from Sigma Chemical Co. (St. Louis, MO). Cercosporin was extracted from cultures of *Cercospora kikuchii* strain PR as previously described (6). All experiments were performed using freshly prepared air-equilibrated solutions at room temperature.

Absorption and fluorescence spectra. Absorption spectra were measured using an HP diode array spectrophotometer model 8452A (Hewlett Packard Co., Palo Alto, CA). Fluorescence spectra were recorded on an SLM SPC 823-SMC 220 spectrofluorometer (SLM Instruments, Urbana, IL) as described previously (7). The relative number of absorbed photons at the excitation wavelength was calculated using the Beer–Lambert law.

Singlet oxygen detection. Singlet oxygen phosphorescence was recorded on a steady-state $^1\text{O}_2$ spectrophotometer (8) featuring an optimized optical system as in our pulse $^1\text{O}_2$ spectrophotometer (9). Samples were excited from a 500 W mercury lamp operating at 300 W through appropriate filter(s). The $^1\text{O}_2$ phosphorescence spectra were recorded over the range of 1200–1350 nm and were normalized to the same number of absorbed photons (10) at the excitation wavelength.

Singlet oxygen lifetimes were measured using a laser pulse spectrometer described in detail elsewhere (9). In this study, the apparatus utilized a Surelite II laser (Continuum, Santa Clara, CA) for excitation. A germanium diode (model 403 HS, Applied Detector Corporation, Fresno, CA) in conjunction with an efficient optical system was used for signal detection. Data were acquired on an HP 54111d digitizing oscilloscope (Hewlett Packard, Colorado Springs, CO) interfaced to a PC computer. Singlet oxygen was produced by single pulse excitation at 532 nm of RB as a photosensitizer. The $^1\text{O}_2$ lifetime was calculated from a monoexponential decay of its phosphorescence.

Oxidation of pyridoxine derivatives. Oxidation of pyridoxine derivatives by $^1\text{O}_2$ was performed at 25°C using a 450 W xenon–mercury lamp and a combination of a cutoff filter (400 nm) and an interference filter (548 nm) for RB excitation. The sample was placed in a closed 1 cm pathlength cuvette and was stirred during irradiation. The absorption spectra were taken at appropriate intervals to obtain up to a 50% decrease in pyridoxine absorption due to photooxidation while the absorption spectrum of RB was unchanged. The photooxidation rate was normalized, if necessary, using the number of photons absorbed by RB, allowing quantitative interpretation of photooxidation data (7,10).

The initial rates of oxidation were taken for calculations that were performed assuming a steady-state approximation for $^1\text{O}_2$ production and decay. The relative rate constant values, $k_r(\text{rel}) = I_x\phi_{\Delta}k_t(P)$ were calculated from the initial consumption rates assuming 1:1 stoichiometry for consumed oxygen and decayed vitamer. The following equation was applied (11):

$$\frac{r(k_d + k_q[P_0])}{[P_0]} = I\phi_{\Delta}k_t^{[P]}$$

where r is the measured oxidation rate; k_d , the rate constant of the natural $^1\text{O}_2$ decay; k_q , the measured total (chemical plus physical) $^1\text{O}_2$ quenching rate constant; $[P_0]$, the initial concentration of pyridoxine substrate; I , the irradiation rate for photons absorbed by RB (not measured here); and ϕ_{Δ} , the quantum yield of $^1\text{O}_2$ production. The values of k_d and k_q were measured in separate experiments in D_2O solutions (Table 1). Because the $I\phi_{\Delta}$ factor was constant in all experiments, $k_r(\text{rel})$ values allow a direct comparison of the oxidation efficacy for all B_6 vitamers.

Electron paramagnetic resonance (EPR) measurements. The EPR spectra were measured using an X-band E-Line Century Series EPR

spectrometer (Varian, Palo Alto, CA) equipped with a TM110 cavity. Samples were irradiated directly inside the microwave cavity of the spectrometer with light from a 1 kW xenon lamp (Krotos) after passing through a cutoff filter transmitting above 300 nm. Spectra were accumulated on a PC and stimulated using software described elsewhere (12).

RESULTS AND DISCUSSION

The structures of pyridoxine (**1**), pyridoxal (**2**), pyridoxal 5-phosphate (**3**) and pyridoxamine (**4**) are shown in Scheme 1. The vitamers have pH-sensitive groups and the reported pK_a values (13) are as follows: 5.0 and 8.96 for **1**; 4.2 and 8.66 for **2**; 2.5, 4.14, 6.2 and 8.69 for **3**; and 3.37, 8.01 and 10.13 for **4**. While it may be expected that pH will influence the spectral and photochemical properties of these vitamers, we mostly focused our attention on the physiologically relevant pH values (Table 1). All vitamers can exist in several ionization forms that may interact differently with singlet oxygen, and there will be a contribution from more than one species at physiological pH. As we feel that the dissociation of the $-\text{OH}$ group has the potential to influence greatly the charge density on the pyridoxine ring (and subsequent interaction with $^1\text{O}_2$) we did not assign the quenching or oxidation rates to a particular ionic species. We believe that the experimentally observed values are sufficient and more relevant to explain the vitamins' oxidation under physiologically relevant conditions.

Absorption and fluorescence

Information on the absorption and fluorescence properties was needed to select suitable conditions for photochemical experiments with the B_6 vitamers. In addition, differences in the intensity and/or position of the spectra in different chemical environments will have utility in determining vitamer localization in the *Cercospora* fungus and in determining the concentration of these important vitamins in fungal extracts using chromatography. The B_6 vitamers absorb light in the UV region and fluoresce. The absorption spectra of **1**, **2** and **4** are very similar (Fig. 1A). However, the spectrum is red shifted for **3**. This shift is probably due to the electron-withdrawing power of the phosphate and aldehyde groups (Scheme 1), which promote the dissociation of the phenol $-\text{OH}$ group in aqueous solutions. In contrast, the pyridoxine spectrum in ethanol is blue shifted by ~ 38 nm (Table 1). The electron-withdrawing effect is even more pronounced in the fluorescence spectra that are strongly red shifted for **3** (Fig. 1B). Although the absorption spectra of **3** in different solvents are similar (Fig. 2A), the fluorescence intensity is strong in alcohol and the spectrum position is distinct compared to the other pyridoxine derivatives (Fig. 2B). This suggests that the fluorescence of **3** will probably not suffer interference from other forms of the vitamin during fluorescence imaging in the fungus. In preliminary experiments we were able to obtain a fluorescence image from the fungus after incubation with pyridoxal phosphate.

Quenching of $^1\text{O}_2$ phosphorescence

We chose RB as a noninterfering photosensitizer to produce $^1\text{O}_2$ for quenching measurements and for pyridoxine oxidation assays because its UV absorption spectrum does not

†Abbreviations: DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; EPR, electron paramagnetic resonance; RB, rose bengal.

Table 1. Quenching of $^1\text{O}_2$ by pyridoxine and derivatives and their oxidation rate by $^1\text{O}_2$

Compound (P)	Buffer or solvent	$k_q \times 10^{-7}$ $M^{-1} s^{-1}$	Observed oxidation rate of P (μM $\times \text{min}^{-1}$)	[P]* μM	Relative oxidation rate constant ($k_r(\text{rel})$ $\times 10^{-3}$)	Extinction coefficient ($M^{-1} \text{cm}^{-1}$ $\times 10^{-3}$)	λ_{max} (nm)
Pyridoxine hydrochloride	pD = 12	24.9 ± 0.1					
	pH = 8.2	12.8^\dagger	54.3	176	77	6.32	322
	pD = 7.4	$10.3 \pm 0.6^\ddagger$					
	pH = 7.2	9.6^\dagger	30.6	123	59	6.50	322
	pH = 6.3	6.3^\dagger	35.9	168	51	6.29	322
Pyridoxine free base	pD = 6.1	5.51 ± 0.04					
	Ethanol	0.345 ± 0.003	5.4	202	2.1	5.87	286
Pyridoxal hydrochloride	pD = 12	60.27 ± 0.02					
	pH = 8.2	12.9^\dagger	27.8	70	94	7.63	316
	pD = 8	10.01 ± 0.06					
	pH = 7.2	8.5^\dagger	13.2	55	56	8.61	316
	pD = 7.4	$8.70 \pm 0.14^\ddagger$					
	pH = 6.3	7.7^\dagger	10.6	65	38	8.59	316
	pD = 6.1	7.5 ± 0.2					
Pyridoxamine dihydrochloride	pH = 8.2	10.6	38.9	72.8	125	6.85	322
	pD = 7.4	$9.9 \pm 0.2^\ddagger$					
	pH = 7.2	9.7^\dagger	15.5	43	83	7.18	322
	pH = 6.3	8.9^\dagger	17.7	84	49	7.21	322
Pyridoxal 5-phosphate	pD = 12	40.0 ± 0.2					
	pD = 6.1	8.79 ± 0.03					
	pH = 8.2	12.2^\dagger	91.2	194	118	4.93	388
	pD = 7.4	$6.2 \pm 0.2^\ddagger$					
	pH = 7.2	5.1^\dagger	36.7	164	53	5.14	388
	pH = 6.3	5^\dagger	22.2	167	31	4.92	388

*Concentrations at which the pyridoxine oxidation rates were measured.

† Values estimated from the k_q -pH dependence in D_2O and used to calculate the relative oxidation rate constants. Quenching and photooxidation were measured using RB to produce $^1\text{O}_2$ in D_2O and H_2O solutions, respectively. In the photooxidation experiments, each time 3.8 mL of solution was used for irradiation. Photooxidation rates were calculated from the absorption spectra using extinction coefficient at the λ_{max} after RB absorption spectrum was subtracted. Observed oxidation rate is presented as the decrease in vitamin concentration in μmol per minute of irradiation. Phosphate buffers (50 mM) were used in all experiments performed at room temperature in air-saturated solutions. Singlet oxygen lifetime measured in ethanol and water was 14.2 μs and 4.4 μs , respectively. Calculation of relative oxidation rates, $k_r(\text{rel.})$, is described in the Materials and Methods.

‡ Reported in Ehrenshaft *et al.* (1).

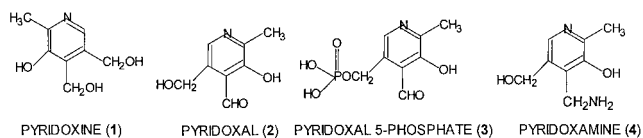
overlap with that of the vitamins, thus making it possible to detect substrate oxidation spectrally. We have found that pyridoxine, pyridoxal, pyridoxal 5-phosphate and pyridoxamine all efficiently quench $^1\text{O}_2$ phosphorescence. The total (physical and chemical quenching) rate constant k_q values are in the range of 10^7 – $10^8 M^{-1} s^{-1}$ (Fig. 3, Table 1). The quenching was more efficient in alkaline solution but decreased by about one order of magnitude in ethanol when pyridoxine free base was used as a quencher (Table 1). Apparently, the dissociation of the $-\text{OH}$ group in water and in alkaline solution produces the phenoxy anion that is a stronger $^1\text{O}_2$ quencher than phenols. However, the higher rate constant of $^1\text{O}_2$ quenching in water compared to ethanol cannot be due solely to the ionization of the $-\text{OH}$ group, because quenching is still faster in water at neutral pH when the $-\text{OH}$ group is not dissociated (Table 1). One possible explanation may be that the less polar alcohol solvent may

affect internal (resonance) charge distribution in the pyridoxine ring, thereby reducing interaction with $^1\text{O}_2$.

The quenching of $^1\text{O}_2$ is considered to be an additive process involving all active functional groups in a quencher molecule (14). However, the k_q value for pyridoxine is similar to the value for the aldehyde and amino derivatives at similar pH, which suggests that rather than these groups, the aromatic pyridoxine core is the main target for $^1\text{O}_2$. Such high reactivity is quite unexpected because other single-ring N-aromatics such as pyridine and most of its derivatives are poorer quenchers of $^1\text{O}_2$ with rate constants in the range of 10^5 – $10^6 M^{-1} s^{-1}$ (11). Apparently, the substituents in the pyridoxine core and their arrangement facilitate the interaction with $^1\text{O}_2$ that can result in the oxidative degradation of vitamin B_6 and all its derivatives.

Oxidation by $^1\text{O}_2$

A significant contribution to total quenching by chemical reactions(s) is suggested by the degradation of all B_6 vitamins during steady-state irradiation with RB (Fig. 4). The absorption spectra of RB and pyridoxine derivatives do not overlap, allowing selective $^1\text{O}_2$ production and a convenient monitoring of photosensitized degradation of pyridoxine *via* its absorption. The vitamin's absorption decreased upon aer-



Scheme 1.

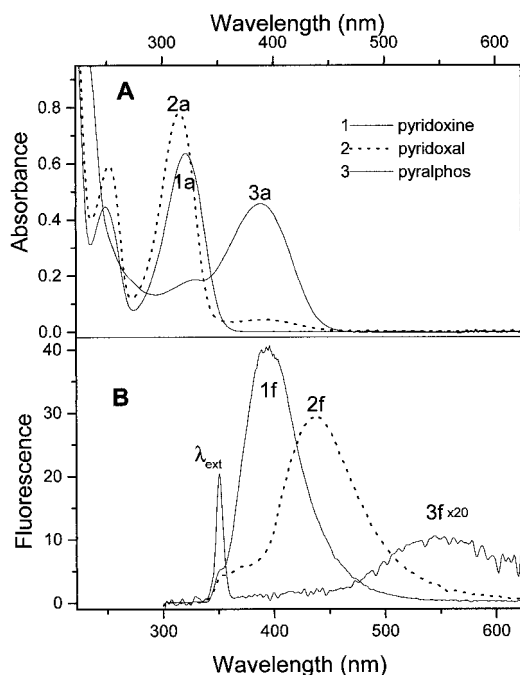


Figure 1. Absorption and fluorescence spectra of pyridoxine derivatives in aqueous solution at pH 7.4, phosphate buffer (25 mM). A: Absorption spectrum of pyridoxine (**1a**), pyridoxal (**2a**) and pyridoxal phosphate (**3a**); the spectrum of pyridoxamine was practically identical to **1a** and is not shown. B: Corrected and normalized fluorescence spectrum of pyridoxine (**1f**), pyridoxal (**2f**) and pyridoxal phosphate (**3f**); the amplitude of spectrum **3f** was increased 20 times for illustration; the spectrum of pyridoxamine was practically identical to **1f** and is not shown. Excitation wavelength 350 nm is marked by the signal from scattered light.

obic irradiation (550 nm) of RB (Fig. 4A), indicating photodegradation that was completely stopped by azide, a known physical quencher of $^1\text{O}_2$ (Fig. 4B). The absorption of RB was unchanged, suggesting that the RB triplet was not involved in the oxidative decomposition of pyridoxine. We confirmed that assumption by sequestering RB inside micelles (15,16) while pyridoxine ions were in the aqueous phase. This assay system separated the RB and pyridoxine moieties from close contact, while oxygen and $^1\text{O}_2$ could diffuse freely between the micellar and aqueous phases. Under these conditions, we observed the same degradation of the pyridoxine moiety while RB absorption was completely unchanged in the micellar solution, even during prolonged irradiation.

All derivatives of vitamin B₆ were degraded by $^1\text{O}_2$ (Table 1), which further supports the idea that the core aromatic moiety in pyridoxine must be mainly responsible for the interaction with $^1\text{O}_2$ and resultant degradation. However, the oxidation rate did not always closely match the quenching rate, depending on vitamer substituents and on pH that must affect ionization status of the -OH group. In addition to pH, the chemical environment strongly influences the interaction of pyridoxine with $^1\text{O}_2$. In ethanol, the rate constant for quenching $^1\text{O}_2$ phosphorescence by the pyridoxine free base was about 10 times lower than in water (Table 1). The resultant degradation rate of the pyridoxine moiety decreased by a factor of 30 times when compared to aqueous solutions (Table 1). This indicates that the oxidation proceeds much

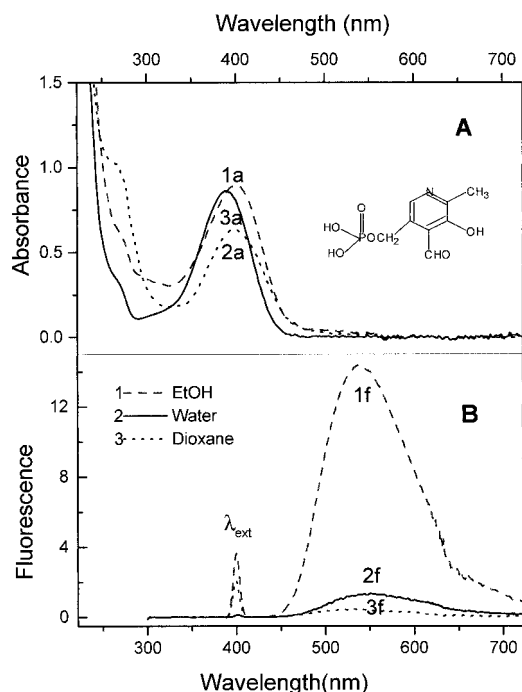


Figure 2. Absorption and fluorescence spectra of pyridoxal 5-phosphate in different solvents. A: Absorption spectrum in ethanol (**1a**), water (**2a**) and 1,4-dioxane (**3a**). B: Corrected and normalized fluorescence spectrum in ethanol (**1f**), water (**2f**) and 1,4-dioxane (**3f**). The spectrum identical to ethanol was observed in 1,2-propanediol. Excitation wavelength, 400 nm, marked.

faster in an aqueous environment, where these vitamins probably reside in biological systems. Nevertheless, the degradation and quenching rates in ethanol are still substantial compared to other biological $^1\text{O}_2$ substrates including vitamins E and C (11).

We measured the oxidation rate for vitamins **1–4** in the pH range 6–8, which is relevant to many physiological conditions. Relative oxidation rate constants presented in Table 1 are directly proportional to the rate constant describing only the efficacy of chemical reaction with $^1\text{O}_2$ and exclud-

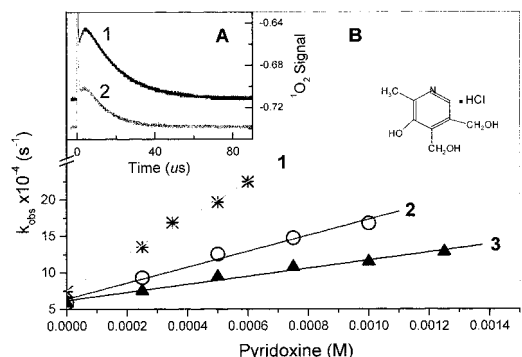


Figure 3. Quenching of $^1\text{O}_2$ phosphorescence by pyridoxine in aerobic D₂O phosphate buffers (20 mM). A: Examples of $^1\text{O}_2$ phosphorescence decay observed at 1270 nm after one-shot laser $^1\text{O}_2$ production in the absence (**1**) and in the presence (**2**) of 0.1 mM pyridoxine. B: Observed rate constant for $^1\text{O}_2$ quenching as a function of the increasing concentration of pyridoxine at pD 12 (**1**), pD 3.4 (**2**) and pD 6 (**3**); the line slopes yield the quenching rate constants. Rose bengal (50 μM) was used to photosensitize $^1\text{O}_2$.

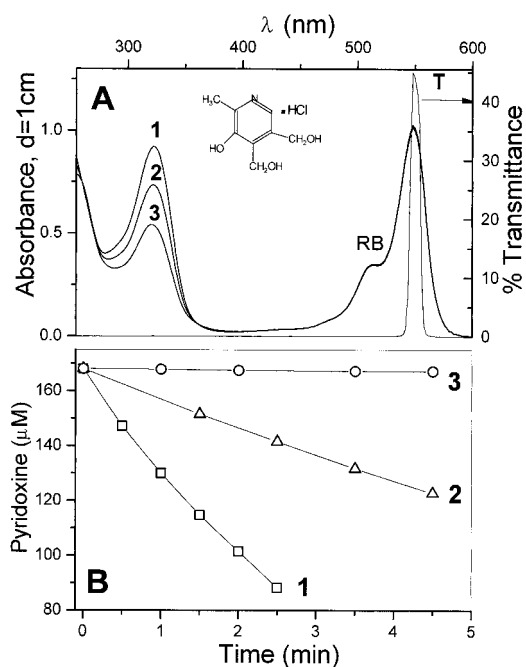


Figure 4. Photosensitized degradation of pyridoxine by $^1\text{O}_2$. A: Subsequent absorption spectra observed during the irradiation of 0.17 mM pyridoxine solutions containing RB; before irradiation (1), after 1 min (2) and after 2.5 min (3) of irradiation. Irradiated solution (3.8 mL) was vigorously stirred with a magnetic bar and air bubbling during irradiation. Transmittance of interference filter used to irradiate RB is shown on the righthand scale. B: Calculated degradation of pyridoxine as a function of irradiation time in the absence of sodium azide (1), in the presence of 0.157 mM (2) and 50 mM (3) of sodium azide. The initial rate of oxidation from plot 1 was used to calculate the oxidation rate (Table 1).

ing physical quenching. A closer examination of these values reveals that at physiological pH 7.4, pyridoxamine is most rapidly oxidized, followed by pyridoxine, pyridoxal and pyridoxal 5-phosphate. The oxidation rate decreases slightly at pH 6.3, mostly preserving the same order, which seems to be determined by the substituents' ability to change the electron density in the pyridoxine ring. However, at pH 8.2, the order is reversed and the oxidation increases, *e.g.* two-fold for pyridoxal 5-phosphate and pyridoxamine (Table 1). This reverse order can be explained by the dissociation of the $-\text{OH}$ group producing a phenol anion. The anion may conjugate with the pyridoxine ring, increasing electron density there, which confirms that the core aromatic moiety in the pyridoxine vitamers is a target for the oxidation by $^1\text{O}_2$ that interacts more efficiently with electron-rich substrates (17–19).

Our results show that all the pyridoxine vitamers are good substrates for $^1\text{O}_2$, because the pyridoxine moiety strongly quenches $^1\text{O}_2$ in different chemical environments in which the pyridoxine ring can be rapidly degraded by $^1\text{O}_2$. The observation may have serious implications for biological systems, suggesting that B₆ vitamers can all be subjected to rapid degradation by $^1\text{O}_2$ whenever this species is produced. As vitamin B₆ is required for all living cells, degradations by $^1\text{O}_2$ may represent an additional mechanism of cellular toxicity by photosensitizers, particularly cells or organisms

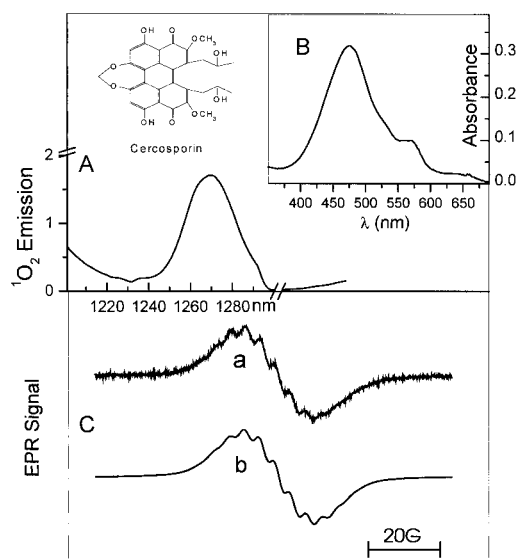


Figure 5. Quenching of excited cercosporin by dissolved oxygen and by pyridoxine. A: Spectrum of $^1\text{O}_2$ phosphorescence photosensitized in dilute solution of cercosporin in micellar solution of benzalkonium chloride illuminated through a 455 nm cutoff filter combination in aerobic water and acquired during one 20 s scan. B: The absorption spectrum of cercosporin in benzalkonium chloride (10 mM) micelles used to obtain the $^1\text{O}_2$ spectrum in A, pathlength 0.5 cm. C: Electron paramagnetic resonance spectrum of cercosporin radical anion (a) produced during the quenching of excited ($\lambda > 400$ nm) cercosporin (0.3 mM) by pyridoxal 5-phosphate (2 mM) in deoxygenated micellar Triton X-100 1% solution at pH 7.4 in phosphate buffer (50 mM). Spectrum simulation (b) using the following splitting constants: $a_{\text{H}}(2) = 0.86\text{G}$, $a_{\text{H}}(2) = 0.32\text{G}$ and $a_{\text{H}}(4) = 0.46\text{G}$.

that do not synthesize pyridoxine but must obtain it from nutritional sources.

Interaction with the cercosporin photosensitizer

Cercosporin, an endogenous $^1\text{O}_2$ -generating photosensitizer produced by *Cercospora* fungi, plays an essential role in the ability of this group of filamentous fungi to parasitize plants (20). That $^1\text{O}_2$ is generated in fungal culture was recently confirmed spectrally by recording the $^1\text{O}_2$ phosphorescence spectrum (25). Although pyridoxine's protective effect in the fungal cell may occur *via* $^1\text{O}_2$ quenching, we have also examined whether pyridoxine has the potential to interact directly with cercosporin. The EPR measurements in anaerobic micellar solutions show that pyridoxine quenches the excited cercosporin by donating an electron, as evidenced by an EPR signal from the cercosporin radical anion (Fig. 5C). In the presence of oxygen, no EPR signal was observed, presumably because the production of $^1\text{O}_2$ was dominant, indicating that the cercosporin triplet was mostly quenched by dissolved oxygen and not by pyridoxine. We confirmed that $^1\text{O}_2$ is efficiently generated (Fig. 5A) by cercosporin in micelles (Fig. 5B). However, at low oxygen concentration, the superoxide radical was detected during the irradiation of cercosporin in dimethylsulfoxide in the presence of pyridoxine and the radical trap DMPO. The superoxide anion is usually produced during photosensitization when oxygen oxidizes the sensitizer radical anion (21).

Cercospora fungi appear to use an elaborate system to

protect themselves against oxidative stress induced by cercosporin and other photosensitizers that are highly toxic to other species. One mechanism of defense may be the chemical reduction of cercosporin (22–24) followed by the localization of reduced cercosporin in the aqueous cytoplasm of the cell, an environment that decreases $^1\text{O}_2$ production by the reduced derivative (25). Another mechanism may involve pyridoxine as a $^1\text{O}_2$ quencher (26). Our data suggest that as a third mechanism, pyridoxine may function as a redox quencher of the cercosporin triplet, leading to superoxide radical production. It is possible that the *Cercospora* fungus may use such reactions to divert cercosporin activation away from the formation of the highly toxic $^1\text{O}_2$ to superoxide and other radical forms of oxygen that are better tolerated by living cells.

Conclusions

Here we have shown that the pyridoxine moiety is a strong quencher of singlet molecular oxygen that initiates rapid oxidation of all B₆ vitamers. The B₆ vitamers are known to play a required role in cells in enzymatic reactions, primarily in amino acid metabolism, and were not previously implicated in $^1\text{O}_2$ or oxidative stress resistance in cells. Our data suggest that pyridoxine and its vitamers can function in photosensitizer resistance by quenching $^1\text{O}_2$. Ironically, our results also indicate that $^1\text{O}_2$ degrades pyridoxine, an observation that has implications for an additional mode of photosensitizer toxicity to cells, primarily animal cells that do not synthesize pyridoxine but must obtain it through nutritional sources. Our data suggest that oxidative degradation of B₆ vitamers can occur during processes in which $^1\text{O}_2$ is produced, such as food processing and storage and during photosensitization in the skin. While it is difficult to separate their potential antioxidant properties from the enzymatic role, pyridoxine appears to contribute strongly to the unusual resilience of *Cercospora* fungi to photooxidative stress. Details for the involvement of vitamin B₆ in complex processes associated with oxidative stress and whether it can function as an antioxidant in other organisms will require more investigation.

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