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Reaction of Singlet Oxygen with Tryptophan in Proteins: A Pronounced Effect of the Local Environment on the Reaction Rate

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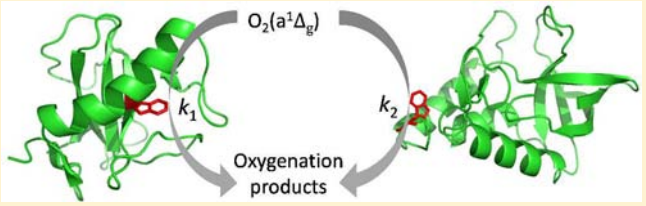
ABSTRACT: Singlet molecular oxygen, $O_2(a^1\Delta_g)$, can influence many processes pertinent to the function of biological systems, including events that result in cell death. Many of these processes involve a reaction between singlet oxygen and a given amino acid in a protein. As a result, the behavior of that protein can change, either because of a structural alteration and/or a direct modification of an active site. Surprisingly, however, little is known about rate constants for reactions between singlet oxygen and amino acids when the latter are in a protein. In this report, we demonstrate using five separate proteins, each containing only a single tryptophan residue, that the rate constant for singlet oxygen reaction with tryptophan depends significantly on the position of this amino acid in the protein. Most importantly, the reaction rate constant depends not only on the accessibility of the tryptophan residue to oxygen, but also on factors that characterize the local molecular environment of the tryptophan in the protein. The fact that the local protein environment can either appreciably inhibit or accelerate the reaction of singlet oxygen with a given amino acid can have significant ramifications for singlet-oxygen-mediated events that perturb cell function.



1. INTRODUCTION

Singlet oxygen, $O_2(a^1\Delta_g)$, is the lowest electronic excited state of molecular oxygen.[1] It has a unique chemistry that results in the oxygenation of organic molecules[2] and this, in turn, has important ramifications in a wide range of disciplines.[3] Of particular interest for the present study is that the production of singlet oxygen in or near a cell can perturb the cell and, at the limit, initiate processes that result in the death of that cell.[4] Although the response of a cell to singlet oxygen can reflect reactions with a variety of molecules (e.g., nucleic acids[5,6] and unsaturated lipids[7]), it is generally considered that the reactions of singlet oxygen with proteins are the most prevalent.[8]

It is well-established that the behavior of a protein can change upon reaction with singlet oxygen, either as a result of a structural alteration and/or a direct chemical modification of an active site.[8−13] We have recently shown that the converse is also true; the behavior of singlet oxygen depends on protein structure (e.g., protein denaturation, crowding of the protein, ligand binding by the protein).[14] Specifically, when a conformational change in a protein either reveals or cloaks selected amino acid residues, the corresponding change in the accessibility of these residues to singlet oxygen is manifested in the overall rate constant for protein-mediated singlet oxygen removal. This latter observation is arguably not surprising given that different amino acids remove singlet oxygen with rate constants that vary over a wide range.[8,15]

Rate constants for the removal of singlet oxygen by native proteins generally fall in the range $\sim 10^8-10^9$ s^{-1} M^{-1}.[15] For a given protein, the magnitude of this bimolecular quenching rate constant reflects the sum of the rate constants for the interaction of singlet oxygen with individual amino acids in the protein.[14] Measurements made on isolated amino acids at physiological pH indicate that, of the 20 natural amino acids, only 5 have appreciable rate constants of interaction with singlet oxygen. Specifically, the sulfur-containing compounds methionine and cysteine, and the unsaturated compounds tyrosine, histidine, and tryptophan have rate constants for singlet oxygen removal that fall in the range $\sim 1-7 \times 10^7$ s^{-1} M^{-1}.[8,15] With the exception of tryptophan, these rate constants principally reflect the chemical reaction channel in which the amino acid is oxygenated. Tryptophan is unique in that it also has a non-negligible physical deactivation channel in which the amino acid promotes the transition $O_2(a^1\Delta_g) \rightarrow O_2(X^3\Sigma_g^-)$ to regenerate the ground state of oxygen.[8]

In light of all the work that has been done thus far on examining interactions between amino acids and singlet oxygen, we were quite surprised to find a specific lack of information on what is arguably the most important point of all; rate constants for the interaction between singlet oxygen and an amino acid when the latter is incorporated in a protein. To our knowledge, there has only been one study on this point and that has been to examine the rate constant for interaction with tryptophan in the protein melittin.[16] However, the tryptophan in melittin is fully exposed to the surrounding solvent and, thus, its behavior is not expected to differ appreciably from that of the isolated amino acid. What is specifically lacking are data on reaction rate constants for amino acids that are "buried" in the protein; this is a significant omission considering that damaging a "buried" amino acid residue generally has a greater effect on protein structure and behavior than does damaging an exposed amino acid residue.[17] The importance of acquiring this rate information is further

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highlighted by the fact that there is already evidence to indicate that the reactions of singlet oxygen with a given amino acid indeed depend on where that amino acid is located in a protein.[10,13,17,18]

For this work, we set out to quantify rate constants for the reaction of singlet oxygen with tryptophan as a function of where this particular amino acid residue is located in a given protein. Five proteins were selected, each containing only a single tryptophan residue. We were particularly interested in decoupling the rate of singlet oxygen diffusion through the protein to the tryptophan residue from local environmental factors at the tryptophan site itself that might influence the oxygenation reaction. We find that the rate constant for tryptophan oxygenation depends not only on the accessibility of the tryptophan residue to singlet oxygen, but also depends significantly on the local protein environment immediately surrounding the tryptophan. These results allow for an important and useful perspective in further elucidating the roles that singlet oxygen can play in influencing cell function.

2. RESULTS AND DISCUSSION

2.1. The Tryptophan-Containing Proteins. Five different proteins, each of which contains a single tryptophan residue, were chosen for this work (Figure 1): (1) bee venom melittin,

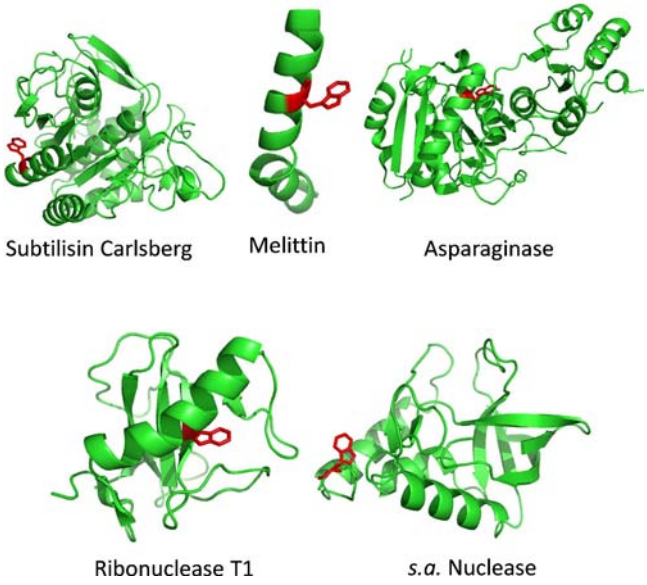

Figure 1. Structures of the five different proteins used in this work. The single tryptophan residue is shown in red. Structures were generated using information provided by the Protein Data Bank (see Experimental Section).

(2) *Bacillus licheniformis* subtilisin carlsberg, (3) *Staphylococcus aureus* nuclease, (4) *Aspergillus oryzae* ribonuclease T1, and (5) *Escherichia coli* asparaginase. On the basis of detailed structural information about these proteins, we choose to establish three "classifications" for the single tryptophan residue: exposed, partially exposed, and buried. In melittin, the tryptophan residue is fully exposed to the solvent. In subtilisin carlsberg and *s. a.* nuclease, the residue is partially exposed to the surrounding solvent-containing environment. In ribonuclease T1 and asparaginase, the residue is buried and access to the site should, at least, be somewhat hindered. We will return to these points in our discussion of the quenching, by oxygen, of tryptophan phosphorescence.

2.2. General Experimental Approach. Our approach to quantify the rate constant for the reaction between singlet oxygen and the tryptophan residue in these proteins is to (1) generate a population of singlet oxygen in the surrounding solvent using a photosensitizer, (2) monitor the extent to which the tryptophan fluorescence is "bleached" in the presence of singlet oxygen, and (3) compare this change in tryptophan fluorescence intensity to the corresponding change in fluorescence intensity of a standard molecule that will kinetically compete with the tryptophan-containing protein for the available singlet oxygen. Through the use of eq 1, we thus obtain the desired rate constant for the reaction of singlet oxygen with the tryptophan residue in a given protein, k_{rxn}.[19]

$$\frac{k_{rxn}}{k_{rxn}^{standard}} = \frac{\log\left(\frac{I_t^{tryp}}{I_0^{tryp}}\right)}{\log\left(\frac{I_t^{stan\ dard}}{I_0^{stan\ dard}}\right)} \quad (1)$$

In eq 1, $k_{rxn}^{standard}$ is the known rate constant for the reaction of singlet oxygen with the standard molecule, I_0 is the fluorescence intensity of the given compound at time = 0 (i.e., before the production of singlet oxygen), and I_t is the fluorescence intensity after some time t of elapsed irradiation of the sensitizer. We address the three points above in turn.

2.2.1. Singlet Oxygen Production. Although singlet oxygen can be produced in a number of ways, it is most conveniently made in a photosensitized reaction wherein the energy of a photoexcited chromophore (i.e., the sensitizer) is transferred to the ground state of oxygen, $O_2(X^3\Sigma_g^-)$.[20] Because all experiments must be performed in aqueous media (we used a phosphate buffer solution), the sensitizer chosen must be hydrophilic. Moreover, we wanted to ensure that the light used to irradiate the sensitizer and generate singlet oxygen would not be absorbed by other chromophores in the system, particularly the reaction standard (vide infra) and amino acids in the protein. To this end, we opted to use Rose Bengal and, in one case, methylene blue as the sensitizers because both absorb light at wavelengths longer than 550 nm.

In all cases, the sensitizer was dissolved such as to yield a solution with an absorbance of ~0.2 at the excitation wavelength (λ > 540 nm) in a 1 cm path length cuvette. Moreover, there was no evidence of sensitizer aggregation in the presence of the protein, nor was there a protein-dependent change in the absorption spectrum of the sensitizer; we only observed a slight protein-dependent change in the spectrum of Rose Bengal when using ribonuclease T1 and, as such, these experiments were performed with methylene blue as the sensitizer.[21] Thus, we infer that the sensitizer is homogeneously distributed in the solvent surrounding the protein, and we likewise generate a homogeneous distribution of singlet oxygen suitable for an experiment based on a kinetic competition. In all cases, there was no irradiation-dependent change in the sensitizer absorbance during the course of our experiment. Although each sensitizer has a very small absorbance at the probe wavelength of 298 nm (vide infra), this does not influence the experiment given the approach used (eq 1).

2.2.2. Bleaching of Tryptophan Fluorescence. The extent of singlet-oxygen-mediated oxygenation of tryptophan was monitored using changes in the intensity of tryptophan fluorescence as a probe. In this experiment, tryptophan was excited at 298 nm with a narrow spectral bandwidth (fwhm ≈ 2 nm). At this wavelength, we specifically avoid excitation of other amino acids in the protein, all of which absorb at shorter wavelengths.[22] Experiments

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were performed at the limit in which the extent of tryptophan oxygenation was small such as to minimize competing absorption by oxygenated products at 298 nm, despite the fact that the latter does not have any adverse effect given our experimental approach (eq 1). Furthermore, if and when products of tryptophan oxygenation do absorb the 298 nm probe light, the resultant fluorescence is sufficiently red-shifted and weak ($\phi_f \approx 10^{-3}$)[23,24] that, this too, will not interfere with the analysis of our data. Finally, irradiation-dependent changes in the spectral profile of tryptophan fluorescence were not observed, indicating that our analysis was not adversely affected by protein denaturation. Thus, upon 298 nm excitation, a decrease in the intensity of tryptophan fluorescence can be directly correlated to a change in the concentration of tryptophan.

2.2.3. The Singlet Oxygen Reaction Standard. Singlet-oxygen-mediated changes in the intensity of tryptophan fluorescence were quantified by comparing the tryptophan data to corresponding fluorescence data obtained from a molecule whose rate constant for reaction with singlet oxygen is known. To this end, we opted to use the water-soluble 9,10-anthracenediyl-bis(methylene)dimalonic acid[25] (ADA, Figure 2)

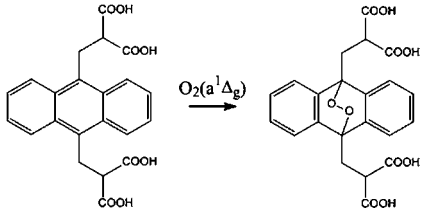

Figure 2. Structure of ADA used as a standard in the kinetic competition to quantify the rate constant for singlet oxygen reaction with the tryptophan-containing proteins. Upon reaction with singlet oxygen, the resultant ADA-endoperoxide does not absorb light at 298 nm and, as such, will not fluoresce.

which is a more hydrophilic variant of related anthracene derivatives used as singlet oxygen traps.[26,27]

Although ADA, like tryptophan, absorbs light at 298 nm, the ADA fluorescence spectrum is sufficiently displaced from that of tryptophan (Figure 3). Moreover, the ADA-endoperoxide formed as a consequence of the reaction with singlet oxygen does not absorb at 298 nm. Thus, relative singlet-oxygen-mediated changes in both tryptophan and ADA concentrations are directly reflected in the respective 298 nm-initiated fluorescence spectra.

In an independent time-resolved $O_2(a^1\Delta_g) \rightarrow O_2(X^3\Sigma_g^-)$ phosphorescence study, where singlet oxygen was produced upon pulsed laser irradiation of Rose Bengal, we ascertained that ADA removes singlet oxygen with a rate constant of $(5.5 \pm 0.5) \times 10^7$ s^{-1} M^{-1}. On the basis of results from related anthracene derivatives,[26,27] we infer that this rate constant accurately reflects the rate constant for the chemical reaction between ADA and singlet oxygen that results in the loss of ADA fluorescence. Indeed, in a control experiment to test the accuracy of our approach, when we use this value of $(5.5 \pm 0.5) \times 10^7$ s^{-1} M^{-1} as our reference point (i.e., $k_{rxn}^{standard}$ in eq 1) to quantify the rate constant for singlet oxygen removal by free, solvated tryptophan, we obtain a rate constant for tryptophan removal of $(3.0 \pm 0.3) \times 10^7$ s^{-1} M^{-1}. The latter is consistent with previously published data.[8,15]

2.3. Rate Constants for Tryptophan Reaction with Singlet Oxygen. Rate constants obtained for the singlet-oxygen-mediated removal of tryptophan in the five selected proteins are listed in Table 1. Examples of the spectral data that give rise to these rate constants are shown in Figure 3. In the least, the data clearly show that, for the two proteins in which the tryptophan residue is buried, the reaction rate constant with singlet oxygen is comparatively small. In this regard, note the correlation between k_{rxn} and the wavelength of the band maximum of tryptophan fluorescence (Table 1). The latter is known to reflect the local environment of the tryptophan residue in the protein matrix.[22,28] We return to a discussion of these data in subsequent sections.

2.4. An Independent Assessment of Oxygen Diffusion through the Protein Matrix. To more completely interpret values of k_{rxn} obtained for the different proteins, we set out to quantify a parameter that would independently reflect the extent to which the protein matrix influences oxygen diffusion to the tryptophan residue. In this regard, we first recognize that, as for any bimolecular process, the reaction between singlet oxygen and tryptophan must occur through a complex formed by the diffusion-dependent encounter of the respective reactants (Scheme 1). In Scheme 1, k_{rem} quantifies the actual bond-making processes that occur within the encounter pair to remove tryptophan from the system.

The overall reaction rate constant, k_{rxn}, can then be expressed in terms of the three rate constants shown in Scheme 1 (eq 2).[29]

$$k_{rxn} = \frac{k_{diff} k_{rem}}{k_{-diff} + k_{rem}} \quad (2)$$

When $k_{rem} \gg k_{-diff}$, $k_{rxn} = k_{diff}$ and we are at the so-called diffusion-controlled limit. When $k_{rem} \ll k_{-diff}$, the magnitude of k_{rxn} is determined by the magnitude of k_{rem} modulated by the diffusion-dependent equilibrium constant (i.e., we are at the so-called pre-equilibrium limit).

The point for the present work is that we would like an independent assessment of how our chosen protein matrices influence k_{diff} for the bimolecular process involving singlet oxygen. To simplify the problem, it is first reasonable to assume that, in a given medium, the diffusion coefficient (and hence k_{diff}) for ground state oxygen will be the same as that for singlet oxygen. With this assumption, and because ground state oxygen is well-known to quench the fluorescence of an organic chromophore at the diffusion-controlled limit,[30] a suitable independent experiment would thus be to monitor the quenching of tryptophan fluorescence by ground state oxygen. Unfortunately, because of the comparatively short lifetime of the tryptophan excited singlet state, these experiments need to be done at high ambient oxygen pressures;[31] and, more importantly, conclusions drawn about oxygen diffusion under these conditions can be incorrect.[32] However, tryptophan phosphorescence from protein matrices is readily monitored at atmospheric pressures.[32] Although oxygen quenching of a triplet phosphorescent state may not occur at the diffusion-controlled limit in a liquid solution (i.e., $k_{diff}(liquid) \approx 3-4 \times 10^{10}$ s^{-1} M^{-1}),[30] the quenching rate constant is often directly proportional to k_{diff} through a factor of ~1/9 that is related to spin statistics.[33] This indeed appears to be the case for tryptophan ($k_q^{phos} \approx 5 \times 10^9$ s^{-1} M^{-1} for free tryptophan,[34] Table 1). Thus, values of the rate constant for the quenching of tryptophan phosphorescence by oxygen, k_q^{phos}, yield the desired information about how our protein matrices influence the value of k_{diff} shown in Scheme 1.

Values of k_q^{phos} are listed in Table 1. To facilitate our discussion of decoupling the protein-dependent rate of encounter between singlet oxygen and the tryptophan residue from the

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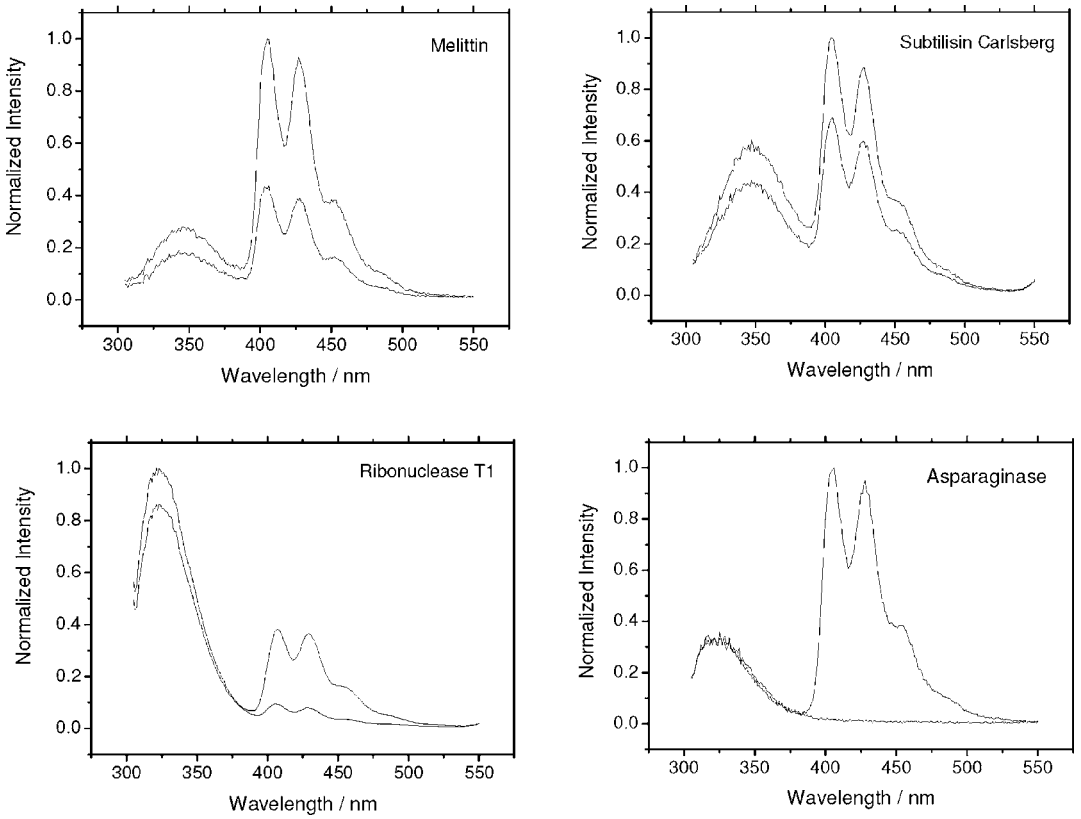


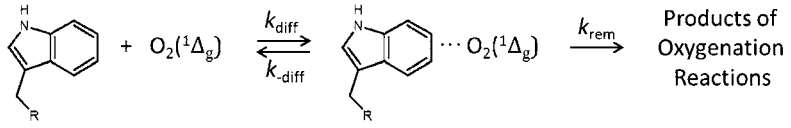
Figure 3. Examples of fluorescence spectra used in association with eq 1 to obtain the rate constant for the reaction of singlet oxygen with the tryptophan residue in a protein. The broad band whose λ_{max}, depending on the protein, falls in the range ~320−350 nm is due to the tryptophan emission. The band with vibronic structure at $\lambda > 400$ nm is due to the emission of ADA also present in the solution. In each panel, data are shown at time = 0 (i.e., before the production of singlet oxygen) and after irradiation of the sensitizer.

Table 1. Rate Constants for Oxygen Quenching of Tryptophan Phosphorescence, k_q^{phos}, and the Reaction of Singlet Oxygen with the Tryptophan Residue, k_{rxn}[a]

compound	tryptophan exposure	λ_{max} tryptophan fluorescence/nm	k_q^{phos} (s^{-1} M^{-1})	k_{rxn} (s^{-1} M^{-1})	k_{rxn}/k_q^{phos} ($\times 10^3$)
free tryptophan	solvated	347	5.0×10^9	3.0×10^7	6.0
melittin	exposed	346	3.0×10^9	2.7×10^7	9.0
sub. Carlsberg	partially exposed	348	$<3 \times 10^9$	3.9×10^7	>13.0
s. a. nuclease	partially exposed	332	9.0×10^8	2.5×10^7	27.8
ribonuclease T1	buried	322	1.4×10^8	6.4×10^6	45.7
asparaginase	buried	318	1.0×10^8	$<1 \times 10^5$	<1.0

[a]Values of k_q^{phos} were obtained from published literature, as cited in the text. Values of k_{rxn}, determined in this study, have an error of ±10%.

Scheme 1. Kinetic Scheme for Tryptophan Removal Mediated by Singlet Oxygen



rate of the actual oxygenation reaction (i.e., k_{rem}), we also present the ratio k_{rxn}/k_q^{phos} in Table 1.

2.5. Protein-Dependent Changes. The data in Table 1 clearly indicate that the rate constant for the reaction of singlet oxygen with the tryptophan residue in these selected proteins depends not only on the accessibility of the tryptophan to oxygen, but also on other factors that must reflect the local environment of the tryptophan in the protein. To expand on this point, we opt to discuss the data from each protein in turn, and include some speculations on the nature of these unique local effects.

2.5.1. Melittin. When compared to data obtained from free solvated tryptophan, the k_q^{phos} data[35] and k_{rxn} data obtained from melittin indicate that the tryptophan residue in this protein is indeed reasonably well exposed. The similarity in λ_{max} of tryptophan fluorescence in these respective samples is likewise consistent with this assessment (Table 1). Although our melittin data differ from those of Vilensky and Feitelson[16] (i.e., $k_{rxn} = (3.9 \pm 0.4) \times 10^7$ s^{-1} M^{-1} and $\lambda_{max}^{fluor} = 350$ nm), the differences are slight and can easily reflect handling-dependent changes in protein structure unique to melittin (e.g., tetramer formation)[16] and/or the purity of the respective protein samples

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(i.e., the purity of commercial bee venom melittin can cover the range ~70 − 90%).

2.5.2. s. a. Nuclease. On the basis of the protein structure, we classified the tryptophan in this case as being "partially exposed". This is certainly reflected in the value of k_q^{phos},[35] which is ~3× smaller than that in melittin. On the basis solely of this protein-dependent change in k_q^{phos}, and an inferred corresponding change in the frequency of collisions between oxygen and the tryptophan residue, one might expect the value of k_{rxn} to change accordingly to yield ~1×10^7 s^{-1} M^{-1} for *s. a.* nuclease. However, our data show otherwise, with $k_{rxn} = 2.5 \times 10^7$ s^{-1} M^{-1}. Specifically, relative to solvated tryptophan and the tryptophan in melittin, the effective reactivity of the tryptophan in *s. a.* nuclease increases appreciably (i.e., k_{rxn}/k_q^{phos} increases).

At present, we have no definitive explanation for this particular protein-dependent increase in tryptophan reactivity. However, as presented throughout the remainder of this section, it is likely that the effects we observe reflect, to a large extent, changes in the local viscosity and/or charge density around the tryptophan residue.

It has independently been shown that, for reactions of singlet oxygen that occur near the pre-equilibrium limit, an increase in the local viscosity around the reaction center can cause an increase in k_{rxn}.[36,37] Although the magnitude of k_{rxn} obtained for *s. a.* nuclease indicates that we are somewhat removed from the pre-equilibrium limit (i.e., $k_{rxn} > 10^5$ s^{-1} M^{-1}), it would nevertheless be prudent to test whether an increased local viscosity in *s. a.* nuclease facilitates more collisions between singlet oxygen and the tryptophan residue in the encounter pair (Scheme 1) and whether this, in turn, contributes to a larger value of k_{rxn}.

The principal products of the reaction between singlet oxygen and tryptophan invariably involve oxygenation of carbons 2 and 3 in the 5-membered ring of the indole system.[38−41] Moreover, it has been established that the interactions between singlet oxygen and tryptophan that lead to these products are characterized by an appreciable amount of charge-transfer character; an electron-rich tryptophan moiety reacts faster and the reaction can be appreciably slowed in a nonpolar solvent.[42−44] With these points in mind, it seems reasonable to expect that a protein-dependent perturbation that shifts more charge out onto carbons 2 and 3 in the indole system will increase the reactivity of the tryptophan residue with electrophilic singlet oxygen. Thus, it would also be prudent to test whether such a phenomenon likewise contributes to the larger value of k_{rxn} in *s. a.* nuclease.

2.5.3. Subtilisin Carlsberg. Solely on the basis of the protein structures shown in Figure 1, we expect that k_q^{phos} for Subtilisin Carlsberg should be similar to that for *s. a.* nuclease and certainly less than that for melittin (to our knowledge, the pertinent data are not available). Even though the tryptophan in Subtilisin Carlsberg is only "partially exposed", we nevertheless obtain a k_{rxn} value (3.9×10^7 s^{-1} M^{-1}) which is greater than that for melittin and free solvated tryptophan. It is difficult to ascribe a difference of this magnitude to local viscosity arguments, particularly since the reaction does not occur at the pre-equilibrium limit.[36,37] Rather, for Subtilisin Carlsberg, it is more likely that local protein-dependent electronic effects account for an increase in k_{rem} and k_{rxn}.

In this regard, we note that the electron-rich nitrogen and oxygen atoms of the amide group in the asparagine117 residue in Subtilisin Carlsberg are appropriately placed near the tryptophan residue to accomplish a protein-induced shift of electron density out onto carbons 2 and 3 in the indole system (Figure 4).

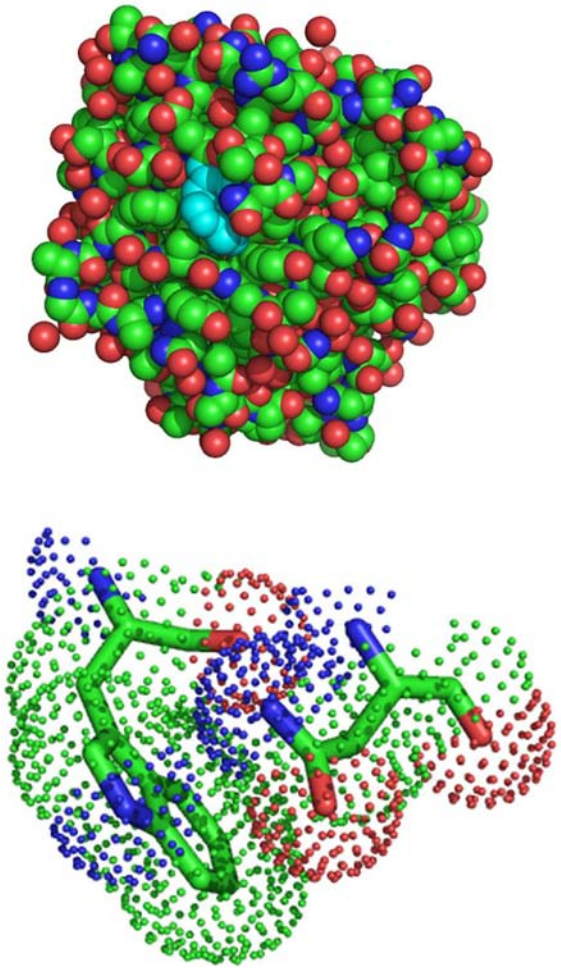

Figure 4. (Top) Model of Subtilisin Carlsberg that shows the partially exposed tryptophan residue in cyan. (Bottom) Expanded view of the tryptophan residue that shows the adjacent asparagine117 residue appropriately placed to perturb the indole moiety and shift electron density out onto the olefinic center that reacts with singlet oxygen. In this illustration, carbon atoms are green, nitrogen atoms are blue, and oxygen atoms are red.

Thus, on this basis, it is indeed reasonable to observe a comparatively large value of k_{rxn} for Subtilisin Carlsberg. A similar effect could likewise be responsible for the arguably unexpected singlet-oxygen-mediated oxygenation of the phenylalanine residue in cytochrome *c* (i.e., under most conditions, singlet oxygen does not react with a singly alkylated phenyl ring).[18]

2.5.4. Ribonuclease T1. The tryptophan residue in ribonuclease T1 is buried in the protein, and this is reflected in the comparatively small k_q^{phos} value of 1.4×10^8 s^{-1} M^{-1}.[45] Despite the poor accessibility to oxygen, the rate constant for oxygenation of this tryptophan residue is nevertheless comparatively large (i.e., of all compounds we have examined, k_{rxn}/k_q^{phos} is the largest, Table 1).

The magnitude of k_{rxn} in this case, 6.4×10^6 s^{-1} M^{-1}, indicates that the reaction approaches the pre-equilibrium limit. As such, the large value of k_{rxn}/k_q^{phos} could reflect the effects of a high local viscosity around the tryptophan residue in the protein which, as outlined above, could facilitate the reaction by increasing k_{rem}.[36] It has been independently shown that the tryptophan in ribonuclease T1 is indeed in a reasonably viscous microenvironment.[46]

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2.5.5. Asparaginase. The tryptophan residue in asparaginase is likewise deeply buried in the protein, and this is also seen in the comparatively small k_q^{phos} value of 1.0×10^8 s^{-1} M^{-1}.[35] Moreover, the local environment of the tryptophan residue in asparaginase is also viscous.[46] As such, one might expect to record values of k_{rxn} and k_{rxn}/k_q^{phos} similar to those recorded for the tryptophan in ribonuclease T1. However, under experimental conditions in which an appreciable singlet-oxygen-mediated change could be observed in the fluorescence of tryptophan in ribonuclease T1, we were not able to observe a corresponding change for the tryptophan in asparaginase (Figure 3). At best, our data allow us to establish an upper limit of 1.0×10^5 s^{-1} M^{-1} for k_{rxn} in asparaginase. To account for these data, we again consider the local environment of the tryptophan residue in asparaginase, much in the same way as we considered the local environment of the tryptophan in Subtilisin Carlsberg (vide supra).

Because reactions between singlet oxygen and tryptophan proceed through an intermediate with appreciable charge-transfer character, a "non-polar" environment can adversely influence the reaction rate.[44] It is thus tempting to infer that the local environment of the tryptophan in asparaginase resembles that, for example, of a hydrocarbon.[44] This interpretation is consistent with the significantly blue-shifted λ_{max} of 318 nm for tryptophan fluorescence in asparaginase (Table 1).[28] In contrast, and based solely on the observed values of k_{rxn}, we infer that the local environment of the tryptophan in ribonuclease T1 must be more "polar" and, as such, would better support the charge-transfer character in the encounter complex between tryptophan and singlet oxygen. It has indeed been established that the local environment of the tryptophan in ribonuclease T1 is surprisingly polar, despite a λ_{max} of tryptophan fluorescence (322 nm) that would imply a "non-polar" environment.[28,47]

This perspective based on the effect of the "polarity" of the local protein environment is consistent with model studies on the effect of solvent on reactions of singlet oxygen with selected amino acids.[44,48,49] One result from these solvent studies could be particularly relevant for a protein in vivo: the protein environment could increase the relative extent to which a given amino acid would physically deactivate rather than react with singlet oxygen and, as such, potentially impart a protective effect in the cell.

3. CONCLUSIONS

The rate constant for the reaction of singlet oxygen with tryptophan depends significantly on the location of this amino acid in a given protein. For singlet oxygen produced in the solvent surrounding the protein, we confirm the expected observation that the reaction rate depends, at least in part, on the extent to which the tryptophan residue is accessible to oxygen. However, and arguably most importantly, we have demonstrated that the reaction rate also depends significantly on the local environment of the tryptophan residue in the protein. The latter can either inhibit or accelerate the singlet oxygen reaction appreciably. We thus conclude that it is overly simplistic, and can possibly be incorrect, to assess the susceptibility of a given amino acid to oxygenation by singlet oxygen solely on the basis of the accessibility of that amino acid to singlet oxygen.[10]

Through the present work on tryptophan, we infer that the local protein environment can appreciably enhance reactions with singlet oxygen by (1) perturbing the electronic distribution in the amino acid, thereby making it more nucleophilic, and (2) increasing the local viscosity, thereby facilitating multiple collisions within the singlet oxygen-amino acid encounter pair. We have also inferred that the local protein environment can inhibit a reaction with singlet oxygen by creating a "non-polar" domain that destabilizes the reaction coordinate characterized by charge-transfer character.

In conclusion, the location of a given amino acid in a protein can have an appreciable effect on the kinetics with which that amino acid reacts with singlet oxygen. This can have important ramifications in singlet-oxygen-mediated processes that influence cell function and, ultimately, cell death.

4. EXPERIMENTAL SECTION

4.1. Materials. *Aspergillus oryzae* Ribonuclease T1 (1 kU/μL, Epicenter), *Staphylococcus aureus* Nuclease (>40%, Sigma), *Escherichia coli* Asparaginase (>60%, Sigma), *Bascillus licheniformis* Subtilisin Carlsberg (Calbiochem), Bee Venom Melittin (>85%, Sigma), Rose Bengal (Sigma-Aldrich), Methylene Blue (Sigma-Aldrich), 9,10-anthracenediyl-bis(methylene)dimalonic acid, ADA (Fluka), and D_2O (>99.9% D, Euriso-Top) were used as received. Although the stated purity of some of the proteins is low (e.g., *s. a.* nuclease is >40%), this is generally due to the presence of additives, not other proteins (e.g., sodium citrate in the case of *s. a.* nuclease). The D_2O-based phosphate buffer was prepared by dissolving 1 PBS tablet (Sigma) in 200 mL D_2O to yield 10 mM phosphate buffer with 2.7 mM KCl and 137 mM NaCl at pD 7.4.

4.2. Methods. The rate constant for ADA-mediated removal of singlet oxygen was obtained in a time-resolved singlet oxygen phosphorescence experiment using an approach and instrumentation that have been previously described.[50,51] The ADA vs protein kinetic competition studies were performed in PBS buffer solutions using steady-state irradiation of the sensitizer (xenon lamp) at wavelengths longer than 540 nm. The sample absorbance was always sufficiently small (i.e., ∼0.2) that homogeneous irradiation was achieved over the entire 1 cm path length of the cuvette used. Tryptophan and ADA fluorescence spectra were recorded using a Fluoromax P spectrometer (Horiba Jobin Yvon) with an excitation wavelength of 298 nm. The concentrations of the protein and ADA used were such that, with a 1 cm path length cuvette, the sample absorbance was ∼0.1 at 298 nm. Samples were housed in open cuvettes and stirred with a magnetic stir-bar.

Structures in Figures 1 and 4 were generated using the PyMOL program[52] using information provided by the Protein Data Bank (PDB): (1) Ribonuclease T1 (PDB ID: 1ygw),[53] (2) *s. a.* Nuclease (PDB ID:1nuc),[54] (3) Asparaginase (PDB ID: 2him),[55] (4) Subtilisin Carlsberg (PDB ID: 1c3l),[56] and (5) Melittin (PDB ID: 2mlt).[57]

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Notes

The authors declare no competing financial interest.

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