Quenching of Singlet Oxygen by Trolox C, Ascorbate, and Amino Acids: Effects of pH and Temperature

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The pH dependence of singlet oxygen quenching by histidine, *N*-acetyltyrosine ethyl ester (ATEE), ascorbic acid, Trolox C, and tryptophan has been observed using time-resolved infrared luminescence measurements in a D_2O/acetonitrile (50:50 v/v) solvent. Deprotonation of ascorbic acid, the protonated imidazole ring of histidine and the phenolic group of ATEE leads to an increase in the quenching rate constants by between 2 and 3 orders of magnitude. Such changes appear to be the basis for wide variations in quoted literature values of singlet oxygen quenching constants for these and related compounds. It is estimated that these pH-dependent quenching rate constants predict a modest (approximately 2- to 3-fold) change in singlet oxygen lifetime between the extremes of cellular pH. Activation data for singlet oxygen quenching show that the enthalpies of activation are low in all cases (between 0 and 11 kJ mol^{-1}) and that substantially negative entropies of activation (between −49 and −116 J K^{-1} mol^{-1}) result in rate constants being much lower than the diffusion-controlled limit. In all cases the data are consistent with quenching via reversible formation of an exciplex, all reactions being at the preequilibrium limit over the available temperature range.

Introduction

The dye-sensitized generation of the excited $^1\Delta_g$ state of oxygen ("singlet oxygen") is widely thought to be a significant mechanism in the photodynamic therapy of tumors.[1,2] This reactive oxidative species is capable of covalently damaging nucleic acids, proteins, and lipids in the cellular environment.[3−5] Chemical quenching of singlet oxygen, which is the cause of biological damage, competes with physical quenching, and several intracellular molecules are known to be effective physical quenchers.[6,7] In many instances both physical and chemical quenching occur through an initial interaction which typically appears to involve the rapidly reversible formation of a charge transfer type of complex.[8,9] The intracellular lifetime of singlet oxygen may therefore be significantly reduced from that in pure water (ca. 4 μs [10]). This may impede efforts to detect and image singlet oxygen in cellular systems by observation of its infrared luminescence in the region of 1270 nm,[11] and may limit the toxicity of singlet oxygen when a significant physical component of the quenching exists. Recent experiments based on acetylcholinesterase inactivation in K562 leukaemia cells suggest a lifetime for singlet oxygen near the cell surface of between approximately 0.5 and 1 μs,[12] whereas in the cell cytoplasm lifetimes of less than 0.5 μs are anticipated largely because of quenching by the high intracellular protein concentration.[13]

The lifetime of singlet oxygen in tissues, biological fluids, and cells will depend on the sum of the products of quencher concentrations and second order quenching rate constants for all quenching species present as has been previously discussed.[13] Second order rate constants for quenching of singlet oxygen have been measured for a very large number of compounds, as in the extensive compilation by Wilkinson et al.,[10] and identify several of the most significant possible intracellular quenchers. These include the amino acids histidine and cysteine, the antioxidants ascorbate (water soluble) and α-tocopherol, and polyenes such as β-carotene (lipid soluble). The rates of both physical and chemical quenching of singlet oxygen by α-tocopherol and related compounds have been shown to vary considerably with solvent.[6,14,15] Deactivation of singlet oxygen by α-tocopherol and several other quenchers has been shown to involve an exciplex with partial charge-transfer character, which is the intermediate leading to both chemical and physical quenching.[8,9,16] For phenols the quenching rate constant therefore depends on the redox potential of the phenol.[15,17] Values presented in the literature[10] for the rate constants for quenching of singlet oxygen by several biologically significant quenchers appear contradictory. A particular example is ascorbate, a water soluble and highly reducing antioxidant, for which quenching rate constants in aqueous solution quoted in the Wilkinson et al. compilation[10] vary between 2.5×10^6 dm^3 mol^{-1} s^{-1} and 1.6×10^8 dm^3 mol^{-1} s^{-1}. This suggests that the rate constant depends critically on the conditions in solution. In particular the importance of the protonation state of the solute is emphasized by studies on the effect of pH on singlet oxygen quenching by phenol[17] and thiols.[18] We now report a detailed study of several amino acids and antioxidants as singlet oxygen quenchers as a function of pH and find that reactivity depends on the protonation state of functional groups in these molecules. For all species investigated the measured Arrhenius activation energies are very close to zero and the rate constants for reaction are under entropic control.

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Materials and Methods

The experiments were undertaken in a 50:50 v/v mixture of D_2O (Atomic Energy of Canada) and acetonitrile (HPLC grade). The pD of this solvent was varied using potassium phosphate buffers. Because of possible effects on pH and pK_as introduced by the acetonitrile, all "pH" values quoted in the text and shown in the figures are the actual pH values measured using a pH meter and glass electrode (radiometer) calibrated with aqueous (H_2O) buffers and are uncorrected for the effect of deuterated solvent. The quenchers were all of the highest purity available from Aldrich or BDH and were used as received.

Singlet oxygen was generated using pulsed laser (frequency tripled Nd:YAG, 355 nm, 10 ns, ca. 5 mJ pulse^{-1}) excitation of duroquinone (5 mmol dm^{-3}) or perinaphthenone (0.1 mmol dm^{-3}) in air-saturated solutions. The singlet oxygen luminescence was detected using either a germanium photodiode−amplifier combination as described previously[9] or a liquid nitrogen cooled germanium detector from Applied Detector Corporation. The latter was found to increase sensitivity by a factor of ca. 10-fold. Variations of quenching rate constants with pH were fitted to pK_a curves using the GRAFIT program (Erithacus Software). Arrhenius data were collected between about 15 and 65 °C using a Peltier-controlled thermostated cell housing providing temperature control to better than 0.5 °C. The second order rate constants were corrected for changes in solute concentration due to variation in solvent density with temperature. Activation parameters were calculated using standard least-squares analysis[19] assuming a standard deviation in the individually measured rate constants of 10%, although routinely the calculated standard error from exponential fitting of the luminescence decays was of the order of 2 to 5%.

Results and Discussion

A D_2O/acetonitrile (50:50 v/v) solvent was used throughout this work. It proved to have several experimental advantages, including a conveniently long singlet oxygen lifetime (ca. 55 to 60 μs) in the absence of quencher and an increased solubility of some of the quenchers investigated. In addition the solutions could be conveniently buffered using either acetate or phosphate. For all quenchers it was demonstrated that for at least one pH value the experimentally obtained first order decay constant of singlet oxygen (k_1) depended linearly on quencher (Q) concentration. Plots of k_1 versus [Q] gave the total second order rate constant for singlet oxygen luminescence quenching (k_t) as the slope and the first order decay rate constant in the absence of quencher (k_0) as the intercept. The effect of pH on k_t was then obtained from values of k_0 and of k_1 at a single concentration of quencher at each pH value. The second order rate constant was then calculated as $(k_1 - k_0)/[Q]$. In all cases the measurements yield the total quenching rate constant representing the sum of rate constants for chemical (k_r) and physical (k_q) quenching and provide no direct means of determining the relative contribution from each component. All solutions in D_2O/acetonitrile were used within 1 h of preparation, and k_0 was invariant within this time. Values of k_0 were determined in the presence of each buffer system and were found to be increased (by less than 20%) only in alkaline solutions.

Second Order Rate Constants for Singlet Oxygen Quenching as a Function of pH. *Ascorbic Acid/Sodium Ascorbate*. Figure 1 shows plots of the first order singlet oxygen luminescence decay plotted against ascorbate concentration at pH values of 7.2 and 4.4 measured using duroquinone as sensitizer. The data show a clear difference in the second order rate constant (slope) at the two pH values: $(4.6 \pm 0.2) \times 10^7$ dm^3 mol^{-1} s^{-1} at pH 4.4 and $(3.2 \pm 0.2) \times 10^8$ dm^3 mol^{-1} s^{-1} at pH 7.2. The reduction in the singlet oxygen luminescence yield immediately following the laser pulse with increasing ascorbate concentration (results not shown) also indicated that the sensitizing triplet duroquinone state was also quenched twice as rapidly at pH 7.2 as at pH 4.4. The full pH dependence for singlet oxygen quenching by ascorbic acid/ascorbate is shown in Figure 2 and follows a typical sigmoidal pK_a curve. Fitting the data by nonlinear least-squares analysis yields a pK_a of 5.4 ± 0.1 and upper and lower second order rate constants of $(1.9 \pm 1.0) \times 10^7$ and $(3.1 \pm 0.1) \times 10^8$ dm^3 mol^{-1} s^{-1}, respectively. The pK_a of 5.4 measured using unadjusted pH values in D_2O/acetonitrile (50:50 v/v) solutions compares with a pK_a of 4.04 for ascorbic acid in H_2O.[20] The difference of 1.4 units is larger than the usual adjustment to convert pH to pD (pD = pH + 0.41)[21] and suggests that the pK_a is most likely perturbed not only by the deuterated solvent but also by the acetonitrile component. The value of $(1.9 \pm 1.0) \times 10^7$ dm^3 mol^{-1} s^{-1} obtained from the pH curve for the reactivity of singlet oxygen with ascorbic acid (pH ≪ pK_a) is somewhat ambiguous and was checked in a further experiment using 1.5 mol dm^{-3} DCl in D_2O/acetonitrile (50:50 v/v). These solutions gave an indicated pH value of −0.6 and the effect of ascorbic acid concentration on k_1 for singlet oxygen quenching is shown in the inset to Figure 2. The slope of this plot gives $k_t = (4.8 \pm 0.1) \times 10^5$ dm^3 mol^{-1} s^{-1}. If it were assumed that ascorbic

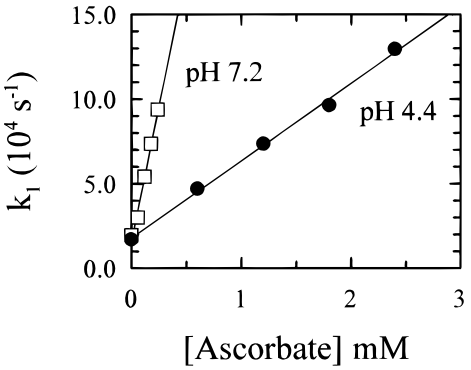

Figure 1. Effect of ascorbic acid/ascorbate concentration on the first order rate constant for the quenching of singlet oxygen luminescence (k_1) at pH 7.2 (□) and at pH 4.4 (●). Aerated solutions were made up in D_2O containing acetonitrile (50% v/v) and duroquinone (5 mmol dm^{-3}) and were buffered with acetate/acetic acid (50 mmol dm^{-3}).

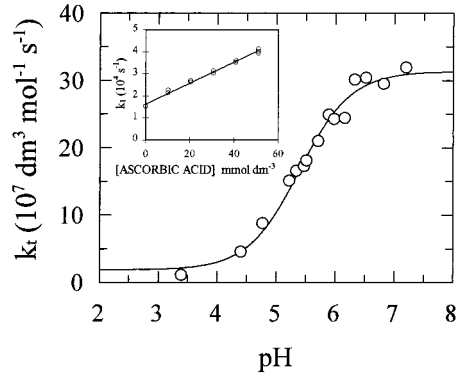

Figure 2. Effect of pH on the second order rate constant for quenching of singlet oxygen luminescence (k_t) by ascorbic acid/ascorbate. The conditions were the same as those described for Figure 1. Inset: The effect of ascorbic acid concentration on the first order rate constant for singlet oxygen quenching in D_2O solutions containing 1.5 mol dm^{-3} DCl and acetonitrile (50% v/v).

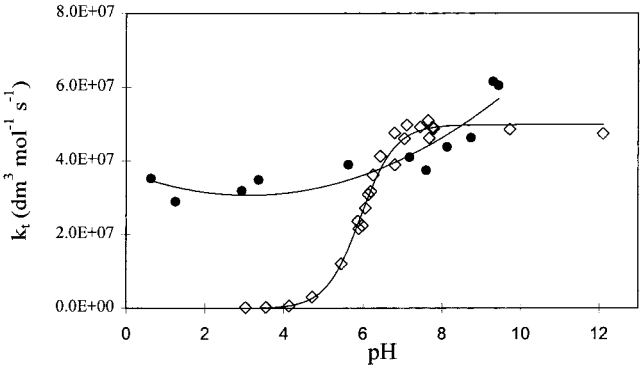

Figure 3. Effect of pH on the second order rate constant for quenching of singlet oxygen (k_t) by histidine (◇) and tryptophan (●). The rates constants were measured in D_2O/acetonitrile (50:50 v/v) buffered with phosphate (40 mmol dm^{-3}) with the addition of NaOH or DCl as required.

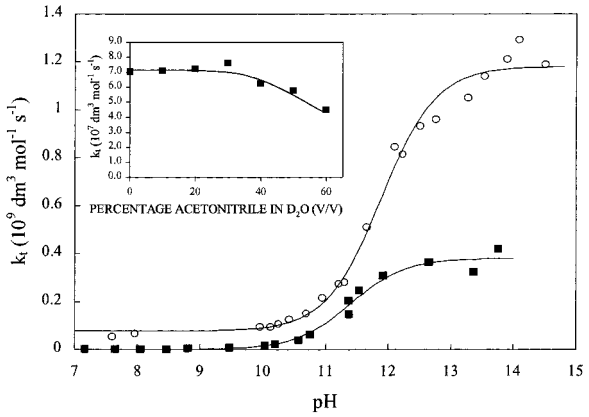

Figure 4. Effect of pH on the second order rate constant for quenching of singlet oxygen (k_t) by 4-methoxyphenol (○) and by ATEE (■). The rate constants were measured in D_2O/acetonitrile (50:50 v/v) buffered with phosphate (40 mmol dm^{-3}) with the addition of NaOH as required. Inset: Effect of acetonitrile addition to D_2O on the second order rate constant for quenching of singlet oxygen by 4-methoxyphenol at pH 7.6.

acid is totally unreactive with singlet oxygen, then at 6 pH units below the pK_a the reactivity of remaining ascorbate ($k_t = 3.1 \times 10^8$ dm^3 mol^{-1} s^{-1} from Figure 2) should give an apparent second order rate constant of about 3×10^2 dm^3 mol^{-1} s^{-1}. This shows that the measured value of $(4.8 \pm 0.1) \times 10^5$ dm^3 mol^{-1} s^{-1} indicates the real reactivity of ascorbic acid itself.

The second order rate constants for singlet oxygen quenching by ascorbate ion quoted in the compilation of Wilkinson et al.[10] span the range of k_t values from 9×10^5 dm^3 mol^{-1} s^{-1} in methyl acetate to 1.6×10^8 dm^3 mol^{-1} s^{-1} in D_2O at pH ~7. It is clear therefore that quenching of singlet oxygen at neutral pH is almost entirely due to the ascorbate ion and that ascorbic acid is comparatively a very weak quencher. It is well recognized that ascorbate is a more powerful reductant than ascorbic acid, and the observations here suggest that quenching of singlet oxygen by ascorbate relies on a charge transfer interaction. The low rate constants reported in the literature for quenching of singlet oxygen by ascorbic acid appear generally to correspond to measurements in solvents where ascorbic acid does not undergo deprotonation. The high rate constant of 1.5×10^8 dm^3 mol^{-1} s^{-1} reported by Scurlock et al.[17] in CD_3OD is surprising if it suggests that ascorbate is ionized in this solvent. Indeed, we have previously measured a rate constant of $\sim 10^6$ dm^3 mol^{-1} s^{-1} for singlet oxygen quenching by palmitoyl ascorbate in ethanol using time-resolved singlet oxygen luminescence decay measurements.[22]

Histidine and Tryptophan. Second-order rate constants for quenching of singlet oxygen (k_t) by histidine over a range of pH are shown in Figure 3. The curve shows the fit to a single pK_a of 5.9 ± 0.1 which is almost identical to the pK_a of 6.0 for deprotonation of the imidazole ring of histidine measured in H_2O. In this case the effect of deuterated solvent on pK_a must be offset by the influence of acetonitrile as cosolvent. The quenching rate constant reaches an upper value of $(5.0 \pm 0.1) \times 10^7$ at pH $\gg$ pK_a. The fit also gives a lower limit of $(-0.1 \pm 1.2) \times 10^6$ dm^3 mol^{-1} s^{-1} at pH $\ll$ pK_a. A further experiment in D_2O/acetonitrile (50:50 v/v) containing DCl (1.5 mol dm^{-3}) and histidine concentrations up to 0.25 mol dm^{-3} showed $k_t \leq 10^4$ dm^3 mol^{-1} s^{-1} for the fully protonated form. The literature surveyed by Wilkinson et al.[10] contains a large number of published rate constants spanning a considerable range, and it seems clear that the variation must in large part reflect solvent effects, principally those governing the protonation state of the imidazole ring of histidine.

Figure 3 also shows the measured value of k_t for quenching of singlet oxygen by tryptophan as a function of pH. There appears to be no pronounced effect of pH between pH 0.6 and pH 9.4, consistent with the lack of ionizable protons over this pH range in the indole nucleus of tryptophan, which is the reactive group of this molecule. At pH 7.6 $k_t = (4.1 \pm 0.4) \times 10^7$ dm^3 mol^{-1} s^{-1}, which is reasonably consistent with many of the previous values quoted by Wilkinson et al.[10]

Tyrosine and Other Phenols. It is well known for phenols that ionization of the phenolic proton results in an increased rate constant for singlet oxygen quenching.[17,23] We have examined the effect of pH on singlet oxygen quenching by phenols representing phenolic antioxidants such as vitamin E and plant polyphenols and the amino acid tyrosine. The experiments serve not only to further demonstrate the importance of ionization of the phenolic proton, but also to establish upper and lower rate constants under well-defined conditions so that they may be compared with one-electron oxidation potential and activation parameters for both phenols and other singlet oxgygen quenchers examined here.

N−Acetyltyrosine Ethyl Ester (ATEE). ATEE is used here to represent the amino acid tyrosine. The solubility of ATEE in the acetonitrile/D_2O solvent used in these experiments has facilitated measurements of singlet oxygen quenching, which are otherwise made difficult by the limited solubility of tyrosine in water at neutral pH. The variation of k_t with pH for ATEE is shown in Figure 4. The fitted curve shows an experimental pK_a of 11.4 ± 0.1 in D_2O/acetonitrile, some 1.3 units higher than the pK_a of tyrosine measured in H_2O. The fitted curve gives a maximum value of k_t at pH $\gg$ pK_a of $(3.8 \pm 0.1) \times 10^8$ dm^3 mol^{-1} s^{-1}. Although the curve indicates a very low value of k_t at near neutral pH, a further experiment over a higher concentration range of ATEE (not shown) indicated k_t to be $(8.5 \pm 0.2) \times 10^5$ dm^3 mol^{-1} s^{-1} at pH 7.6. As argued above in the case of ascorbic acid, this value is about fourteen times larger than might be expected of the contribution from quenching by residual phenolate at this pH and therefore represents a real rate of quenching by the neutral phenol group of ATEE.

4-Methoxyphenol and Trolox C. The effect of pH on singlet oxygen quenching by 4-methoxyphenol shown in Figure 4 clearly shows the reactivity of the neutral form of 4-methoxyphenol to be $k_t = (5.3 \pm 0.2) \times 10^7$ dm^3 mol^{-1} s^{-1} at pH 7.6. The effect of the amount of acetonitrile in the solvent on the quenching by 4-methoxyphenol at pH 7.6 was also investigated, and the results are shown as the inset to Figure 4. There is only

TABLE 1: Second Order Rate Constants (k_t) and Activation Parameters for Quenching of Singlet Oxygen (All Rate Constants Measured in Solutions of D_2O/Acetonitrile mixtures (50:50 v/v))

solute	k_t (298 K) dm^3 mol^{-1} s^{-1}	pK_a (obs)	pK_a (H_2O)	E_{exp} kJ mol^{-1}	$\Delta H^{\ddagger}$ kJ mol^{-1}	$\Delta S^{\ddagger}$ J K^{-1} mol^{-1}	E'_7 (mV)
ascorbic acid	$(4.8 \pm 0.1) \times 10^5$ (pH = −0.6)	5.4 ± 0.1	4.04[e]	8.3 ± 1.4	5.9 ± 1.4	-116 ± 5	
ascorbate	$(3.1 \pm 0.1) \times 10^8$			13.1 ± 1.8	10.6 ± 1.8	-49 ± 6	300[a]
histidine (ImH^+)	$\leq 10^4$ (pH = −0.6)	5.9 ± 0.1	6.02[e]				
histidine (Im)	$(5.0 \pm 0.1) \times 10^7$			9.5 ± 1.8	7.0 ± 1.8	-74 ± 6	>770[b]
N−acetyltyrosine, ethyl ester	$(8.5 \pm 0.2) \times 10^5$ (pH = 7.6)	11.4 ± 0.1	10.13[f]	9.8 ± 1.7	7.4 ± 1.7	-107 ± 5	760[c,d]
N−acetyltyrosinate, ethyl ester	$(3.8 \pm 0.1) \times 10^8$			8.8 ± 1.4	6.3 ± 1.4	-59 ± 5	
4-methoxyphenol	$(5.3 \pm 0.2) \times 10^7$ (pH = 7.6)	11.9 ± 0.1	10.2[g]	-0.6 ± 1.8	-3.1 ± 1.8	-104 ± 6	600[a]
4-methoxyphenolate	$(1.2 \pm 0.1) \times 10^9$			4.9 ± 1.5	2.5 ± 1.5	-65 ± 5	
Trolox C	$(4.4 \pm 0.1) \times 10^8$ (pH=7.6)		11.92[a]	3.3 ± 1.7	0.8 ± 1.7	-77 ± 5	430[a]
tryptophan	$(4.1 \pm 0.4) \times 10^7$ (pH = 7.6)			7.0 ± 1.8	4.5 ± 1.8	-82 ± 6	1080[c]

[a] From ref 31. [b] From ref 32. [c] From ref 33. [d] Value shown is for phenol. [e] From ref 20. [f] Value shown is for tyrosine. [g] Value quoted in ref 23.

a small decrease in the rate constant as the proportion of acetonitrile is increased. At 50% D_2O, as used in all of the other experiments reported here, the rate constant is reduced by only 18% compared with that in D_2O alone. The value of k_t increases to a fitted maximum of $(1.2 \pm 0.1) \times 10^9$ dm^3 mol^{-1} s^{-1} with a pK_a of 11.9 ± 0.1. Trolox C, the water soluble analogue of α-tocopherol (vitamin E), is a much more efficient quencher of singlet oxygen at near neutral pH than 4-methoxyphenol and was found to have $k_t = (4.4 \pm 0.1) \times 10^8$ dm^3 mol^{-1} s^{-1} at pH 7.6.

For the quenchers which are weak acids (4-methoxyphenol, ATEE, and ascorbic acid) the observed pK_as in D_2O/acetonitrile (Table 1) are approximately 1.4 to 1.7 units higher than the literature values quoted for aqueous solution. This is larger than the difference of 0.4 units previously observed between solutions in H_2O and D_2O alone,[17,18,21] and is ascribed to the effect of acetonitrile, which would be expected to stabilize the neutral uncharged form of the quencher (the conjugate acid). Consequently more alkaline conditions are required to deprotonate the molecule, and the pK_a is increased. In contrast, the observed pK_a for singlet oxygen quenching by histidine in D_2O/acetonitrile is almost the same as the pK_a of histidine in H_2O. Acetonitrile would be expected to stabilize the neutral conjugate base form of the imidazole ring in histidine, and hence the effect on the pK_a is opposite to that observed with the weak acids and presumably cancels out the pD correction of 0.4 units.

The rate constants for singlet oxygen quenching by phenols obtained here by the time-resolved luminescence method may be compared with an extensive set of data obtained by Tratnyek and Holgne.[23] These are based on measurements of phenol oxidation in H_2O solutions and therefore measure the chemical quenching rate constant, k_r. Values of k_r measured at neutral pH for phenol itself and for 4-methylphenol were $(2.6 \pm 4) \times 10^6$ and $(9.6 \pm 2.8) \times 10^6$ dm^3 mol^{-1} s^{-1}, respectively, and are both higher than our value of $(8.5 \pm 0.2) \times 10^5$ dm^3 mol^{-1} s^{-1} for ATEE at neutral pH, although clearly the value for phenol is not reliable. In alkaline solution (pH > pK_a) the rate constants measured by Tratnyek and Holgne[23] for phenol and for 4-methylphenol, $(1.55 \pm 0.05) \times 10^8$ and $(3.50 \pm 0.08) \times 10^8$ dm^3 mol^{-1} s^{-1}, respectively, are quite similar to our value of $(3.8 \pm 0.1) \times 10^8$ dm^3 mol^{-1} s^{-1} for ATEE at high pH. The rate constants obtained by Tratnyek and Holgne[23] for 4-methoxyphenol at pH < pK_a ($(2.2 \pm 2.2) \times 10^7$ dm^3 mol^{-1} s^{-1}) and pH > pK_a ($(6.67 \pm 0.25) \times 10^8$ dm^3 mol^{-1} s^{-1}) were both about one half of our values. These results imply that a much greater fraction of the overall quenching interaction in aqueous solution (≥ 50%) leads to chemical destruction of the phenol compared with the situation in methanol and hydrocarbon solvents where physical quenching overwhelmingly predominates.[14,15] Considering that the quenching interaction between singlet oxygen and phenol involves an intermediary charge transfer complex (vide infra), these results suggest that reactive quenching is favored for the more oxidizable phenols in a polar environment such as an aqueous solution. Gorman and co-workers[16] have attempted and failed to observe oxidation of α-tocopherol to the tocopheroxyl radical by singlet oxygen in toluene solution, but Thomas and Foote[15] have reported transient absorption indicative of the phenoxyl radical of 2,4,6-triphenylphenol on quenching of singlet oxygen in methanol. Consequently we have attempted to detect phenoxyl radical formation from Trolox C and 4-methoxyphenol in aqueous solution. Figure 5 shows the transient absorption spectra from laser flash photolysis (355 nm) of a deaerated solution (D_2O/acetonitrile, 50:50 v/v) containing perinaphthenone (PN, 0.1 mmol dm^{-3}) and Trolox C (400 μmol dm^{-3}) at pH 7.6. Under these conditions it was found that triplet perinaphthenone (^{3}PN*, λ_{max} 480−500 nm) reacts with Trolox C with a rate constant of ca. 2×10^9 dm^3 mol^{-1} s^{-1} to give the Trolox phenoxyl radical (λ_{max} 430 nm). Saturation of the same solution with oxygen leads to rapid quenching of ^{3}PN* and energy transfer to oxygen. The singlet oxygen luminescence lifetime under these conditions is about 5 μs. The inset to Figure 5 shows the transient absorption recorded at 430 nm for this solution when both deaerated and oxygen saturated. In the presence of oxygen a small amount of phenoxyl radical is formed immediately ($t < 0.5$ μs) by direct reaction of Trolox C with ^{3}PN in competition with energy transfer to oxygen.

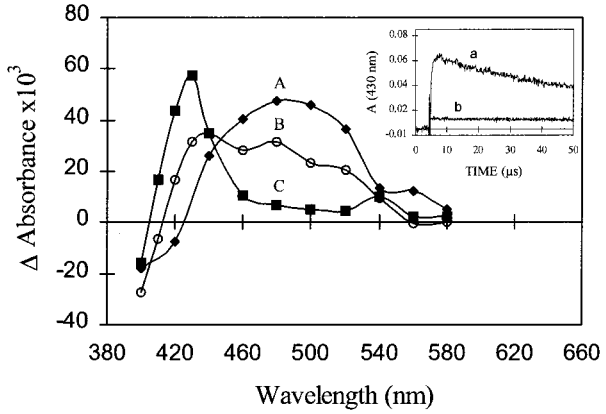

Figure 5. Transient absorption spectra measured on laser flash excitation (355 nm, 15 mJ per pulse) of solutions (D_2O/acetonitrile, 50:50 v/v) of perinaphthenone (PN) and Trolox C. The transient spectrum of ^{3}PN* was measured 1 μs (curve A) after the laser flash for a deaerated solution of PN (0.1 mmol dm^{-3}). Curves B and C show the spectra measured 1 μs and 10 μs, respectively, after laser excitation of a deaerated solution containing PN (0.1 mmol dm^{-3}) and Trolox C (0.4 mmol dm^{-3}). Inset: Absorption transients measured at 430 nm from a solution of PN (0.1 mmol dm^{-3}) and Trolox C (0.4 mmol dm^{-3}) when (a) deaerated and (b) oxygen saturated.

However there is no subsequent formation of phenoxyl radical by reaction of singlet oxygen. The experiment shows that the yield of phenoxyl radical from quenching of singlet oxygen by Trolox C at pH 7.6 is less that 2%. A similar experiment with 4-methoxyphenol at pH ~12 also failed to detect phenoxyl radical formation. Although the products of the putative full electron transfer reaction, the Trolox phenoxyl radical and superoxide, react rapidly ($k = 4.5 \times 10^8$ dm^3 mol^{-1} s^{-1}),[24] it was anticipated that should the reaction proceed in this way at least a fraction of the products would escape from the solvent separated radical ion pair. The results would appear to confirm that reactive quenching of singlet oxygen by phenols proceeds by reactions not involving electron transfer, e.g., endoperoxide and hydroperoxide formation.[16,25]

Effect of Temperature on Singlet Oxygen Quenching−Activation Parameters. The effect of temperature on the quenching rate constant, k_t, was measured between 15 and 65 °C in the D_2O/acetonitrile solvent containing appropriate buffers. The second order rate constants were adjusted both for changes in the decay rate of singlet oxygen in the absence of solute (k_0) and for the change in density of the D_2O/acetonitrile solvent as a function of temperature. In D_2O/acetonitrile (50:50 v/v) containing sodium phosphate (40 mmol dm^{-3}, pH 7.6) the effect of temperature on k_0 was small, corresponding to an apparent Arrhenius activation energy of 2.0 ± 0.2 kJ mol^{-1}. This compares with a reported value of 6.7 kJ mol^{-1} in D_2O alone.[26] Activation parameters are shown in Table 1 together with a summary of the kinetic data described above. The Arrhenius plots were linear and were used to determine enthalpies ($\Delta H^{\neq}$) and entropies ($\Delta S^{\neq}$) of activation. In all cases the enthalpies of activation were low and entropies of activation were highly negative. For ascorbic acid and ascorbate anion the enthalpies of activation (5.9 ± 1.4 and 10.6 ± 1.8 kJ mol^{-1}, respectively) differ by less than a factor of 2, despite k_t differing by a factor of over 500. As discussed in section 1a, these two rate constants represent the actual differing reactivities of ascorbate and ascorbic acid, and the entropies of activation are therefore true values and do not simply reflect a change in concentration of the reactive anion with pH. The rate constants for these reactions are therefore controlled not by enthalpy but by the entropies of activation which are (−116 ± 5) and (−49 ± 6) J K^{-1} mol^{-1} for ascorbic acid and ascorbate, respectively. A similar situation is observed for 4-methoxyphenol and 4-methoxyphenolate where the enthalpies of activation at pH 7.7 and pH 13.2 are quite similar, whereas the entropies of activation are (−104 ± 6) and (−65 ± 5) J K^{-1} mol^{-1}, respectively. Almost identical differences in the entropies of activation are measured for ATEE at neutral and alkaline pH. A further comparison of the reactivities of 4-methoxyphenol and Trolox C at around pH 7.6 shows that the higher reactivity of Trolox C by nearly 1 order of magnitude is also due to a less negative entropy of activation (−77.0 ± 5 J K^{-1} mol^{-1} compared with −104 ± 6 J K^{-1} mol^{-1} for 4-methoxyphenol). In contrast the lower reactivity of ATEE at pH 7.6 compared with 4-methoxyphenol appears to be a result of a somewhat higher enthalpy of activation.

Activation enthalpies measured at pH 7.6 for histidine and tryptophan were also low and comparable with values determined for ascorbic acid and ATEE. Entropies of activation were measured to be −74 ± 6 J K^{-1} mol^{-1} for histidine and −82 ± 6 J K^{-1} mol^{-1} for tryptophan and are consistent with the two molecules having similar values of k_t. Activation parameters for the fully protonated form of histidine could not be obtained since it was extremely unreactive and it was not possible to obtain reliable kinetic data in acid solutions.

The activation parameters determined here are in excellent agreement with earlier conclusions that reactions of this type take place via reversible formation of an exciplex.[9,16] At the available temperatures, the reactions are clearly at the preequilibrium limit where, although the activation enthalpies are close to zero, the rate constants are lower by several orders of magnitude than that for diffusion, cf. 1.2×10^{10} dm^3 mol^{-1} s^{-1} for the oxygen quenching of tryptophan fluorescence.[27] This is because of the large negative entropy factor associated with a highly ordered product-forming transition state (chemical quenching) or intersystem crossing from the exciplex to the ground-state complex (physical quenching). The results here say nothing concerning the balance between the two processes in any individual case. However, it is of interest that the deprotonated forms, ascorbate, *N*-acetyltyrosinate ethyl ester, and 4-methoxyphenolate, all exhibit markedly lower negative activation entropies than do the corresponding conjugate acids. Since, at the preequilibrium limit, the overall experimental entropy of activation is the entropy difference between the transition state for quenching, i.e., the second step, and the reactants, changes in the exciplex entropy are irrelevant. Therefore, whether the aforementioned experimental entropy differences reflect real mechanistic differences, e.g., changes in the balance between chemical and physical quenching and/or solvation entropy factors, is impossible to determine at this stage.

A Hypothetical pH Curve for Singlet Oxygen Quenching in a Cellular Environment. The results described above show that temperature effects on the overall quenching of singlet oxygen by the compounds investigated are quite small and in line with current views on the mechanisms of such reactions. In contrast, the pH effects are very large and merit discussion in the cellular context. Baker and Kanofsky[13] have concluded that proteins are the most significant singlet oxygen quenchers, and residues of histidine, tryptophan, and tyrosine within proteins are expected to be major quenchers of singlet oxygen. In addition our results show that ascorbate, which in cells such as lymphocytes may reach an intracellular concentration[28] of 3 mmol dm^{-3}, may contribute significantly. The studies of Rougée et al.[18] also indicate that thiol compounds may also be important in quenching singlet oxygen, and in particular reduced glutathione for which an intracellular concentration of 5 mmol dm^{-3} is fairly typical.[29] The effective concentrations of histidine and tyrosine residues, which might be shielded from reaction with singlet oxygen by their protein environments, are more difficult to assess. To reach a qualitative result, the work of Zimmerman and Trach[30] may be taken to indicate an approximate cytoplasmic protein concentration of around 230 to 300 g dm^{-3}. Taking an average molecular weight of 110 for an amino acid and that each amino constitutes about 5% of the total amino acid composition, it is calculated that the equivalent molarity of amino acids in the cytoplasm is of the order of 100 mmol dm^{-3}. Assuming that the protein environment might reduce reactivity by a factor of 5 for histidine and tyrosine and by a factor of 10 for the more hydrophobic tryptophan residues, then we estimate an effective cytoplasmic molarity of 20 mmol dm^{-3} for histidine and tyrosine and 10 mmol dm^{-3} for tryptophan. The presence of cysteine in proteins is ignored for the present since it occurs mainly in the oxidized form and it will be assumed that glutathione is present at 5 mmol dm^{-3}. These concentrations and appropriate rate constants for tyrosine, histidine, and ascorbate from Table 1 have been used together with rate constants for glutathione[18] (pK_a (−*SH*) = 8.75) of $k_{lower} = 4 \times 10^4$ dm^3 mol^{-1} s^{-1} as an upper limit when pH ≪ pK_a and $k_{upper} = 1.9 \times 10^8$ dm^3 mol^{-1} s^{-1} when pH ≫ pK_a. The calculated

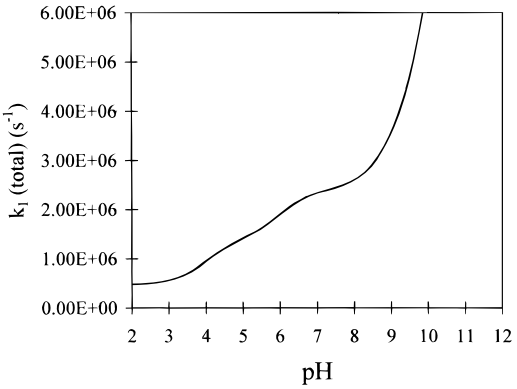

Figure 6. A plot of the overall first order rate constant for singlet oxygen quenching (k_1(total)) as a function of pH for a solution containing tryptophan (10 mmol dm^{-3}), histidine (20 mmol dm^{-3}), tyrosine (20 mmol dm^{-3}), ascorbate (3 mmol dm^{-3}), and reduced glutathione (5 mmol dm^{-3}) in which the concentrations are taken to be representative of an intracellular environment.

TABLE 2: Contributions of Individual Singlet Oxygen Quenchers to the Overall First Order Decay Constants at pH 4.0 and pH 7.5 for a Mixture of Amino Acids and Antioxidants Taken to Represent Concentrations Appropriate to Intracellular Conditions

	concn (mmol dm^{-3})	k_1 at pH 4.0 (s^{-1})	k_1 at pH 7.5 (s^{-1})
ascorbate	3.0	4.70×10^5	9.39×10^5
histidine	20	1.47×10^4	9.69×10^5
glutathione, reduced	5.0	2.17×10^2	5.08×10^4
tryptophan	10	4.50×10^5	4.50×10^5
tyrosine	20	1.70×10^4	3.60×10^4
total		9.52×10^5	2.44×10^6

[a] The first order decay constants are the products of concentrations and second order rate constants at each pH value.

overall first order pH-dependent rate constant, k_1(total), for quenching of singlet oxygen calculated from the sum of the individual contributions of each quencher according to eq 1 is shown in Figure 6.

$$k_1(\text{total}) = \sum_i \left\{ \left[\frac{k_{i,\text{lower}} \times c_i}{1 + 10^{(\text{pH}-pK_i)}} \right] + \left[\frac{k_{i,\text{upper}} \times c_i \times 10^{(\text{pH}-pK_i)}}{1 + 10^{(\text{pH}-pK_i)}} \right] \right\} \quad (1)$$

The contributions to k_1(total) at pH 4.0 and pH 7.5 are listed in Table 2, taken as encompassing the range of pH from lyzosomes to the cell cytoplasm. The calculations have been undertaken to demonstrate the effect of pH on the overall quenching rate constant, rather than to establish an absolute lifetime for singlet oxygen under these conditions. Nevertheless, the overall first order decay constant at pH 7.5 of 2.4×10^6 s^{-1} is some 10-fold higher than that in pure water and equivalent to a lifetime of 0.4 μs, in accord with the conclusions of Baker and Kanosky.[13] At pH 4.0 the overall decay constant is 9.5×10^5 s^{-1} mainly because of the reactivity of tryptophan and residual ascorbate, each contributing almost 50% to the total. At pH 7.5 the overall decay constant is some 2.5-fold higher, largely because of deprotonation of ascorbic acid and histidine with pK_a's of 4.0 and 6.0 and the consequent increased reactivity of ascorbate and histidine (deprotonated imidazole ring) which now each contribute approximately 40% to the total quenching. The ability of singlet oxygen to react with and to damage critical targets will therefore depend on the pH and concentrations of potential quenchers at a particular cellular location. Likewise the ability to detect singlet oxygen generated at an intracellular site will also be controlled by the pH and quencher concentration within the microenvironment.

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