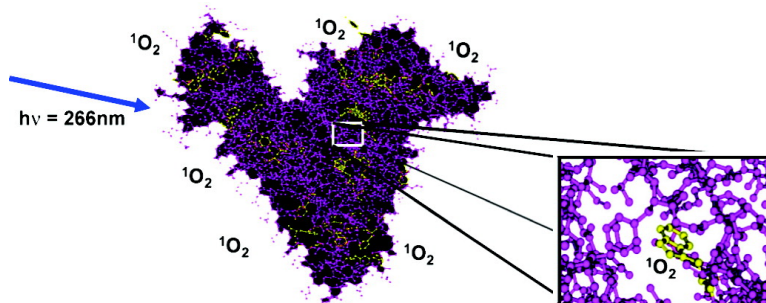


Quantitative Determination of Singlet Oxygen Generated by Excited State Aromatic Amino Acids, Proteins, and Immunoglobulins

Khin K. Chin, Colleen C. Trevithick-Sutton, Jeremy McCallum, Steffen Jockusch, Nicholas J. Turro, J. C. Scaiano, Christopher S. Foote, and Miguel A. Garcia-Garibay

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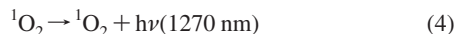
Khin K. Chin,[†] Colleen C. Trevithick-Sutton,[‡] Jeremy McCallum,[§] Steffen Jockusch,^{||} Nicholas J. Turro,^{*,||} J. C. Scaiano,^{*,‡} Christopher S. Foote,[†] and Miguel A. Garcia-Garibay^{*,†}

Department of Chemistry and Biochemistry, University of California, Los Angeles, California 90095-1569, Department of Chemistry, University of Ottawa, Ottawa, Ontario K1N 6N5, Canada, Department of Chemistry and Biochemistry, Loyola Marymount University, 1 LMU Drive, MS 8225, Los Angeles, California 90045, and Department of Chemistry, Columbia University, 3000 Broadway, Mail Code 3119, New York, New York 10027

Received February 5, 2008; E-mail: mgg@chem.ucla.edu

Singlet oxygen, $^1\text{O}_2$, is a highly reactive electronically excited state of oxygen invoked in many physiological and pathological processes.¹ While its generation in biological systems is mainly traced back to photosensitization by sunlight-absorbing cofactors and dark enzymatic pathways, $^1\text{O}_2$ can also form by photosensitization with aromatic amino acids such as tryptophan (Trp), tyrosine (Tyr), and phenylalanine (Phe), which are abundant light absorbers in the UV-B range (290–320 nm).² Notably, though the role of $^1\text{O}_2$ in the formation of H_2O_2 by in vitro UV irradiation of aromatic amino acids in immunoglobulins was reported by Wentworth et al.,³ the generation of $^1\text{O}_2$ by aromatic amino acids and biological macromolecules has not been analyzed quantitatively.⁴ In this manuscript we report the quantum yields of $^1\text{O}_2$ generation upon excitation of Trp, Tyr, and Phe in their zwitterionic forms, as methyl esters, and within a few test proteins and immunoglobulins.

The sensitized generation of $^1\text{O}_2$ starts by excitation of the amino acid by absorption of UV light, followed by intersystem crossing to the triplet state (eqs 1 and 2), and is completed by energy transfer to oxygen in its triplet ground state (eq 3). The quantum yields of $^1\text{O}_2$ can be obtained in air-saturated solutions by measuring the quantum yield of its near IR emission (eq 4 and Figure 1).



The amino acids Phe, Trp, and Tyr, the N-acetylated amino acids NAc-Phe, NAc-Trp, and NAc-Tyr, the proteins bovine serum albumin (BSA) and ovalbumin (OVA), and the immunoglobulins bovine-IgG, human-IgG, and sheep-IgG were dissolved in either D_2O or MeCN. Samples were irradiated with a 266 nm pulse from a frequency quadrupled continuum Nd:YAG laser (8 ns, 3 mJ). The near-IR luminescence from $\text{O}_2(^1\Delta_g)$ was collected at right angles to excitation after focusing onto the variable slit of a SPEX 1681 monochromator and detected using a Hamamatsu PMT sensitive in the near-IR region.

The near-IR luminescence spectrum of singlet oxygen photosensitized by phenylalanine in an air-saturated D_2O solution is shown in Figure 1. There is a strong peak at about 1270 nm, characteristic of singlet oxygen phosphorescence. A time-resolved decay trace of the observed phosphorescence has a lifetime (τ_Δ) of

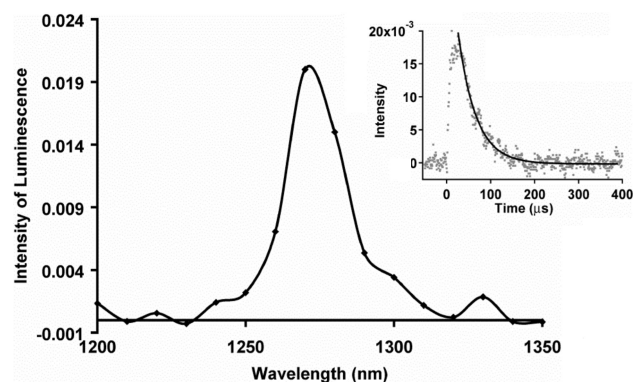


Figure 1. Near-infrared luminescence of singlet oxygen sensitized by UV irradiation of phenylalanine in D_2O . The emission spectrum shows a peak at about 1270 nm. Inset: Decay of singlet oxygen phosphorescence as a function of time (in μs), sensitized by phenylalanine. The black line is a curve fit of the decay.

about 48 μs , consistent with reported values of $^1\text{O}_2$ in D_2O . Solutions of tryptophan, tyrosine, bovine serum albumin (BSA), ovalbumin (OVA), the immunoglobulins bovine IgG, human IgG, and sheep IgG in D_2O , and the N-acetyl amino acids in MeCN were irradiated under similar conditions to emit spectra consistent with singlet oxygen luminescence. Argon-purged solutions of each compound showed no near-IR luminescence.

The quantum yields of oxygen (Φ_Δ) were determined using methylene blue in D_2O ($\Phi_\Delta = 0.52$) or MeCN ($\Phi_\Delta = 0.52$) as a reference.⁵ Triplicate kinetic analysis of decay traces for Tyr and Trp gave lifetimes for singlet oxygen of 35–40 μs in D_2O , which are about 20% shorter than those observed with Phe. This may be attributed to quenching, in agreement with the high reactivity of singlet oxygen with these two amino acids. To ensure that singlet oxygen emission measured was generated by energy transfer from free amino acids and proteins in their native states, and not from photooxidation products, a fresh solution of each compound was used for acquisition after each laser shot.

The quantum yields of singlet oxygen emission are summarized in Table 1. The $^1\text{O}_2$ quantum yields (Φ_Δ) for phenylalanine, tyrosine, and tryptophan ($\Phi_{\Delta,\text{Trp}} = 0.065$, $\Phi_{\Delta,\text{Tyr}} = 0.138$, $\Phi_{\Delta,\text{Phe}} = 0.062$) are only a fraction of the corresponding quantum yields of triplet formation ($\Phi_{\text{T},\text{Trp}} = 0.18$, $\Phi_{\text{T},\text{Tyr}} = 0.50$, $\Phi_{\text{T},\text{Phe}} = 0.40$).^{6,7} Given that the decrease in Φ_Δ with respect to Φ_{T} is greater than the decrease in $^1\text{O}_2$ lifetimes, we discount a significant amount of quenching by the original sensitizer.⁸ In fact, aromatic amino acids are known to undergo triplet state electron transfer, electron ejection, and photodissociation, all of which may contribute to the difference

[†] University of California, Los Angeles.

[‡] University of Ottawa.

[§] Loyola Marymount University.

^{||} Columbia University.

Table 1. Quantum Yields of Singlet Oxygen Generated by Photosensitized Aromatic Amino Acids, N-Acetyl-Amino Acids, Proteins, and Immunoglobulins^a

compound	solvent	Φ_{Δ}
phenylalanine	D ₂ O	0.065 ± 0.004
tyrosine	D ₂ O	0.138 ± 0.007
tryptophan	D ₂ O	0.062 ± 0.011
N-acetyl-phenylalanine	MeCN	0.083 ± 0.02
N-acetyl-tyrosine	MeCN	0.19 ± 0.05
N-acetyl-tryptophan	MeCN	0.11 ± 0.02
bovine serum albumin	D ₂ O	0.037 ± 0.008
chick ovalbumin	D ₂ O	0.049 ± 0.005
bovine IgG	D ₂ O	0.043 ± 0.008
human IgG	D ₂ O	0.030 ± 0.010
sheep IgG	D ₂ O	0.036 ± 0.007

^a All experiments were conducted at room temperature and excited at 266 nm. Reported values are averages of 5 or more measurements.

between the triplet quantum yield (Φ_T) and the singlet oxygen quantum yield (Φ_{Δ}).⁶

The protonation state of the α -amino group in aromatic amino acids has a significant impact on photophysical properties.^{6,7} N-Acetylated amino acids have lower photoionization efficiencies and longer triplet lifetimes than native aromatic amino acids. The quantum yields of singlet oxygen formation of N-acetyl amino acids were indeed slightly greater than those of the native amino acids. The longer triplet lifetimes may allow for more efficient energy transfer to oxygen and the lower reactivity of singlet oxygen toward N-acetylated amino acid also diminishes quenching (data not shown).^{6,7}

The impact of peptide linkages and three-dimensional structures on ¹O₂ quantum yields was investigated by analysis of the proteins BSA and OVA and the immunoglobulins bovine-IgG, human-IgG, and sheep-IgG. Native proteins and immunoglobulins with characteristic tertiary and quaternary structures may alter the formation of singlet oxygen by entrapping it within hydrophobic pockets or by preventing it from accessing amino acids buried deep within their 3D structure.¹⁰ Their structures could also affect the lifetime of singlet oxygen, as compared to that observed in free amino acid solutions, by offering additional reactive paths and quenching processes. Indeed, while an equimolar mixture of free aromatic amino acids in solution would have a weighted average singlet oxygen quantum yield of 0.08, the proteins and immunoglobulins sampled in this work have quantum yields that are less than half this value (Table 1). Because the decay of ¹O₂ emission fit well to a single exponential that is similar to that observed with the free amino acids in water, we discount populations of ¹O₂ that are rapidly quenched in a specific protein or IgG environment. It seems more likely that the diminished quantum yield results from a fraction of aromatic residues unavailable for energy transfer.¹⁰

In support of the latter hypothesis, previous reports on the quenching of tryptophan phosphorescence by molecular oxygen by Strambini et al.⁹ and Vanderkooi et al.¹⁰ have shown that the quenching rate constant is closely related to the flexibility of the protein matrix. Rigid polypeptide environments surrounding the sensitizing chromophores can hinder the migration of oxygen and reduce the quenching of tryptophan phosphorescence within a protein. The barrier for diffusion of oxygen in water (ca. 3 kcal mol⁻¹) is much smaller than the average barrier for diffusion through the internal matrix of a protein (ca. 10 kcal mol⁻¹).^{9,10}

Significant differences in the availability of aromatic amino acids and the diffusivity of oxygen into the protein environment can be inferred by a close analysis of the structures of OVA and BSA. Ovalbumin possesses only a small fraction (ca. 18%) of aromatic residues on the surface of its native structure.¹¹ If only surface residues could sensitize oxygen, the quantum yield of singlet oxygen

would be smaller than that obtained with an equivalent number of free aromatic amino acids in solution. However, the quantum yield of 0.049 (Table 1) for OVA is greater than that predicted by the number of surface residues (ca. 0.016), and implies energy transfer from residues buried within the protein. In contrast, structural data from Human Serum Albumin,¹¹ which is homologous to BSA, suggests a larger fraction (ca. 40%) of aromatic residues located on the surface, which can easily account for the observed quantum yield.¹²

In conclusion, we have quantified the singlet oxygen quantum yields generated by excited-state aromatic amino acids tryptophan, tyrosine, and phenylalanine by time-resolved phosphorescence measurements. In addition, we have measured the singlet oxygen quantum yield from N-acetylated amino acids and from selected proteins and immunoglobulins. The three-dimensional conformation found in proteins and immunoglobulins results in decreased quantum yields. A crude analysis of the fraction of surface and internal residues in BSA and OVA suggests that molecular oxygen can diffuse through the polypeptide matrix and can be sensitized by residues buried within the folds of the protein structure. In agreement with previous tryptophan phosphorescence studies, singlet oxygen generation is hindered and limited by the increased viscosity of the protein matrix.

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Supporting Information Available: Experimental details and a detailed analysis of the number and location of aromatic residues in OVA and HSA. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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