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Singlet Oxygen's Response to Protein Dynamics

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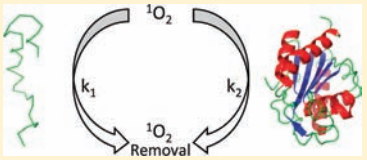
ABSTRACT: Singlet molecular oxygen, $O_2(a^1\Delta_g)$, is an intermediate in a variety of processes pertinent to the function of biological systems, including events that result in cell death. Many of these processes involve a reaction between singlet oxygen and a given protein. It is acknowledged that the behavior of a protein can change upon reaction with singlet oxygen, as a result of a structural alteration and/or a direct chemical modification of an active site. However, the converse, where one considers how the behavior of singlet oxygen can be altered by changes in protein structure, has received little attention. In this report, we use a variety of proteins to demonstrate how the rate constant for singlet oxygen removal by a protein responds to (a) protein denaturation, (b) macromolecular crowding of the protein, (c) ligand binding by the protein, and (d) polymerization of the protein. From one perspective, the data show that the kinetics of singlet oxygen removal can be used to monitor protein dynamics. Most importantly, however, the data indicate that protein structural changes that either reveal or cloak a given amino acid residue can have a measurable effect on the overall rate constant for singlet oxygen removal which, in turn, can have ramifications for singlet-oxygen-mediated intracellular events that perturb cell function.



INTRODUCTION

Singlet oxygen, $O_2(a^1\Delta_g)$, is the lowest electronic excited state of molecular oxygen.[1] Although singlet oxygen can be produced in a number of ways, it is most commonly made in a photosensitized reaction wherein the energy of a photoexcited chromophore (i.e., the sensitizer) is transferred to the triplet ground state of oxygen, $O_2(X^3\Sigma_g^-)$.[2] Although efficient photosensitizers are commonplace in our sunlight-filled world, this process is also of significance for the controlled production of singlet oxygen using lasers and lamps. Most importantly, singlet oxygen has a unique chemistry that results in the oxygenation of organic molecules,[3] and this, in turn, has important ramifications in disciplines that range from polymer science to cell biology.[4] Indeed, the controlled production of singlet oxygen in or near a cell can, among other things, initiate processes that result in the death of that cell.[5] This has been exploited in photodynamic therapy (PDT),[6] a medical procedure whereby undesired cells (e.g., cancer cells) can be destroyed.

The chemical reactions of singlet oxygen kinetically compete with processes wherein singlet oxygen is simply deactivated to generate $O_2(X^3\Sigma_g^-)$.[2] For solution-phase systems, this deactivation could be the result of collision-dependent interactions with (a) solvent molecules and/or (b) solutes, including the molecule that can also react with singlet oxygen. The overall rate constant for singlet oxygen removal, k_Δ, can thus be expressed as a sum of bimolecular terms as shown in eq 1,

$$\tau_\Delta^{-1} = k_\Delta = k_{nr}[S] + k_r[S] + k_{q1}[Q1] + \ldots + k_{rxn1}[R1] + \ldots \quad (1)$$

where S represents the solvent, R1 a given chemical reactant, and Q1 a so-called "quencher" that promotes the deactivation of singlet oxygen to $O_2(X^3\Sigma_g^-)$. The solvent-dependent deactivation terms are further subdivided into a nonradiative process, k_{nr}, and a radiative process, k_r. The latter corresponds to the $O_2(a^1\Delta_g) \rightarrow O_2(X^3\Sigma_g^-)$ phosphorescence at ~1275 nm, which provides a convenient tool by which singlet oxygen can be monitored in systems that range from liquid solutions to biological cells.[4] The reciprocal of the first-order rate constant k_Δ defines the lifetime of singlet oxygen, τ_Δ, in the given system.

In any system where singlet oxygen is produced in a localized spatial domain, the combination of τ_Δ and the diffusion coefficient of oxygen defines a spatially resolved sphere in which the initial activity of singlet oxygen will be confined.[4] In a cell, molecules that interact with singlet oxygen, and thus influence τ_Δ, include nucleic acids,[7,8] unsaturated lipids,[9] and proteins.[10−12] These interactions include both reaction and quenching components.[13] For the present study, we focus only on selected aspects of the interaction between singlet oxygen and proteins. On the basis of their prevalence and the magnitudes of the rate constants for singlet oxygen removal, proteins are considered to be the most significant class of intracellular biomolecules that interact with singlet oxygen.[10,14]

It is well-established that the behavior and activity of a protein can change upon reaction with singlet oxygen, as a result of a structural alteration and/or a direct chemical modification of an active site.[10−12,15−18] Although the converse has been inferred (i.e., that protein structure may influence τ_Δ),[19] to our knowledge, there have been only two studies that explicitly address how changes in protein structure influence the rate constant for protein-dependent singlet oxygen removal. Specifically, it was shown by Kanofsky in 1990 that the rate constant for singlet oxygen removal by native human serum albumin (HSA) was a

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factor of 2 greater than the rate constant for singlet oxygen removal by HSA that had been denatured by the anionic detergent sodium dodecyl sulfate (SDS).[20] Independently, Michaeli and Feitelson[21,22] showed that, upon urea-mediated chemical denaturation, the five proteins studied become *more* effective at removing singlet oxygen, not *less* as seen in Kanofsky's experiment. Although the proteins studied by these respective groups were admittedly different, the data nevertheless present an apparent contradiction. In both studies, the observed effects were attributed to denaturation-dependent structural alterations that change the extent to which selected amino acids become accessible to interaction with singlet oxygen.

There is a need to expand upon the *in vitro* studies of Kanofsky and Michaeli/Feitelson, particularly in light of current work that examines the behavior of singlet oxygen *in vivo*.[4] It has been established that the site of singlet oxygen production in a cell influences both τ_Δ[23] and the susceptibility of that cell to processes that result in loss of function and, ultimately, death.[4,5] It has also been established that, upon the photosensitized production of intracellular singlet oxygen, τ_Δ in selected subcellular domains increases with an increase in the elapsed irradiation time[24,25] and that these changes in τ_Δ are coincident with an increase in the viscosity of localized intracellular domains.[24] Although it is a nontrivial exercise to ascertain the origin of this irradiation-dependent change in τ_Δ, it is reasonable to associate such a viscosity change with singlet-oxygen-dependent changes in the structure of intracellular proteins.[4,26]

It is incumbent upon us now to quantify and better understand how dynamic changes in the structure and conformation of a given protein influence the kinetics of singlet oxygen removal. It is also important to explicitly consider whether any change in the kinetics of singlet oxygen removal directly reflect a change in protein conformation or whether one is observing singlet oxygen's response to a correlated effect (e.g., protein-dependent change in solution viscosity and/or the presence of other compounds added to initiate protein change). Presented herein are the results of *in vitro* studies in which the kinetics of singlet oxygen removal were examined as a function of different ways to influence protein structure and conformation. The latter include (a) temperature- and chemical-dependent protein denaturation, (b) macromolecular crowding, (c) ligand binding by a protein, and (d) protein polymerization. In the least, the data obtained allow us to resolve the apparent contradiction between the Kanofsky and Michaeli/Feitelson experiments. On a more global scale, our data provide the perspective needed to examine how protein dynamics can influence the behavior of singlet oxygen. In turn, this should give us better insight into mechanisms of cell death mediated by singlet oxygen.

RESULTS AND DISCUSSION

1. Effects of Protein Denaturation. Protein denaturation can be triggered by a number of externally controlled stimuli. In their singlet oxygen studies, Michaeli and Feitelson[21,22] added a chemical agent in high concentration to initiate denaturation; in the presence of 6 M urea, all proteins in their systems were presumably denatured. Because urea at 6 M is itself an effective quencher of singlet oxygen for experiments performed in D_2O, it is also necessary to account for this extra deactivation channel in the analysis of the Michaeli and Feitelson protein data. [We have independently ascertained that urea removes singlet oxygen with a rate constant of $9.0 \times 10^3\ s^{-1}\ M^{-1}$ in D_2O. Thus, at 6 M urea, the first-order removal term is $5.4 \times 10^4\ s^{-1}$, which is significantly larger than the D_2O-dependent term of $\sim 1.5 \times 10^4\ s^{-1}$.] In Kanofsky's study, HSA was denatured upon the addition of 70 mM SDS.[20]

Another convenient approach to initiate denaturation is to increase the temperature of a solution containing the protein. With this approach, protein structural stability has been characterized using, for example, circular dichroism,[27] tryptophan fluorescence,[28] or differential scanning calorimetry[29] as experimental probes. An obvious advantage here is that one avoids potential complications associated with the addition of a chemical reagent.

We first set out to ascertain if temperature-dependent protein denaturation could be accurately monitored using changes in τ_Δ for a photosensitized reaction performed in a solution of that protein. With the Kanofsky[20] and Michaeli/Feitelson[21,22] experiments in mind, one would expect τ_Δ to remain roughly unchanged with an increase in temperature until a given temperature is reached at which a sufficient fraction of the proteins in solution undergo a structural change. This denaturation-dependent structural change could either (a) cloak amino acid residues and thereby render them inaccessible to singlet oxygen or (b) reveal amino acid residues that were previously inaccessible to singlet oxygen. In any event, starting at this critical temperature, one would expect to observe a change in τ_Δ with an increase in temperature up to the point that all of the protein in solution has been denatured.

We opted to use the whey protein bovine α-lactalbumin for our experiments because it conveniently denatures at ~62 °C in pH 6 aqueous solutions[30] (i.e., 62 °C is the so-called melting temperature, T_M, of this protein). We used D_2O instead of H_2O as the solvent to take advantage of the fact that singlet oxygen has an inherently longer lifetime in D_2O than in H_2O[31] and, as such, it is easier to more accurately quantify the effect of the protein on the rate constant for singlet oxygen removal.[4] The singlet oxygen sensitizer used for these experiments was phenalen-1-one-2-sulfonic acid (PNS). In an independent series of control experiments, we established that the absorption spectrum of PNS and the quantum yield for PNS-sensitized singlet oxygen production were independent of the presence of the protein. Thus, although other singlet oxygen sensitizers have been observed to bind to a given protein,[32] we infer that, in our experiments, PNS is not associated with bovine α-lactalbumin. As in all of the studies reported herein, values of τ_Δ were obtained in time-resolved $O_2(a^1\Delta_g) \rightarrow O_2(X^3\Sigma_g^-)$ phosphorescence experiments.

In contrast to what was expected, τ_Δ steadily decreased from a value of ~60 μs to a value of ~25 μs as the temperature of the bovine α-lactalbumin solution was increased over the range 10−80 °C (Figure 1A). The absence of a change in τ_Δ was first observed only at temperatures above ~80 °C.

In an attempt to explain these data, a control experiment was performed in which τ_Δ was measured from a protein-free solution of D_2O over the same temperature range of ~10−90 °C (Figure 1B). Over the years, it has generally been considered that the solvent-dependent lifetime of singlet oxygen does not change much with a change in temperature (i.e., the term $(k_r + k_{nr})[S]$ in eq 1 has been considered to be effectively temperature independent).[2] However, as seen in Figure 1B, we found that τ_Δ in D_2O decreased from ~72 μs at 15 °C to ~50 μs at 90 °C.

The data shown in Figure 1B prompted an independent study of the effect of temperature on the solvent-dependent

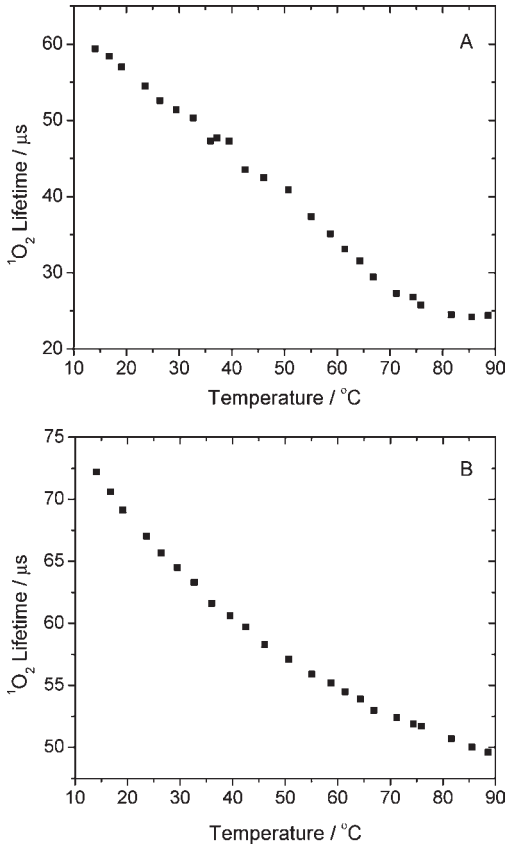

Figure 1. (A) Plot of the singlet oxygen lifetime, τ_Δ, observed in a photosensitized reaction from a D_2O solution of bovine α-lactalbumin (pD 6.4) as a function of temperature. (B) Plot of the singlet oxygen lifetime, τ_Δ, observed in a photosensitized reaction from a protein-free D_2O solution as a function of temperature.

deactivation of singlet oxygen.[33] For the present discussion, we simply note that τ_Δ in neat D_2O varies substantially with temperature, and it is therefore necessary to account for this variation when interpreting the data recorded in the protein denaturation experiment. To this end, the temperature-dependent τ_Δ data recorded from protein-free D_2O were converted to rate constants (i.e., $k_{D_2O} = (k_{nr} + k_r)[D_2O]$ from eq 1), and for data at each given temperature, this pseudo-first-order rate constant, k_{D_2O}, was subtracted from the corresponding rate constant obtained from the bovine α-lactalbumin experiment, k_Δ. As shown in eq 2, this difference was then divided by the concentration of the protein to yield an apparent second-order rate constant for protein-dependent singlet oxygen removal, $k_{protein}$. [The molecular weight of the protein is 14178 g/mol and, at the concentrations of the protein used, α-lactalbumin is completely dissolved. Thus, it is not necessary to consider a temperature-dependent change in protein solubility.] With reference to eq 1, note we do not discriminate between the reaction and quenching channels of singlet oxygen removal in this experiment (i.e., $k_{protein} = k_q + k_{rxn}$).

$$k_{protein}(T) = \frac{k_\Delta(T) - k_{D_2O}(T)}{[protein]} \qquad (2)$$

A plot of $k_{protein}$ against temperature now better corresponds to what was initially expected for the temperature-dependent denaturation of bovine α-lactalbumin as monitored in a singlet

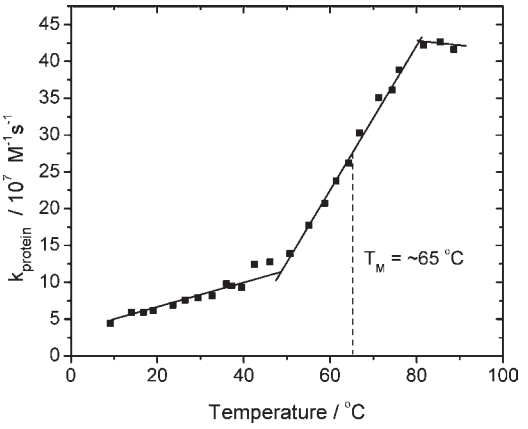

Figure 2. Bimolecular rate constant for the removal of singlet oxygen by bovine α-lactalbumin plotted as a function of temperature (D_2O solution at pD 6.4). The solid lines are linear fits to selected portions of the data. In an attempt to be consistent with the standard assignment of the melting temperature, T_M, we have assumed that the midpoint of the section in which data changes most rapidly will be accurate.

oxygen experiment (Figure 2). We first note that the magnitude of $k_{protein}$ increases slightly over the temperature range of 10−50 °C. It is reasonable to ascribe this to a combination of (a) the inherent temperature-dependent change seen in rate constants for singlet oxygen removal by both reaction (k_{rxn}) and quenching (k_q) channels[34,35] and (b) the temperature-dependent increase in confined, local motions of the macromolecule that change the extent to which singlet oxygen interacts with selected amino acids in the protein. However, over the temperature range 50−80 °C, the temperature-dependent increase in $k_{protein}$ is more pronounced, changing by a factor of ~5. We ascribe this observation to the fact that we are now observing the effects of protein denaturation. The midpoint of the unfolding transition, which is the temperature at which half of the protein population is denatured, is denoted the melting temperature, T_M. Our present data yield $T_M \approx 65$ °C.

In comparing our value of T_M to values obtained using other techniques, it is important to first consider that our experiments were performed in D_2O, not H_2O. With this change of solvent, exchangeable protons in the protein can be replaced by deuterons which can influence the hydrogen bonds that impart structure and stability to the protein, among other things.[36] For bovine serum albumin and hen egg white lysozyme, for example, the exchange of H_2O by D_2O results in a systematic increase of ~2−3 °C in T_M over a broad range of pH.[36] On the basis of differential scanning calorimetry (DSC) experiments, a value of ~62 °C has been reported for the T_M of bovine α-lactalbumin in H_2O at pH 6 (note: pD = pH + 0.4).[30] Considering the effects of H_2O/D_2O solvent exchange, this value correlates well with the value of $T_M \approx 65$ °C obtained in our D_2O-based experiment (Figure 2).

Using temperature-dependent data, we have ascertained that there can indeed be a pronounced difference in the efficiency with which folded and unfolded proteins remove singlet oxygen from a given system. Moreover, our data on bovine α-lactalbumin are consistent with the data reported by Michaeli and Feitelson[21,22] for five different proteins (melittin, neuropeptide Y, insulin, bovine pancreatic trysin inhibitor, and ribonuclease A): in all cases, the denatured, unfolded form of the protein is more effective at removing singlet oxygen than is the folded, native form of the protein (i.e., $k_{protein}$(unfolded) > $k_{protein}$(native)).

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We now consider the data of Kanofsky where the report of $k_{protein}$(unfolded) < $k_{protein}$(native) for HSA provides an apparent contradiction.[20] We first repeated Kanofsky's experiments, as published using 70 mM SDS as the chemical denaturing agent, and fully confirmed his "contradictory" results. We also ascertained that, when using a water-soluble sensitizer, τ_Δ in a D_2O solution of 70 mM SDS (61 μs) is not appreciably different from τ_Δ in a solution of neat D_2O (67 μs). We then performed an SDS-free temperature-dependent study on HSA and obtained $k_{protein}(23\ ^\circ C) = (7.2 \pm 0.4) \times 10^8\ s^{-1}\ M^{-1}$ and $k_{protein}(85\ ^\circ C) = (2.8 \pm 0.2) \times 10^9\ s^{-1}\ M^{-1}$. The T_M reported for HSA at pH 7.4 is 63 °C;[37] thus, the proteins in our sample should all be unfolded at 85 °C. It is clear that these latter temperature-dependent HSA data are consistent with our bovine α-lactalbumin results and the independent results of Michaeli and Feitelson in which the unfolded protein is more effective than the native protein at removing singlet oxygen. On this basis, it is reasonable to suggest that SDS itself cloaks amino acids in the unfolded form of the protein and, as such, reduces the reactivity of the protein with singlet oxygen.

The *in vitro* data presented here are important when evaluating the effects of singlet oxygen on mechanisms of cell death.[4] Functional proteins are in their folded conformation. Nevertheless, cells contain unfolded proteins which can be randomly distributed in the cytosol or can be localized in specific organelles (e.g., in the lysosomes and in the endoplasmic reticulum).[38] Moreover, the ratio of folded to unfolded proteins in a given subcellular domain may change as a cell responds to a given stimulus or perturbation.[38]

2. Effect of Macromolecular Crowding. It is well-established that the structure and activity of a given protein in dilute solution can differ markedly from those in the crowded conditions of a cell.[39−42] At the limit, an unfolded protein in dilute solution can be forced to fold into a specific structure as a consequence of macromolecular crowding.[41,43−45] As in our temperature-dependent denaturing study, this should change the accessibility of selected amino acids in the protein and, in turn, affect the rate constant for protein-mediated singlet oxygen removal.

To test the effects of macromolecular-crowding-induced protein conformational changes on the kinetics of singlet oxygen removal, we first used the mitochondrial protein cytochrome c. In acidic solutions, this protein is denatured. However, upon the addition of a crowding agent, the protein collapses to a structure that resembles the molten-globule state.[44,45]

For our experiments, we opted to use 5,10,15,20-tetrakis(*N*-methyl-4-pyridyl)-21*H*,23*H*-porphine (TMPyP) as the sensitizer because, unlike many other sensitizers, it produces a sufficient amount of singlet oxygen under the acidic conditions required for this study (pD 1.6). Admittedly, the absorption spectrum of the porphyrin inherent in cytochrome c has some overlap with that of TMPyP. Nevertheless, upon irradiation at 420 nm which coincides with the maximum of the TMPyP Soret band, we were not able to observe a singlet oxygen phosphorescence signal upon irradiation of cytochrome c alone; the presence of TMPyP was required to see a signal and quantify the effect of the protein on the rate of singlet oxygen removal.

The crowding agent we opted to use was Ficoll 70, which is a polymer of sucrose. In an independent control experiment, we established that this Ficoll removes singlet oxygen with a rate constant of $4 \times 10^6\ s^{-1}\ M^{-1}$. Thus, at the Ficoll concentrations used (4.3 mM, 300 mg mL^{-1}), the pseudo first-order rate constant for Ficoll-mediated singlet oxygen removal ($1.7 \times 10^4\ s^{-1}$) is similar to that for D_2O-mediated singlet oxygen removal ($1.5 \times 10^4\ s^{-1}$), and we can readily subtract the Ficoll-dependent term to isolate the rate constant for protein-mediated singlet oxygen removal.

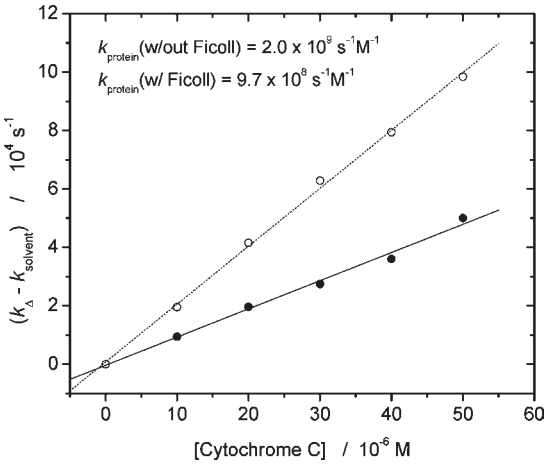

Figure 3. Plot of the pseudo-first-order rate constant for singlet oxygen removal, k_Δ, from which the solvent/Ficoll deactivation term has been subtracted, against the concentration of cytochrome *c* in D_2O at pD 1.6 with (●) and without (○) Ficoll 70. The slopes of the respective lines yield $k_{protein}$.

As can be seen from the data in Figure 3, the rate constant for protein-mediated singlet oxygen removal in the absence of the crowding agent ($2.0 \times 10^9\ s^{-1}\ M^{-1}$) is a factor of ~2 greater than that for singlet oxygen removal in the presence of 4.3 mM of the crowding agent ($9.7 \times 10^8\ s^{-1}\ M^{-1}$). The phenomenon we observe in this experiment is thus consistent with that observed in our temperature study (Figure 2) and again points to a model in which a given protein can better deactivate/remove singlet oxygen when it is unfolded, presumably due to the fact that, when unfolded, pertinent amino acids are more accessible to singlet oxygen.

3. Effect of Ligand Binding by the Protein. For many proteins the structural change between an "open" and "closed" form can be more subtle than that seen for cytochrome *c* in the previous section. Upon the addition of a crowding agent to such a protein, the corresponding change in the exposed surface area of the amino acids may be small enough to preclude a perceptible change in the protein-dependent rate constant for singlet oxygen removal. In these cases, differences in $k_{protein}$ may be overshadowed by the change due solely to the direct Ficoll-mediated deactivation. As such, it would be advantageous to monitor a subtle crowding-related conformational change that could be achieved without the addition of an agent such as Ficoll. To this end, we investigated the effect of a protein structural change induced by the binding of a ligand. Because ligand binding by a protein is an event that routinely occurs *in vivo*, this also becomes a relevant and informative exercise.

Adenylate kinase (also known as myokinase) catalyzes phosphoryl transfer reactions.[46] Upon binding the ligand denoted Ap_5A (i.e., a molecule with two ADP moieties connected by a fifth phosphate group), a subtle conformational change in the protein occurs.[41,46] For example, it has been estimated that, upon Ap_5A binding, there is only an 11% decrease in the solvent accessible surface area of the protein.[41] Thus, we do not expect to see a large Ap_5A-dependent change in $k_{protein}$.

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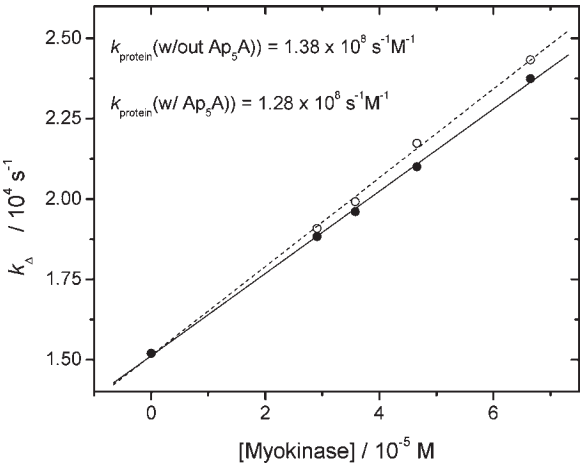

Figure 4. Plot of the overall rate constant for singlet oxygen removal, k_Δ, against the concentration of myokinase added to a D_2O solution. Experiments were performed without (○) and with (●) the ligand Ap_5A. The slope in this plot yields $k_{protein}$.

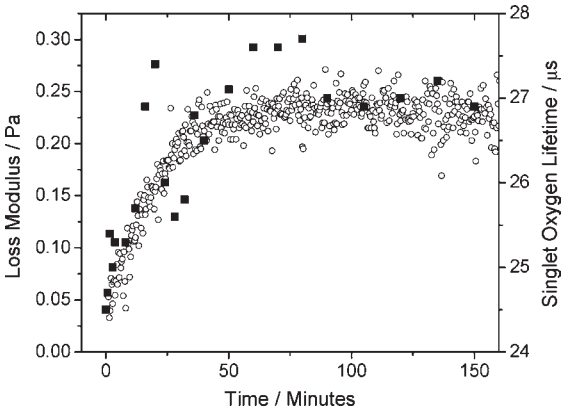

Figure 5. Data recorded from a D_2O solution of 25 μM actin as a function of the elapsed time after initiating actin polymerization. Singlet oxygen was produced upon irradiation of the sensitizer PNS, and its lifetime was recorded in a time-resolved experiment (■). Independently, a rheometer was used to quantify polymerization-dependent changes in the loss modulus of the solution (○). The storage modulus of this solution did not change appreciably over this same period of time (data not shown).

Rate constants for myokinase-mediated singlet oxygen removal were measured in the presence and absence of Ap_5A. In an independent experiment, we ascertained that, at the concentrations used in our study, Ap_5A does not influence τ_Δ in D_2O. As shown in Figure 4, there is a subtle yet perceptible difference in the rate constant for protein-mediated singlet oxygen removal upon the addition of Ap_5A to the system. The magnitude of this change in $k_{protein}$, which has been systematically reproduced in independent experiments, is consistent with our expectation based on the binding-dependent change in the solvent accessible surface area of the protein (*vide supra*). We observe that $k_{protein}$ decreases upon binding the ligand (i.e., upon formation of the "closed" structure and a decrease in the exposed surface area of the protein). This result is likewise consistent with those obtained in our temperature study and Ficoll-mediated crowding study.

4. Actin Polymerization. Like structural changes associated with denaturation and macromolecular crowding, specific interactions of a given protein with another biomolecule (e.g., DNA, membranes, another protein) can also influence structure which, in turn, could influence the rate constant for protein-mediated singlet oxygen removal. To this end, we examined singlet oxygen's response to the effect of a particularly important interaction: the protein−protein interaction, as exemplified by actin polymerization.

Actin, a cytoskeletal protein, is abundant in mammalian cells.[38] This is pertinent for the present τ_Δ study because we have independently shown that, upon the production of intracellular singlet oxygen, one response of the cell is the "collapse" of cytoskeletal proteins to yield local domains of what appear to be cross-linked protein aggregates.[4,26]

A buffered solution of 25 μM monomeric bovine cardiac muscle actin was prepared and divided into two portions. One portion was used to monitor the effect of actin polymerization on singlet oxygen kinetics, whereas the second portion was used to concurrently monitor the polymerization-dependent changes in the sample viscosity (obtained using a rheometer to quantify the loss modulus of the solution which, in turn, is proportional to the viscosity[47,48]).

The data obtained indicated that, after chemically initiating actin polymerization to yield double-stranded filaments (by adding 0.1 mM $CaCl_2$ and 0.5 mM $MgCl_2$),[49] both the sample viscosity and τ_Δ increase over a period of ~1 h (Figure 5). Although the change in τ_Δ is subtle, it is nevertheless systematic. Moreover, the change in τ_Δ clearly correlates with the polymerization-dependent change in solution viscosity. In the least, these data indicate that, like experiments based on fluorescent probes,[50] optical measurements of τ_Δ can be used as a tool to monitor polymerization-dependent dynamic changes in proteins. [As an aside, the clear absence in Figure 5 of a nucleation or "lag phase" upon salt addition to our solution indicates that, as is sometimes the case, our solution of actin "monomer" also contains oligomeric fragments of actin.[38,51]]

The two most likely origins for the polymerization-dependent increase in τ_Δ (i.e., decrease in k_Δ) are (a) a viscosity-dependent decrease in the frequency of collisions between singlet oxygen and the reactive/active sites on the protein and (b) a polymerization-dependent cloaking of some of the reactive/active sites on the protein such that they are no longer readily accessible to singlet oxygen. Although the phenomenon we observe may be a combination of these two mechanistic scenarios, we can provide some substantive comments to discount the viscosity-dependent scenario.

The data in Figure 5 indicate that, prior to initiating actin polymerization, the lifetime of singlet oxygen in this D_2O solution is ~24.5 μs. Considering that the lifetime of singlet oxygen in neat D_2O is 67 μs and that we used 25 μM of the actin monomer, this corresponds to a bimolecular rate constant for singlet oxygen removal by the actin monomer of $\sim 1 \times 10^9$ s^{-1} M^{-1}. In short, singlet oxygen removal by actin is reasonably efficient and is characteristic of a process that occurs near the diffusion-controlled limit. It has independently been shown in a number of experiments that, as expected, when the bimolecular rate constant for singlet oxygen removal is characteristic of that for a diffusion-limited reaction, the magnitude of this rate constant decreases (and hence τ_Δ increases) with an increase in the viscosity of the medium.[23,52−54]

However, the "apparent" rate constant of $k_{protein} = 1 \times 10^9$ s^{-1} M^{-1} to which we refer actually reflects the sum of rate constants

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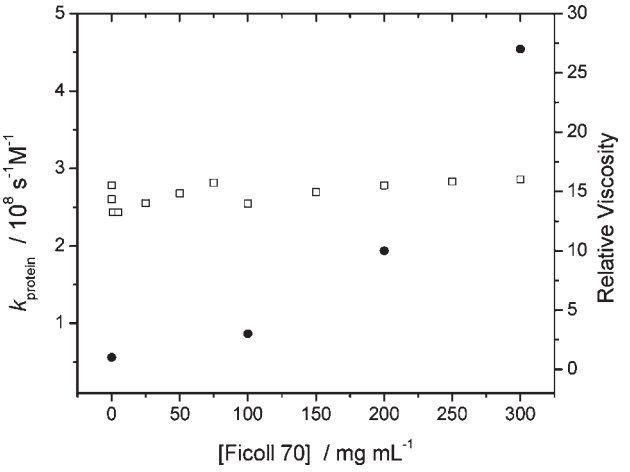

Figure 6. Bimolecular rate constant for singlet oxygen removal by the protein Subtilisin Carlsberg (□) plotted against the concentration of Ficoll 70 in the D_2O solution. Also shown is the viscosity of the solution (●) relative to that in Ficoll-free water.

for the interaction of singlet oxygen with individual amino acids in the protein (i.e., see eq 1). Thus, each of these latter rate constants must, by definition, be smaller than 1×10^9 s^{-1} M^{-1} (this statement is indeed consistent with independent measurements of rate constants for the reaction of singlet oxygen with selected amino acids which fall in the $\sim(1-9) \times 10^7$ s^{-1} M^{-1} range[11]). Therefore, with rate constants for singlet oxygen removal by components of the protein that are appreciably smaller than the rate constant for a diffusion-limited process, it is reasonable to suggest that the correlation between τ_Δ and viscosity shown in Figure 5 is, in part, "coincidental" and the result of a transitive effect (i.e., τ_Δ principally reflects the polymerization-dependent cloaking of reactive amino acids in the protein and this cloaking, in turn, correlates to the viscosity of the system).[55]

This conclusion about the effect of solution viscosity on $k_{protein}$, and hence τ_Δ, is further supported by the result of an independent experiment (Figure 6). In this study, we determined $k_{protein}$ for the natively folded protein Subtilisin Carlsberg as a function of the concentration of Ficoll 70 added to the solution. Because this protein is relatively compact, the addition of the crowding agent should not have a pronounced effect on the surface area of exposed amino acids.[56] On the other hand, upon increasing the Ficoll concentration over the range 0−300 mg mL^{-1}, the viscosity of the solution increases by a factor of approximately 30.[57] (Note that for a cell, which is inherently inhomogeneous, one must refer to an apparent viscosity.[58] Nevertheless, the range covered by our Ficoll experiments adequately represents the viscosity in a range of intracellular domains.) The data in Figure 6 clearly show that $k_{protein}$ for Subtilisin Carlsberg is independent of the pronounced change in solution viscosity.

For the data in Figure 5, our conclusion of "coincidence" in the correlation between protein-dependent values of τ_Δ and the solution viscosity is pertinent with respect to recently published data on the intracellular lifetime of singlet oxygen.[24] For a photosensitized reaction, the lifetime of intracellular singlet oxygen has been shown to increase with an increase in the elapsed irradiation time of the sensitizer.[24,25] It has also been shown that the viscosity of selected intracellular domains likewise increases with an increase in the elapsed irradiation time of the singlet oxygen sensitizer.[24] On the basis of images of cells recorded before and after irradiation, it is reasonable to assume that these changes in intracellular viscosity may, for example, derive from photooxidative-initiated structural changes in cytoskeletal proteins.[4,26] In any event, in light of our present data, we suggest that the reported irradiation-dependent changes in intracellular τ_Δ do not reflect the direct effect of a change in intracellular viscosity on $k_{protein}$. Rather, the reported changes in intracellular τ_Δ are a response to other phenomena, one of which may be an irradiation-induced structural-dependent "cloaking" of amino acids in selected intracellular proteins.

To summarize this section, we conclude that the actin polymerization data are consistent with the temperature, macromolecular crowding, and ligand binding data presented in the previous sections.

EXPERIMENTAL SECTION

Singlet oxygen lifetimes were obtained in time-resolved experiments upon pulsed-laser excitation of a photosensitizer using an approach and instrumentation that has previously been described.[59] When evaluating the time-resolved $O_2(a^1\Delta_g) \rightarrow O_2(X^3\Sigma_g^-)$ phosphorescence traces, events that result in singlet oxygen formation were decoupled from events that influence singlet oxygen removal by using a difference of two exponential functions to fit the data.[4,60] For all experiments, the temperature of the sample was controlled using instrumentation that has likewise been described.[33] The viscosity (i.e., loss modulus) data were recorded on an Anton Paar Physica MCR 501 rheometer using a 25 mm parallel plate measurement system with 1% strain and a frequency of 1 Hz; the viscosity was sampled every 20 s.

The singlet oxygen sensitizer phenalen-1-one-2-sulfonic acid (PNS) was synthesized according to the method of Nonell et al.[61] Calcium-depleted bovine α-lactalbumin (85%, Sigma-Aldrich), equine cytochrome c (>95%, Sigma-Aldrich), bovine cardiac muscle actin (>99%, Cytoskeleton Inc.), yeast myokinase (activity of 312 U/mg, Calbiochem), human serum albumin (97−99%, Sigma-Aldrich), *Bascillus licheniformis* Subtilisin Carlsberg (Calbiochem), Ap_5A (96%, Sigma), Ficoll 70 (Sigma), TMPyP (98%, Porphyrin Systems), sodium dodecyl sulfate (>99%, Sigma-Aldrich), D_2O (>99.9% D, Euriso-Top), adenosine triphosphate (ATP, >99%, Sigma Aldrich), dithiothreitol (>99.5%, Sigma-Aldrich), $CaCl_2$ (>97%, Sigma-Aldrich), and $MgCl_2$ (>99%, Sigma-Aldrich) were used as received. The D_2O-based phosphate buffer was prepared by dissolving 1 PBS tablet (Sigma) in 200 mL of D_2O to yield 10 mM phosphate buffer with 2.7 mM KCl and 137 mM NaCl at pD 7.8.

For the temperature-dependent protein denaturation study, a 50 μM solution of bovine α-lactalbumin with 500 μM $CaCl_2$ and PNS ($A_{400\ nm} \approx 0.1$) was prepared in D_2O. The pD of the solution was measured to be 6.4. Subsequently, the solution was transferred to a 1 cm path length quartz cuvette which was then placed in our temperature-controlled cuvette holder.[33] For these experiments, the temperature was increased at the rate of ~2 °C min^{-1}.

For the myokinase experiments, 2 mg of the protein were dissolved in 1.5 mL of D_2O-based phosphate buffer at pD 7.8. This solution was divided into two portions and transferred to separate cuvettes for the optical experiments. Identical volumes of buffer and Ap_5A solution were added to the respective cuvettes, thus ensuring identical protein concentrations in each cuvette. The Ap_5A/protein ratio was 10. The singlet oxygen sensitizer PNS was irradiated at 400 nm.

Bovine cardiac muscle actin was obtained as a lyophilized powder and used for the polymerization experiments (the powder supplied also contained sugars and a buffer). A pD 7.6 D_2O solution of the protein was prepared with 1.3 mM ATP and 0.2 mM dithiothreitol. Prior to use, this solution was centrifuged at 10 000 rpm at 4 °C for 30 min in an attempt

to separate monomers from most of the oligomers. Following centrifugation, the supernatant was removed and the pellet was discarded. To initiate polymerization, 0.1 mM $CaCl_2$ and 0.5 mM $MgCl_2$ were added to the solution immediately before the experiments were commenced. The rheometric and optical experiments were performed using actin from the same stock solution to ensure identical protein concentrations.

CONCLUSIONS

We have demonstrated that, for a variety of different protein systems, the overall rate constant for protein-dependent singlet oxygen removal, and hence the lifetime of singlet oxygen, depends on the dynamics of that protein. Specifically, if a conformational change in the protein either reveals or cloaks selected amino acid residues, the corresponding change in the accessibility of these residues to singlet oxygen will be manifested in the singlet oxygen lifetime.

The results obtained point to several important caveats that must be considered when interpreting singlet oxygen kinetic data. First, we have demonstrated that the rate of a protein-dependent reaction with singlet oxygen can also depend significantly on the presence of other compounds in solution (e.g., singlet oxygen's response to SDS-mediated denaturation of a given protein is significantly different from the response to the temperature-mediated denaturation of the same protein). Carrying this point further, it is established that, *in vivo*, proteins not only change conformation and structure, they can also exist in forms not routinely found *in vitro* (e.g., complexes with other intracellular molecules and/or crowded conditions). Thus, at least when dealing with singlet oxygen, caution must always be exercised when using *in vitro* results as a model to quantify intracellular phenomena.

In conclusion, protein dynamics can have a measurable effect on the kinetics of singlet oxygen removal. This can have important ramifications in singlet-oxygen-mediated processes that influence cell function and, ultimately, cell death. Moreover, when singlet oxygen is selectively produced in spatially confined and discrete subcellular domains, the subtle effects of a given protein could be amplified by subsequent processes in the cascade of events that characterize apoptotic cell death.

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