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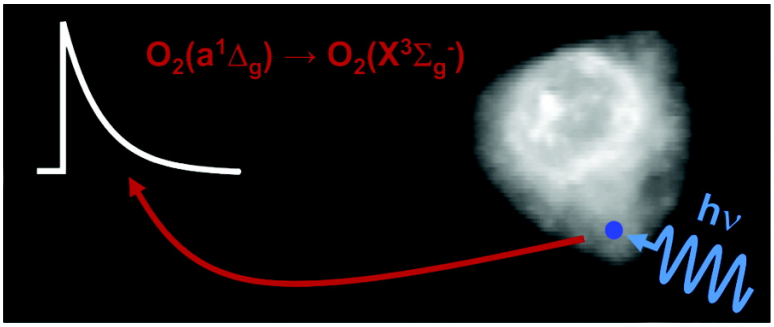

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Subcellular, Time-Resolved Studies of Singlet Oxygen in Single Cells

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Singlet oxygen, $O_2(a^1\Delta_g)$, is the lowest excited electronic state of molecular oxygen. It is an important cytotoxic agent involved in photoinduced cell death in aerated systems.[1] Singlet oxygen is readily produced in cells upon the absorption of UV or visible light. The production is facilitated by a so-called photosensitizer, a molecule that can either be endogenous or that is added to the system for this specific purpose. The energy absorbed by the sensitizer is transferred to ground-state oxygen, $O_2(X^3\Sigma_g^-)$, thereby generating $O_2(a^1\Delta_g)$. This process forms the basis for photodynamic therapy, a medical procedure used to destroy undesired tissue in treatments for cancer[2] and macular degeneration.[3]

The specific roles played by $O_2(a^1\Delta_g)$ in photoinduced cell death are not fully understood. This is particularly true in "programmed" cell death or apoptosis.[4] On the basis of indirect photobleaching experiments,[5] $O_2(a^1\Delta_g)$ phosphorescence experiments on bulk samples of lysed cells,[6] and $O_2(a^1\Delta_g)$ phosphorescence experiments on bulk samples both in vitro and in vivo,[7] the lifetime of $O_2(a^1\Delta_g)$, τ_Δ, inside a cell has been estimated to be in the range of 10−300 ns. Given that τ_Δ in pure H_2O is ∼3.5 μs,[8] these cell data were presumed to reflect the effects of endogenous quenchers such as proteins. This assessment of intracellular τ_Δ has significant implications with respect to the distances within a cell over which $O_2(a^1\Delta_g)$ activity will be manifested.

To provide insight into the behavior of $O_2(a^1\Delta_g)$ in a cell and into the mechanisms of oxygen-dependent cell death, we set out to develop optical techniques to directly monitor $O_2(a^1\Delta_g)$ in time-resolved experiments with subcellular resolution.[9] On the basis of our first experiments performed on single neurons from the hippocampus of Wistar rats,[9b] we ascertained that τ_Δ in the cell nucleus is significantly longer than that suggested by the studies mentioned above. Specifically, spatially resolved experiments demonstrated that $O_2(a^1\Delta_g)$ deactivation within the cell nucleus is dominated by interactions with the solvent, and not with cellular components.

Although our experiment differs significantly from those of the other investigators in several ways, the issues of spatial resolution and location of the $O_2(a^1\Delta_g)$ being studied stand out. Specifically, the sensitizers used by others localize mainly in membranes within the cytoplasm where endogenous quenchers may well differ from those in the nucleus. As such, it is difficult to place our results in the context of cumulative work in the field. We therefore set out to quantify τ_Δ in the cytoplasm and to compare the data obtained to the data we obtained from the nucleus.

The photosensitizer 5,10,15,20-tetrakis(*N*-methyl-4-pyridyl)-21*H*,23*H*-porphine (TMPyP) was incorporated into cultured hippocampal neurons and associated glial cells using a procedure already described.[9a] Although TMPyP preferentially localizes in the nuclei, a sufficient amount of dye remains in the cytoplasm (Figure 1).

The spectroscopic transition used to monitor $O_2(a^1\Delta_g)$ is the weak 1270 nm $O_2(a^1\Delta_g) \rightarrow O_2(X^3\Sigma_g^-)$ phosphorescence. It is well established that the medium in which $O_2(a^1\Delta_g)$ is dissolved significantly influences a $\rightarrow$ X experiments.[10] In particular, deuteration of the surrounding solvent can result in an appreciable increase in τ_Δ, which in turn increases the quantum efficiency of $O_2(a^1\Delta_g)$ phosphorescence.[10] Therefore, experiments were performed with cells that had first been equilibrated with D_2O.[9a] Note, however, that if the system contains quenchers that dominate $O_2(a^1\Delta_g)$ deactivation, this solvent isotope effect would be mitigated, and possibly even obscured totally.

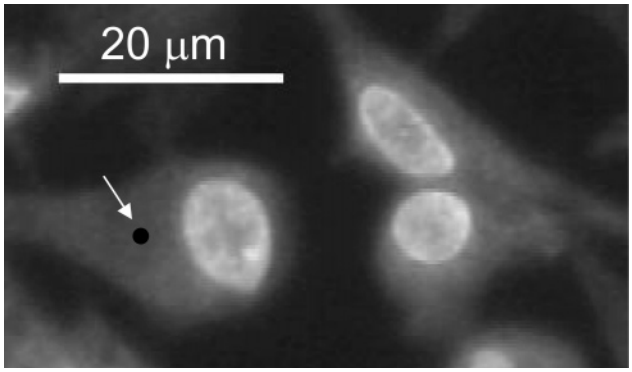

Figure 1. Fluorescence image of hippocampal cells that had been incubated with TMPyP. Although TMPyP preferentially localizes in the nuclei (bright structures), the cytoplasm nevertheless contains significant amounts of TMPyP. The superimposed spot (white arrow) in the cytoplasm approximates the cross-sectional area at the waist of our focused laser beam which was selectively positioned in the cell by translating the sample on the microscope stage.

Using techniques and instruments described previously,[9b] a single cell containing TMPyP was irradiated at 420 nm in a spatially resolved experiment. At the point of intersection with the cell, which is lying flat on the polylysine-coated coverslip, the cross-sectional diameter of the focused laser beam used for irradiation was ∼1.4 μm (see Figure 1).[9b] Near-IR emission from the cell was monitored in a single photon counting experiment.[9b] On the basis of control experiments previously described,[9b] this emission was assigned to $O_2(a^1\Delta_g)$ phosphorescence.

A time-resolved $O_2(a^1\Delta_g)$ phosphorescence signal recorded upon selective irradiation of TMPyP in the nucleus of a cell incubated with D_2O is shown in Figure 2. A single-exponential fit to the data yields $\tau_\Delta = 46 \pm 3$ μs. In neat D_2O, τ_Δ is 68 μs.[11] Even if there exist domains with varying τ_Δ within our system, the single-exponential nature of the observed decay indicates that the rate of $O_2(a^1\Delta_g)$ equilibration between these domains is rapid compared to the rate of decay.[12] This interpretation is reasonable given the relatively large value of τ_Δ observed.

A similar recording made from the cytoplasm of a neuron is shown in Figure 3. These data yield a lifetime of 37 ± 2 μs, which is comparable to that recorded in the nucleus.

With such long lifetimes, an appreciable fraction of the $O_2(a^1\Delta_g)$ produced in the cell could diffuse across the cell membrane, and

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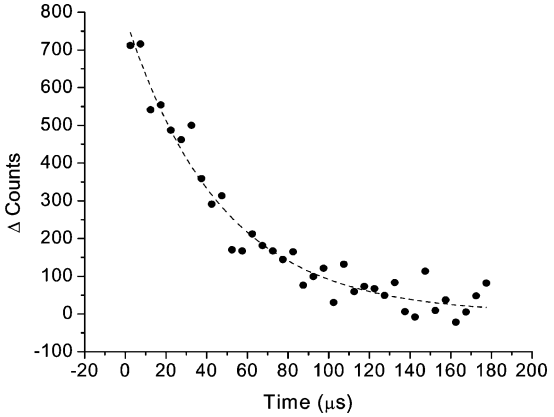

Figure 2. Time-resolved singlet oxygen phosphorescence signal recorded upon selective irradiation of TMPyP in the nucleus of a neuron incubated in D_2O. The dashed line is a single-exponential fit to the data and yields $\tau_\Delta = 46 \pm 3$ μs. A lifetime of 35 ± 3 μs is obtained when the medium surrounding the cell contains 0.75 mM BSA. In neat D_2O, $\tau_\Delta = 68$ μs.

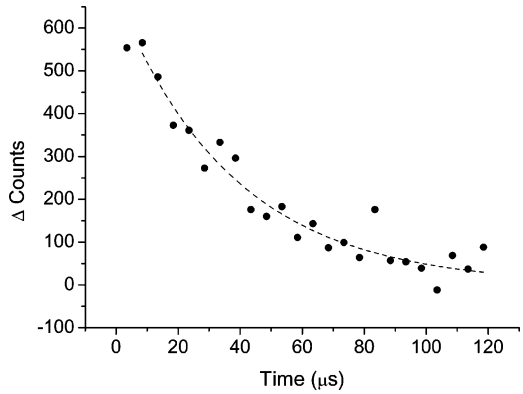

Figure 3. Time-resolved singlet oxygen phosphorescence signal recorded upon selective irradiation of TMPyP in the cytoplasm of a neuron incubated with D_2O. The dashed line is a single-exponential fit to the data and yields $\tau_\Delta = 37 \pm 2$ μs. A lifetime 30 ± 3 μs was recorded in the presence of 0.75 mM extracellular BSA.

the optical signal observed would partly reflect an extracellular population of $O_2(a^1\Delta_g)$. However, we have previously demonstrated that any extracellular contribution to our signal can be selectively removed by adding bovine serum albumin (BSA) to the extracellular medium.[9b] BSA is an efficient $O_2(a^1\Delta_g)$ quencher ($k_q = 5 \times 10^8$ M^{-1} s^{-1})[13] but is too large (MW ~65 kDa) to penetrate the cell membrane. Thus, values of τ_Δ obtained in the presence of BSA only reflect the behavior of $O_2(a^1\Delta_g)$ within the cell.

Upon the addition of 0.75 mM BSA to the extracellular medium, the lifetime of $O_2(a^1\Delta_g)$ produced upon irradiation into the cytoplasm decreased to 30 ± 3 μs, while that recorded upon irradiation into the nucleus decreased to 35 ± 3 μs. When compared to the lifetimes obtained in the absence of BSA, these data indicate that a significant fraction of our signal originates from $O_2(a^1\Delta_g)$ inside the cell.

Equation 1 can be used to estimate the first-order contribution of any $O_2(a^1\Delta_g)$ quenchers present in the cytoplasm of our cells:

$$\tau_\Delta^{-1} = k_{D_2O} + k_q[Q] \qquad (1)$$

where τ_Δ is the observed $O_2(a^1\Delta_g)$ lifetime in the cytoplasm, 30 ± 3 μs, and k_{D_2O} is the pseudo first-order rate constant for $O_2(a^1\Delta_g)$ deactivation by D_2O (i.e., $k_{D_2O} = (68\ \mu s)^{-1}$). Solving for $k_q[Q]$, a value of 1.8×10^4 s^{-1} is obtained. This value clearly indicates that, in H_2O-equilibrated cells ($k_{H_2O} = (3.5\ \mu s)^{-1} = 2.9 \times 10^5$ s^{-1}), other material present in the cytoplasm will not be particularly effective at deactivating singlet oxygen.

In support of the time-resolved experiments, we also monitored changes in the intensity of the integrated $O_2(a^1\Delta_g)$ signal from the cytoplasm. It is important to note that the intensity of the $O_2(a^1\Delta_g)$ phosphorescence signal is proportional to the product $k_r\tau_\Delta$, where k_r is the radiative rate constant for a $\rightarrow$ X emission.[9a] Because k_r is independent of solvent deuteration,[14] the intensity of our signal will scale according to τ_Δ.

For experiments performed in the presence of 0.75 mM extracellular BSA, the intensity of the $O_2(a^1\Delta_g)$ signal recorded from the cytoplasm of a cell in a 1:1 H_2O-D_2O mixture (by volume) was one-fifth of that recorded from the same cell in a pure D_2O medium. Given that $[H_2O] \sim [D_2O] \sim 55$ M, these results are entirely consistent with a $k_q[Q]$ value of 1.8×10^4 s^{-1}, $\tau_\Delta(H_2O) = 3.5$ μs, and $\tau_\Delta(D_2O) = 68$ μs. In short, deactivation of $O_2(a^1\Delta_g)$ in both the cytoplasm and nucleus of our cells is dominated by interactions with the solvent and not by cellular constituents such as proteins.

In conclusion, we have demonstrated that, irrespective of subcellular domain, $O_2(a^1\Delta_g)$ has a much longer lifetime in a cell than previously believed and can consequently diffuse over much greater distances. These results have significant implications for elucidating the potential role(s) of $O_2(a^1\Delta_g)$ in intracellular signaling,[15] as well as in mechanisms of cell death.

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