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Uric Acid: A Less-than-Perfect Probe for Singlet Oxygen†

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ABSTRACT

Uric acid and/or its mono anion have long been used as chemical trapping agents to demonstrate the presence of singlet oxygen, $O_2(a^1\Delta_g)$, in aqueous systems. "Oxidative bleaching" of uric acid, generally monitored through changes in the uric acid absorption spectrum, is often used in giort of claims for the intermediacy of $O_2(a^1\Delta_g)$. The bleaching of uric acid has also been used to quantify photosensitized $O_2(a^1\Delta_g)$ yields in selected systems.

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Unfortunately, experiments performed to these ends often neglect processes and phenomena that can influence the results obtained. For the present study, we experimentally examined the behavior of uric acid under a variety of conditions relevant to the photo-initiated creation and subsequent removal of $O_2(a^1\Delta_g)$. Although the oxidative destruction of uric acid can indeed be a useful tool in some cases, we conclude that caution must be exercised such as not to incorrectly interpret the data obtained.

INTRODUCTION

Uric acid, UA, has long been used as a hydrophilic probe to assess whether or not singlet oxygen, $O_2(a^1\Delta_g)$, is involved in a given reaction. To this end, the "oxidative bleaching" of UA is commonly monitored through changes in the absorption spectrum of the sample. Indeed, this practice is so prevalent, as reflected in a plethora of published papers, that it is arguably inappropriate to support this statement with only one or two references to the literature. Unfortunately, in many of these published experiments, the data obtained are treated rather superficially leaving ample room for misinterpretation. In short, UA is assumed to be the consummate quencher of $O_2(a^1\Delta_g)$ and much is often taken for granted. For example, in photosensitized systems, UA could also influence the production of $O_2(a^1\Delta_g)$ by quenching the sensitizer as well. Although some substantive studies of UA chemistry in photo-initiated oxidizing environments have been published (1-3), many of the fundamental concepts reported therein are often overlooked by the community. As such, we feel it is appropriate to better clarify aspects of uric acid behavior under conditions in which $O_2(a^1\Delta_g)$ is produced.

The fully protonated neutral form of uric acid, UA^n, present under acidic conditions, is shown in Figure 1. Reported pK_a values for the production of the more hydrophilic mono-anion, UA^{ma}, range from 5.4 to 5.8, with some disagreement as to which proton is actually removed (1, 4-7). In Figure 1, we show the structures of the two mono-anions for which there appears to be substantive support.

>Figure 1<

Once formed in an aqueous system that contains uric acid, $O_2(a^1\Delta_g)$ can be removed/deactivated by several kinetically competing processes (Figure 2). Over the years, it has been established that electron-rich compounds are particularly adept at deactivating/removing $O_2(a^1\Delta_g)$ (8, 9). In general, among other things, the magnitude of rate constants for chemical reaction, k_{rxn}, reflect the electrophilicity of $O_2(a^1\Delta_g)$ and can be as large as ~ 10^9 s^{-1} M^{-1} (8, 10). Independently, the magnitude of rate constants, k_q, for the quenching of $O_2(a^1\Delta_g)$ to the ground state, $O_2(X^3\Sigma_g^-)$, are generally larger for molecules Q that can readily donate charge and facilitate mixing with the $Q^{+\bullet}O_2^{-\bullet}$ charge-transfer state (8, 9). Likewise, values of k_q can be as large as ~ 10^9 s^{-1} M^{-1}. On this basis, one would expect that the rate constants $k_{rxn}{}^n$ and $k_q{}^n$ for UA^n would be smaller than $k_{rxn}{}^{ma}$ and $k_q{}^{ma}$ for the more electron-rich UA^{ma}. Specifically, as one changes the pH of an aqueous solution of uric acid, bracketing the UA pK_a value of ~ 5.6, one will change the relative concentrations of UA^n and UA^{ma} and, as such, should change measured values for the rate constants of $O_2(a^1\Delta_g)$ removal/deactivation. This expectation has been confirmed by Montaña, *et al*. (1), who showed that measured values of k_{rxn} and k_q for the removal and deactivation of $O_2(a^1\Delta_g)$, respectively, indeed increase as a solution of UA is made more alkaline. Given that the pK_a value of ~5.6 is sufficiently close to the range of pH values found in a mammalian cell (11),

this phenomenon can have significant ramifications in the interpretation of data recorded from a host of biological systems.

> Figure 2 <

A variety of oxygenated products can be envisioned as a consequence of reactions between $O_2(a^1\Delta_g)$ and UA^{ma} and UA^n, certainly when one takes secondary and tertiary reactions into account (12, 13). In Figure 1, we show one molecule, parabanic acid, that has recently been identified as a specific product of the reaction between $O_2(a^1\Delta_g)$ and UA (12). With respect to the use of UA as a probe for the presence of $O_2(a^1\Delta_g)$, a key point in this regard is that these reactions change the absorption spectrum of the given sample over the range ~ 200 – 350 nm. This is a spectral range over which many molecules M have appreciable absorption that likewise changes as a result of oxidation reactions with $O_2(a^1\Delta_g)$ to yield oxygenated products, MO_2 (Figure 2).

Carrying this latter point further, it is important to recognize that UA will also react with other reactive oxygen species (ROS) often found in $O_2(a^1\Delta_g)$-containing systems (1, 2, 12, 14). In short, UA is not a $O_2(a^1\Delta_g)$-selective probe, and these competing reactions can appreciably complicate the system being examined. This point is often overlooked to the detriment of mechanistic conclusions that may be drawn from a given system.

There is a great need for molecules that can selectively probe for $O_2(a^1\Delta_g)$ under a variety of conditions. The advantages of using an optical spectroscopic approach in this regard should be apparent. Although activity to this end is as old as $O_2(a^1\Delta_g)$ itself, it remains an active field of research (15-20), certainly with respect to the goal of elucidating the intracellular behavior of $O_2(a^1\Delta_g)$ in time- and spatially-resolved experiments. Sadly, it is our impression that UA has acquired the stature of an "ideal" water-soluble probe for $O_2(a^1\Delta_g)$, likely by virtue of longevity in its use. As such, UA is often used in indiscriminate ways without concern for potential complications that could adversely influence data interpretation.

On the basis of these points, we set out to perform selected experiments to further illustrate and establish guidelines for the effective use of uric acid as a probe for $O_2(a^1\Delta_g)$.

MATERIALS AND METHODS

Materials. Uric acid (UA), 9,10-anthracenediyl-bis(methylene)dimalonic acid (ADA), L-tryptophan, *meso*-Tetrakis(4-sulfonatophenyl)porphine (TPPS), Flavin mononucleotide (FMN), Rose Bengal (RB), and Methylene Blue (MB) were obtained from Sigma-Aldrich and used as received. The sulfonated derivative of 1*H*-phenalenone (PNS) was synthesized as described by Nonell *et al.* (21). D_2O (99.9% D) was obtained from EurisoTop and H_2O was purified by filtration (Milli-Q system by Millipore Corporation).

H_2O- and D_2O-based phosphate buffer solutions (PBS) were prepared using commercially available PBS tablets (Sigma-Aldrich). (Note: pD = pH + 0.4) The pD of D_2O-based solutions was adjusted with deuterochloric acid (DCl) and sodium deuteroxide (NaOD). The pH of H_2O-based solutions was similarly adjusted using HCl and NaOH.

Methods. In previous publications (22, 23), we describe the time-resolved instruments and approaches used to (a) generate and optically detect $O_2(a^1\Delta_g)$, and (b) monitor sensitizer excited states. All reported quantum yields of $O_2(a^1\Delta_g)$ production were measured using PNS in D_2O-PBS as the standard ($\phi_\Delta = 0.97 \pm 0.06$) (24).

Selected experiments were performed in D_2O-based solutions, instead of H_2O-based solutions, to exploit the fact that the lifetime of $O_2(a^1\Delta_g)$ in D_2O is appreciably longer than that in H_2O (8, 25). For time-resolved $O_2(a^1\Delta_g)$ phosphorescence experiments, the use of D_2O as a solvent thus makes it easier to accurately discern quencher-dependent kinetics on the rising portion of the phosphorescence signal (*i.e.*, events that influence the decay of the $O_2(a^1\Delta_g)$ precursor).

RESULTS AND DISCUSSION

Absorption and Fluorescence Spectra

The absorption and fluorescence spectra of UA dissolved in a phosphate-buffered H_2O solution (pH = 7.4) are shown in Figure 3. Note that, at this pH, the spectra reflect contributions from both UA^n and UA^{ma}. This point is substantiated by the pH-dependent data in Figure 4, where the absorption spectra obtained are consistent with a system of two species in equilibrium.

> Figure 3 <

> Figure 4 <

As seen in Figure 3, the absorption spectra show three clearly resolved bands. This is consistent with spectra recorded by Montaña, *et al*. (1), but differs from spectra recorded by Fischer *et al.* (14), likely because the latter did not extend their measurement down to 200 nm using a more dilute solution.

The recorded absorbance values scale linearly over the UA concentration range of ~ 0 – 300 μM and pH range of ~ 2 – 10. Thus, under these conditions, solute aggregation does not appear to be a problem. The data yield a molar extinction coefficient for the band with λ_{max} = 292 nm of (1.17 ± 0.07) × 10^4 $M^{-1}cm^{-1}$ at pH = 7.4.

The fluorescence spectrum we record has a band maximum at ~ 375 nm (Figure 3). Using tryptophan in H_2O-PBS (ϕ_f = 0.12 ± 0.01) (26) as a fluorescent standard, we estimate that the quantum efficiency of the fluorescent signal recorded from the UA solution at this pH is < 0.0005.

The comparatively small fluorescence quantum yield from UA is consistent with the fact that, over the years, the photo-oxidative bleaching of UA has commonly been monitored in absorption experiments. The latter are generally performed using the UA band centered at ~ 292 nm. As we outline further below, this can be complicated given that many molecules apt to be present in the system of interest likewise absorb light in this spectral region.

UA-Sensitized Production of $O_2(a^1\Delta_g)$

Upon irradiation of an air-saturated phosphate buffered solution of UA at 295 nm, we were not able to detect a time-resolved phosphorescence signal at 1275 nm characteristic of the $O_2(a^1\Delta_g)$ - $O_2(X^3\Sigma_g^-)$ transition. For this experiment, we specifically prepared our buffer solution using heavy water (pD = 7.8) to exploit the fact that the lifetime of $O_2(a^1\Delta_g)$ in D_2O is appreciably longer than that in H_2O and, as such, the quantum efficiency of $O_2(a^1\Delta_g)$ phosphorescence is greater (8, 25). Using the corresponding $O_2(a^1\Delta_g)$ phosphorescence signal from a PNS sensitized reaction as a standard, we estimate that the quantum yield of $O_2(a^1\Delta_g)$ production sensitized by UA under these conditions is < 0.001. These results, combined with the weak fluorescence signal, lead us to infer that non-radiative deactivation of UA excited states is comparatively rapid and efficient. This inference is consistent with the results of recent computations (27).

Overall Rate Constant for UA-Mediated Removal of $O_2(a^1\Delta_g)$

We performed experiments to quantify the rate constant, k_{total}, for the overall deactivation/removal of $O_2(a^1\Delta_g)$ mediated by UA. With reference to Figure 2, k_{total} is equal to the sum of the reactive and physical deactivation channels of $O_2(a^1\Delta_g)$ (*i.e.*, $k_{total} = k_{rxn} + k_q$) . As outlined above, k_{rxn} and k_q will be different for UA^n and UA^{ma}, and the measured value of k_{total} at any given pH will thus depend on the respective fractions, χ^n and χ^{ma}, of UA^n and UA^{ma} present in the solution (eq 1).

$$k_{total} = k_{total}^{n}\,\chi^{n} + k_{total}^{ma}\,\chi^{ma} = \frac{k_{total}^{n}}{1 + 10^{pH-pK_a}} + \frac{k_{total}^{ma}}{1 + 10^{pK_a-pH}} \qquad (1)$$

We performed these experiments in the standard way using the time-resolved 1275 nm phosphorescence of $O_2(a^1\Delta_g)$ as a probe. Specifically, we recorded the $O_2(a^1\Delta_g)$ lifetime, τ_Δ, as a function of the UA concentration in both D_2O-based and H_2O-based phosphate buffer solutions and, independently, as a function of pD. In separate experiments, $O_2(a^1\Delta_g)$ was produced using RB, MB, TPPS, PNS, and FMN as the sensitizers. Representative data are shown in Figure 5.

> Figure 5 <

In D_2O-PBS at pD = 7.8, our experiments yield $k_{total} = (3.6 \pm 0.2) \times 10^8\ M^{-1}s^{-1}$. This number is in excellent agreement with the number reported by Kanofsky for experiments also performed in a D_2O-based buffer at pD = 7.8 ($k_{total} = (3.6 \pm 0.2) \times 10^8\ M^{-1}s^{-1}$) (28). A corresponding measurement in H_2O-PBS yields an identical value of $k_{total} = (3.5 \pm 0.3) \times 10^8\ M^{-1}s^{-1}$ confirming the expected absence of a solvent isotope effect on k_{total}. These numbers differ only slightly from the value $5.1 \times 10^8\ M^{-1}s^{-1}$ published by Montaña, *et al.* (1) for a reported pH value of 7 in H_2O using a different experimental technique. At pD ~ 3, where UA is present as UA^n, k_{total} drops to a value of $(2.3 \pm 0.3) \times 10^7\ M^{-1}s^{-1}$ (Figure 5c). The value of k_{total} reported by Montaña, *et al.* (1), $1.8 \times 10^8\ M^{-1}s^{-1}$, for an experiment at pH = 5 (*i.e.*, pD = 5.4) is somewhat larger. Nevertheless, this number of Montaña, *et al.* is consistent with our titration curve shown in Figure 5c.

The comparatively large rate constant of $k_{total}^{ma} = (3.6 \pm 0.2) \times 10^8\ M^{-1}s^{-1}$ for neutral and moderately alkaline solutions indicates that, as a probe added in modest concentrations, UA will indeed kinetically compete with many other molecules in processes that remove/deactivate $O_2(a^1\Delta_g)$ (8).

Rate Constants for the Removal of $O_2(a^1\Delta_g)$ by Reaction with UA: Sensitized Experiments

As established above, monitoring UA disappearance in an absorption experiment can be complicated because UA absorbs light at wavelengths where many other molecules also absorb. For example, in protein-containing systems, tryptophan has a distinct absorption band with λ_{max} at ~ 298 nm, and the intensity of this band likewise changes upon the facile reaction of tryptophan with $O_2(a^1\Delta_g)$ (29). Many $O_2(a^1\Delta_g)$ sensitizers also absorb at these same wavelengths, and these molecules may likewise "bleach" upon irradiation.

However, even if other molecules in the system remain stable over the reaction period, the spectral changes due to the reactions of UA alone can render analysis difficult. We illustrate this point with the data shown in Figure 6. In these experiments, we monitored the photosensitized bleaching of UA in both D_2O- and H_2O-based buffer solutions. We ascertained that, over the course of the reaction, the sensitizer remained stable. As such, we could subtract the absorption spectrum of the sensitizer to reveal the dynamic changes related to UA alone.

> Figure 6 <

On the basis of the kinetic scheme in Figure 2, and the knowledge that the solvent-mediated $O_2(a^1\Delta_g)$ deactivation channels are less efficient in D_2O than in H_2O (8, 9, 25), one would expect that the rate of $O_2(a^1\Delta_g)$-mediated UA change should be greater in the D_2O-based buffer. The data in Figure 6 confirm this expectation. Most importantly, however, it is not just the reaction rates that distinguish the D_2O-based data from the H_2O-based data; the spectra themselves show pronounced differences as a function of elapsed photolysis time.

We infer that these spectral differences reflect the rate at which the reaction products appear and subsequently disappear. Specifically, the D_2O/H_2O isotope effect will principally affect the rate of $O_2(a^1\Delta_g)$-mediated UA disappearance and, hence, the rate of initial product formation (*e.g.*, hydroperoxides). However, it is reasonable to assume that many secondary and tertiary oxidation products will be formed via thermal reactions that do not depend on the solvent isotopic composition (*e.g.*, hydroperoxide decomposition and subsequent rearrangement/fragmentation reactions) (1, 12, 13). We indeed find that, after an initial period of irradiation, samples stored in the dark continue to show changes in the absorption spectrum. These subsequent reactions will contribute differently to the spectra in the D_2O- and H_2O-based experiments because the observation times are different (Figure 6). We observed no changes in the absorption spectrum for an unirradiated control sample kept in the dark for 24 h (data not shown).

On this basis, we argue that any attempt to use data such as those in Figure 6 to quantify k_{rxn} for $O_2(a^1\Delta_g)$ and UA can be subject to error. Indeed, in such an attempt, we obtain appreciably different results upon analysis of the spectra in Figures 6a and 6b. In this regard, and to our knowledge, there is no precedence for a solvent isotope effect on k_{rxn} for a reaction involving $O_2(a^1\Delta_g)$. As outlined in subsequent sections, more accurate information can only be obtained from such absorption spectra under the following conditions:

(a) Ensure that the spectra principally reveal changes in UA concentration alone.

(b) Ensure that the rate and amount of $O_2(a^1\Delta_g)$ production is known and that UA does not also quench the photosensitizer.

(c) Ensure that, in the nascent reactions of a photoinitiated process, $O_2(a^1\Delta_g)$ is selectively produced at the expense of other ROS.

Rate Constants for the Removal of $O_2(a^1\Delta_g)$ by Reaction with UA: Sensitizer-Free Experiments

As indicated above, if one wants to accurately quantify $O_2(a^1\Delta_g)$-dependent changes in UA concentration, one should ideally perform the experiment under conditions where $O_2(a^1\Delta_g)$ is exclusively produced in controlled amounts at the expense of other ROS. Because many sensitizers are known to concomitantly produce more than one ROS upon excitation (*i.e.*, $O_2(a^1\Delta_g)$ via energy transfer and the superoxide ion via electron transfer) (9, 30), one would ideally want an independent sensitizer-free approach to make $O_2(a^1\Delta_g)$. Moreover, removing the sensitizer decreases the potential complexity of the experiment; even if the sensitizer does not immediately react with $O_2(a^1\Delta_g)$, it may respond to secondary reactions (*e.g.*, radical propagation processes) and still influence the spectra recorded.

We have recently shown that experimentally useful amounts of $O_2(a^1\Delta_g)$ can be selectively produced in sensitizer-free experiments by excitation of oxygen to its second excited state, $O_2(b^1\Sigma_g^+)$, through the $O_2(X^3\Sigma_g^-) \rightarrow O_2(b^1\Sigma_g^+)$ transition at 765 nm (23, 31, 32). $O_2(b^1\Sigma_g^+)$ subsequently decays with near unit efficiency to $O_2(a^1\Delta_g)$.

In Figure 7, we show absorption spectra of a sensitizer-free sample of UA in D_2O-PBS recorded as a function of elapsed irradiation into the spectrally narrow $O_2(X^3\Sigma_g^-) \rightarrow O_2(b^1\Sigma_g^+)$ band at 765 nm. Identical samples that were irradiated at 800 nm, or kept in the dark, did not show detectable changes in the absorption spectrum over the course of 24 hours. Thus, we infer that our results from the samples irradiated at 765 nm are not the result of thermal reactions or multi-photon processes involving UA. The data in Figure 7 also show that, for sensitizer-free samples irradiated over a comparatively long time period at 765 nm, oxidation products contribute much less to the absorption spectra.

> Figure 7 <

Using the extinction coefficient of oxygen at 765 nm in water ($(13.4 \pm 1.6) \times 10^{-4}$ $M^{-1}cm^{-1}$) (31), and the concentration of oxygen in air-saturated D_2O-PBS at 23 °C (0.29 ± 0.01 mM) (33), the data in Figure 7 yield a rate constant for the reaction of $O_2(a^1\Delta_g)$ with UA of $(1.5 \pm 0.2) \times 10^8$ $M^{-1}s^{-1}$. Control experiments in which we monitored the $O_2(a^1\Delta_g)$-mediated bleaching of UA relative to the bleaching of ADA ($k_{rxn} = 5.6 \pm 0.3 \times 10^7$ $M^{-1}s^{-1}$) (31), gave identical results in both H_2O- and D_2O-based buffered solutions. This latter observation is consistent with our expectations regarding a solvent isotope effect on k_{rxn} (*vide supra*).

> Table 1 <

Our values of k_{rxn} thus obtained (Table 1) are similar to those reported by Montaña, *et al.* (1) for UA in H_2O at pH = 5 (1×10^7 $M^{-1}s^{-1}$) and pH = 7 (1.2×10^8 $M^{-1}s^{-1}$). The data of Montaña, *et al.* were obtained from a RB-sensitized system using an approach based on oxygen consumption with furfuryl alcohol as a reference reactant.

The Quenching of Sensitizer Triplet States by Uric Acid

Under ideal circumstances, the optimal probe for $O_2(a^1\Delta_g)$ will selectively quench and/or react with $O_2(a^1\Delta_g)$, and $O_2(a^1\Delta_g)$ alone. Thus, in a photosensitized reaction, where the sensitizer triplet state is the immediate precursor of $O_2(a^1\Delta_g)$, an ideal probe for $O_2(a^1\Delta_g)$ will not quench the sensitizer triplet state. If such quenching occurs, it will influence both the yield and kinetics of $O_2(a^1\Delta_g)$ formation.

We performed a series of time-resolved $O_2(a^1\Delta_g)$ phosphorescence studies to investigate the effects of UA on the $O_2(a^1\Delta_g)$ precursor. We were particularly interested in the effect of UA on the kinetics of $O_2(a^1\Delta_g)$ formation (*i.e.*, the decay rate of the sensitizer triplet state). In Figure 8, we show pertinent $O_2(a^1\Delta_g)$ phosphorescence data obtained for a PNS-sensitized system in a D_2O-based buffer solution at pD = 7.8. We recorded data as function of the incident laser power and the concentration of UA in the solution. In all cases, we could fit the phosphorescence trace using a difference of two exponential functions (25): one function describing the rise of the $O_2(a^1\Delta_g)$ signal and a second describing the fall of the signal. As such, the decay of the $O_2(a^1\Delta_g)$ precursor (*i.e.*, the sensitizer triplet state) followed first order kinetics, and the decay of $O_2(a^1\Delta_g)$ itself likewise followed first order kinetics. Using standard procedures (34, 35), we also used the integral of these traces to obtain the quantum yield of photosensitized $O_2(a^1\Delta_g)$ production, ϕ_Δ, as a function of UA concentration.

> Figure 8 <

In these studies, we ascertained that the kinetics of $O_2(a^1\Delta_g)$ formation and decay did not change upon increasing the incident laser power. Furthermore, the absorption spectrum of the sample did not change appreciably over the course of the experiment (*i.e.*, the change in

absorbance at the UA λ_{max} of 292 nm was always less than 2%). As such, transport of UA in and out of the irradiated volume was sufficiently facile in our stirred cuvette to maintain a stable concentration of UA throughout the experiment.

We plotted the kinetic parameters obtained from the data in Figure 8 against the concentration of UA (Figure 9). From these plots, we obtain (a) the rate constant for total quenching of $O_2(a^1\Delta_g)$ by UA, k_{total}, from the UA-dependent changes in the fall of the $O_2(a^1\Delta_g)$ signal (*e.g.*, Figure 5b), (b) the rate constant for the quenching of the sensitizer triplet state by UA, k_T, from the UA-dependent changes in the rise of the $O_2(a^1\Delta_g)$ signal (Figure 9a), and (c) the concomitant changes in the quantum yield of $O_2(a^1\Delta_g)$ production, ϕ_Δ, from the integrated signal intensity normalized by the $O_2(a^1\Delta_g)$ lifetime (Figure 9b).

> Figure 9 <

As expected for a system in which UA quenches the sensitizer triplet state, the yield of $O_2(a^1\Delta_g)$ drops appreciably as the concentration of added UA is increased. Specifically, ϕ_Δ for PNS drops from ~ 1 to ~ 0.5 in the presence of just 200 μM of UA. Moreover, the measured rate constant for quenching of the sensitizer triplet state (just like the rate constant for $O_2(a^1\Delta_g)$ quenching) varies considerably as a function of pD (Figure 9c). Thus, we infer that UA^n and UA^{ma} show different quenching capabilities toward ^{3}PNS. From the data in Figure 9c, we find $k_T^n = (2.7 \pm 0.3) \times 10^9\ M^{-1}s^{-1}$ and $k_T^{ma} = (1.5 \pm 0.2) \times 10^9\ M^{-1}s^{-1}$.

We performed experiments analogous to those with PNS using other common water-soluble sensitizers. Data obtained from these experiments are shown in Table 2.

> Table 2 <

Flavins are important $O_2(a^1\Delta_g)$ photosensitizers with a host of biological ramifications (6). It has been shown that UA quenches the riboflavin triplet state, and that this process involves electron transfer from UA to riboflavin (3). Among other things, the resultant riboflavin radical anion will reduce $O_2(X^3\Sigma_g^-)$ to the superoxide radical anion, which, in turn, can react with UA (1). Thus, in a riboflavin-sensitized experiment, the use of UA as a probe for $O_2(a^1\Delta_g)$ will not only disturb the yields and kinetics of $O_2(a^1\Delta_g)$, it will also result in the production of other oxidizing species whose reactions with UA will complicate the system even further.

We obtain a value of k_T for FMN at pD = 7.8 of $(1.6 \pm 0.2) \times 10^9$ $M^{-1}s^{-1}$. Given that the chromophore in FMN is identical to that in riboflavin, it is reassuring to note that our number is similar to that reported by Cardoso, *et al*. (3), $(2.90 \pm 0.14) \times 10^9$ $M^{-1}s^{-1}$, for riboflavin at pH = 6.4. It is important to note that the mechanism by which UA quenches ^{3}FMN (*i.e.*, electron transfer) yields radical ions that can ultimately result in the production of other ROS (specifically $O_2^{-\cdot}$) (1, 3). As such, at least in FMN photosensitized systems, care must be exercised in interpreting relative UA-dependent yields of $O_2(a^1\Delta_g)$ and $O_2^{-\cdot}$ (38).

Of the five $O_2(a^1\Delta_g)$ sensitizers examined, we find that k_T for TPPS is uniquely small (Table 2). Although we were unable to find literature data for the triplet state energy, E_T, of UA, this value of k_T could nevertheless reflect an energy mismatch between values of E_T for UA and TPPS (39). In contrast, k_T for MB is comparatively large, despite an E_T similar to that of TPPS. In this case, it is reasonable to speculate that the unique redox properties of MB (40, 41) play a more dominant role in the interaction with UA. The important point in this regard is that the rate of UA-mediated triplet state deactivation depends on the sensitizer.

Our data clearly show that care must be exercised if one intends to obtain the quantum yield of photosensitized $O_2(a^1\Delta_g)$ production using a procedure that depends on quenching by UA. In this regard, we note that such a procedure, in fact, has been promoted (14, 42, 43). However, our results indicate that one should expect such a procedure to yield values of ϕ_Δ that depend on (a) the rate constant for sensitizer triplet state quenching by UA, (b) the initial concentration of UA used, and (c) the pH of the solution.

Uric Acid as a Probe for $O_2(a^1\Delta_g)$

UA shows properties that, at a first glance, appear to make it a suitable water-soluble probe for $O_2(a^1\Delta_g)$: it has a high reactivity toward $O_2(a^1\Delta_g)$, is generally biocompatible, and does not itself sensitize the production of $O_2(a^1\Delta_g)$.

However, as we have established, UA has a number of undesirable features:

First, with the common approach of monitoring UA disappearance via absorption spectroscopy, the data obtained can be appreciably influenced by absorbance changes due to the time-dependent composition of UA oxidation products and/or other molecules in the system. As such, one may have to revert to other, more elaborate, quantification techniques.

Second, UA is not a probe specific for $O_2(a^1\Delta_g)$; it reacts with a host of other species, including many common ROS (1, 2, 12, 14). Thus, the bleaching of UA alone cannot be used to claim the intermediacy of $O_2(a^1\Delta_g)$ in a given reaction. This is a major drawback, certainly

if one intends to use UA in a complicated biological system where other ROS are apt to be present.

Third, UA can perturb and quench the excited states of other molecules present in the system. Specifically, when UA is present, the photophysics of selected sensitizers may not be well defined, and care must be exercised to obtain an accurate quantum yield of photosensitized $O_2(a^1\Delta_g)$ production.

Finally, with all of the above points in mind, the extent to which UA perturbs a given system depends on pH. Since the pertinent pK_a value of UA is ~ 5.6, this can have appreciable ramifications for experiments performed "indiscriminately" in the biologically relevant pH range of ~ 5-7.

CONCLUSIONS

Uric acid is often regarded as one of the consummate water soluble quenchers of $O_2(a^1\Delta_g)$. As such, it is frequently used to assess whether or not $O_2(a^1\Delta_g)$ is involved as a reactive intermediate. Moreover, the rate of uric acid removal, as recorded through changes in its absorption spectrum, has been touted as a convenient method to quantify yields of $O_2(a^1\Delta_g)$ in selected systems. Despite the level of certainty now often taken for granted in this regard, we hope to have established that care must be exercised when using uric acid as a quantitative probe for $O_2(a^1\Delta_g)$, certainly in selected photosensitized reactions.

Although other water-soluble probes for $O_2(a^1\Delta_g)$ are available (16, 17, 19, 20, 31), these molecules likewise have selected disadvantages. As such, there is still a need to develop and characterize a wider range of $O_2(a^1\Delta_g)$ probes.

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Table 1. Measured rate constants for the interaction of UA^n and UA^{ma} with $O_2(a^1\Delta_g)$ in D_2O.

Compound	**Total $O_2(a^1\Delta_g)$ deactivation k_{total} ($M^{-1}s^{-1}$)**	**Reactive $O_2(a^1\Delta_g)$ deactivation k_{rxn} ($M^{-1}s^{-1}$)[a]**	**Physical $O_2(a^1\Delta_g)$ deactivation k_q ($M^{-1}s^{-1}$)[b]**
UA^n (pD = 4)	$(2.3 \pm 0.3) \times 10^7$	$(2.3 \pm 0.3) \times 10^7$	~ 0
UA^{ma} (pD = 7.8)	$(3.6 \pm 0.2) \times 10^8$	$(1.5 \pm 0.2) \times 10^8$	~ 2×10^8

[a]Obtained in sensitizer-free experiments.

[b]Obtained from $k_{total} - k_{rxn}$.

Table 2. Sensitizer triplet state energy, E_T, rate constant for sensitizer triplet state quenching by UA, k_T, and the quantum yield of $O_2(a^1\Delta_g)$ production, ϕ_Δ, at two different UA concentrations.

Sensitizer	**Triplet state energy, E_T (kJ/mol)**	**Rate constant of triplet state quenching by UA, k_T (10^9 $M^{-1}s^{-1}$)[a]**	**Quantum yield of $O_2(a^1\Delta_g)$ production, φ_Δ[a]**	
			[UA] = 0 µM	[UA] = 200 µM
FMN	209[b]	1.6 ± 0.2	0.67 ± 0.05	0.32 ± 0.03
PNS	182[c]	1.5 ± 0.2	0.97 ± 0.06	0.51 ± 0.04
RB	164[d]	1.4 ± 0.2	0.76 ± 0.05	0.52 ± 0.04
MB	138[d]	3.9 ± 0.3	0.52 ± 0.05	0.26 ± 0.03
TPPS	138[e]	< 0.05	0.73 ± 0.06	0.72 ± 0.06

[a]Data were recorded in D_2O-PBS at pD = 7.8 and thus reflect the effects of UA^{ma}.

[b]Data for riboflavin (36).

[c]Data for unsulfonated 1*H*-phenalenone (PN) (37).

[d]From reference (36).

[e]Data for unsulfonated tetraphenyl-porphyrin (TPP) (36).

Figure Captions:

Figure 1. Chemical structures of the neutral form of uric acid (left), the most likely mono-anions (right), and the reported principal product of reaction with $O_2(a^1\Delta_g)$, parabanic acid (bottom).

Figure 2. Scheme illustrating the kinetically competing pathways for the removal/deactivation of $O_2(a^1\Delta_g)$ in a solvent, S, that contains both a reactive molecule, M, the neutral form of uric acid, UA^n, and the uric acid mono-anion, UA^{ma}. Note that the bimolecular rate constants for physical quenching, k_q, and chemical reaction, k_{rxn}, depend on the molecule involved. In most solvents, certainly in H_2O, the solvent-mediated channel for non-radiative $O_2(a^1\Delta_g)$ deactivation dominates over the channel for radiative deactivation (*i.e.*, $k_{nr} >> k_r$).

Figure 3. Normalized absorption and fluorescence spectra recorded from a phosphate buffered H_2O-solution of uric acid (pH = 7.4). The fluorescence spectrum has a λ_{max} of ~ 375 nm (excitation wavelength = 265 nm).

Figure 4. Absorption spectra of uric acid in H_2O recorded as a function of pH over a range that brackets the first pK_a value of ~ 5.6. The spectrum of UA^{ma} is red-shifted relative to the spectrum of UA^n, and isosbestic points are clearly visible. The latter are consistent with an equilibrium between UA^n and UA^{ma}.

Figure 5. (a) Representative time-resolved $O_2(a^1\Delta_g)$ phosphorescence traces recorded at 1275 nm upon 400 nm pulsed laser irradiation of FMN in D_2O-PBS at pD = 7.8. (b) The inverse lifetime of $O_2(a^1\Delta_g)$, τ_Δ^{-1}, obtained from the time-resolved traces plotted as a function of UA concentration (Note: $\tau_\Delta^{-1} = (k_r + k_{nr})[\text{solvent}] + k_{total}[UA]$, see text). (c) The rate constant for total $O_2(a^1\Delta_g)$ removal, k_{total}, as a function of pD in D_2O-based solutions. The solid line is a numerical fit to eq 1. The dashed lines indicate the values of k_{total} for the neutral and mono-anionic forms of UA, respectively. The inflection point in this titration curve yields a pK_a of 5.7 ± 0.2. The data point at pD = 9.6 is likely influenced by the start of the titration curve for the second pK_a, and was excluded from the fit.

Figure 6. Spectra of phosphate buffered aqueous solutions of UA. Spectra were recorded as a function of the elapsed irradiation time of the $O_2(a^1\Delta_g)$ sensitizer methylene blue (MB). We independently ascertained that MB does not bleach under these conditions. Hence, the MB spectrum was subtracted to yield the data shown. (a) Experiment performed in D_2O-PBS (pD = 7.8). (b) Experiment performed in H_2O-PBS (pH = 7.4).

Figure 7. Absorption spectra of UA in D_2O-PBS as a function of elapsed irradiation into the $O_2(X^3\Sigma_g^-) \rightarrow O_2(b^1\Sigma_g^+)$ band at 765 nm (fs laser spectral bandwidth of ~ 8 nm fwhm). The incident laser power was 2.52 W.

Figure 8. Representative time-resolved $O_2(a^1\Delta_g)$ phosphorescent traces recorded upon pulsed laser excitation of PNS in D_2O-PBS. (a) Traces recorded at six different concentrations of added UA. (b) Expanded portion of the traces in Figure 8a to more clearly show the UA-dependent changes on the rising portion of the signal (*i.e.*, the part of the trace that principally reflects the lifetime of the $O_2(a^1\Delta_g)$ precursor).

Figure 9. Pertinent parameters for the UA-mediated quenching of ^{3}PNS in D_2O. (a) The inverse of the PNS triplet state lifetime, $\tau_T^{-1} = k_T$, obtained from the rise of the $O_2(a^1\Delta_g)$ phosphorescence signal in Figure 8. (b) The quantum yield of $O_2(a^1\Delta_g)$ production, ϕ_Δ, obtained from the integral of the $O_2(a^1\Delta_g)$ phosphorescence signal normalized by the $O_2(a^1\Delta_g)$ lifetime. (c) The rate constant of triplet state quenching, k_T, obtained from the slope of plots such as the one shown in panel (a), as a function of pD. The data point at pD = 9.6 is likely influenced by the start of the titration curve for the second pK_a, and was excluded from the fit.