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Exerting better control and specificity with singlet oxygen experiments in live mammalian cells ☆

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ABSTRACT

Singlet molecular oxygen, $O_2(a^1\Delta_g)$, is a Reactive Oxygen Species, ROS, that acts as a signaling and/or perturbing agent in mammalian cells, influencing processes that range from cell proliferation to cell death. Although the importance of $O_2(a^1\Delta_g)$ in this regard is acknowledged, an understanding of the targets and mechanisms of $O_2(a^1\Delta_g)$ action is inadequate. Thus, methods that better facilitate studies of $O_2(a^1\Delta_g)$ in mammalian cells are highly desired. This is particularly important because, as a consequence of its chemistry in a cell, $O_2(a^1\Delta_g)$ can spawn the generation of other ROS (*e.g.*, the hydroxyl radical) that, in turn, can have a unique influence on cell behavior and function. Therefore, exerting better control and specificity in $O_2(a^1\Delta_g)$ experiments ultimately reduces the number of variables in general studies to unravel the details of ROS-dependent cell dynamics.

In this article, we summarize our recent efforts to produce $O_2(a^1\Delta_g)$ with increased control and selectivity in microscope-based single-cell experiments. The topics addressed include (1) two-photon excitation of a photosensitizer using a focused laser to create a spatially-localized volume of $O_2(a^1\Delta_g)$ with subcellular dimensions, (2) protein-encapsulated photosensitizers that can be localized in a specific cellular domain using genetic engineering, and (3) direct excitation of dissolved oxygen in sensitizer-free experiments to selectively produce $O_2(a^1\Delta_g)$ at the expense of other ROS. We also comment on our recent efforts to monitor $O_2(a^1\Delta_g)$ in cells and to monitor the cell's response to $O_2(a^1\Delta_g)$.

1. Introduction

Singlet oxygen, $O_2(a^1\Delta_g)$, is the lowest excited electronic state of molecular oxygen [1,2]. It has a unique and characteristic chemistry that results in the oxygenation/oxidation of organic molecules [3], proteins [4], lipids [5] and nucleic acid bases [6,7]. In this way, $O_2(a^1\Delta_g)$ is acknowledged as a Reactive Oxygen Species, ROS, that can initiate a plethora of responses in both plant and animal cells [8–13]. ROS are indeed important in cell function and signaling [9,11,13–17], influencing processes that range from the protection and proliferation of cells to events that result in cell death [9,18–22]. Nevertheless, many details of ROS action are still not understood and this, in turn, limits our ability to exploit ROS-dependent pathways through the development, for example, of more efficient drugs for the prevention and/or treatment of diseases.

In this article, we elaborate on some of our recent work, including unpublished data, involving the development and implementation of tools that allow us to exert control and specificity in studies of $O_2(a^1\Delta_g)$ in mammalian cells. From the perspective of understanding the general behavior of ROS in mammalian cells [8,22,23], the ability to exert control and specificity with $O_2(a^1\Delta_g)$ becomes important, in part, because many reactions of $O_2(a^1\Delta_g)$ lead to the generation of other ROS (*e.g.*, the hydroxyl and the hydroperoxyl radicals) [24–26]. Thus, with our focused work on $O_2(a^1\Delta_g)$, we enable more systematic general studies and interpretations of ROS action in living cells.

2. Use of photosensitizers to produce $O_2(a^1\Delta_g)$: advantages and limitations

The use of a photosensitizer to produce $O_2(a^1\Delta_g)$ is common in many fundamental studies as well as practical applications. In this

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process, light absorbed by a given molecule (the sensitizer) produces an excited electronic state which, upon colliding with ground state oxygen, $O_2(X^3\Sigma_g^-)$, transfers its energy of excitation to oxygen to produce $O_2(a^1\Delta_g)$ (Fig. 1) [1,27].

One of the biggest advantages of using a photosensitizer to produce $O_2(a^1\Delta_g)$ is that the quantum yield of this process, ϕ_Δ, can be quite high. Indeed, a wide variety of molecules have been used as $O_2(a^1\Delta_g)$ photosensitizers in biological systems [28] and, depending on the sensitizer, quantum yields ranging from ~0.3 to 1.0 can be readily obtained. Note that, when $\phi_\Delta = 1.0$, every photon absorbed by the sensitizer results in $O_2(a^1\Delta_g)$ production. Moreover, the very fact that the $O_2(a^1\Delta_g)$ precursor is an excited electronic state of a discrete molecule allows for certain aspects of control, with respect both to the light-absorbing molecule used and the light delivered to the system [10]. We provide selected examples of such control in our discussion below.

Unfortunately, the photosensitized production of $O_2(a^1\Delta_g)$ also has many disadvantages and limitations, certainly with regard to its use as a mechanistic tool to better understand the behavior of $O_2(a^1\Delta_g)$ in cells. One disadvantage is that, depending on the sensitizer used and its ultimate location in the cell, photoinduced electron transfer reactions that produce other ROS can kinetically compete with the desired energy transfer process to produce $O_2(a^1\Delta_g)$ (*e.g.*, production of the superoxide ion which, when protonated, yields the hydroperoxyl radical) [29]. Thus, in these cases, a mechanistically undesired complication is introduced at the onset of an experiment because more than one ROS is produced. Another disadvantage of using a photosensitizer to produce $O_2(a^1\Delta_g)$ is the fact that $O_2(a^1\Delta_g)$ is created immediately adjacent to the sensitizer and, as such, it is readily available to react with the sensitizer. Thus, sensitizer degradation (or "bleaching") becomes a problem and this, in turn, is manifested in (a) the "dose" of $O_2(a^1\Delta_g)$ delivered over time, (b) the creation of other reactive species (*e.g.*, sensitizer hydroperoxides) that can also perturb the cell, and (c) the possible relocation of the sensitizer in the cell [30]. In our discussion below, we further address these issues and provide methods by which these problems can be solved.

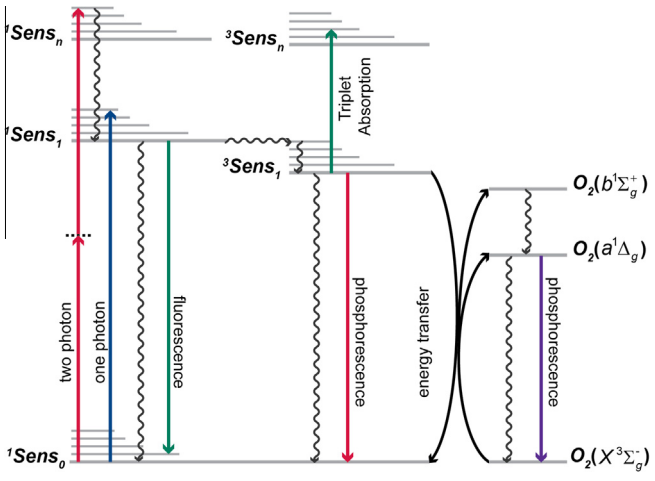

Fig. 1. Diagram illustrating how one- and two-photon excitation schemes can be used to create an excited state of a sensitizer (Sens). With sensitizers for $O_2(a^1\Delta_g)$ production, the $^1Sens_1 \rightarrow {}^3Sens_1$ interconversion ideally occurs efficiently. Because the $^3Sens_1$ state has a comparatively long lifetime, it has a higher probability for colliding with ground state oxygen, $O_2(X^3\Sigma_g^-)$, which is required for $O_2(a^1\Delta_g)$ production. Sensitizer photophysics can be studied by fluorescence, phosphorescence and/or transient absorption experiments, and $O_2(a^1\Delta_g)$ is ideally monitored by its $O_2(a^1\Delta_g) \rightarrow O_2(X^3\Sigma_g^-)$ phosphorescence. Transitions involving the $O_2(b^1\Sigma_g^+)$ state of oxygen are discussed later in this article. This figure is reprinted from Westberg et al. [10].

3. Exerting control and selectivity in the production of $O_2(a^1\Delta_g)$

In this section, we briefly discuss methods by which one can overcome some of the limitations associated with the photosensitized production of $O_2(a^1\Delta_g)$ for experiments performed with single cells. Descriptions of these techniques have been published (references presented below in the respective sections), and we focus here on the specific advantages/disadvantages of these methods within the context of our discussion in Section 2 above.

3.1. Two-photon excitation of a sensitizer for spatial control

The excitation of $O_2(a^1\Delta_g)$ photosensitizers is most often achieved through a one-photon process (Fig. 1). For such experiments in which spatial localization of excitation is desired, one can indeed use an objective to focus this exciting light down to a diffraction-limited spot in the image plane of a microscope (*i.e.*, lateral cross-sectional diameter at the beam waist of ~0.5–1.0 μm, depending on the wavelength of light and the numerical aperture of the objective) [10,31,32]. However, a mammalian cell is an inhomogeneous medium that scatters light. Thus, when using light whose wavelength is resonant with a one-photon transition in a sensitizer, scattered light will be absorbed by these molecules distributed throughout the cell, with a concomitant loss in the spatial localization of $O_2(a^1\Delta_g)$ production [33]. A corollary to this is that sensitizer molecules located along the entire path of light propagation through the sample (*i.e.*, axial direction orthogonal to the image plane) will also absorb light [10].

A $O_2(a^1\Delta_g)$ sensitizer can also be excited via a two-photon transition when the incident irradiance is sufficiently high (Fig. 1) [1]. In the present context, the principal advantage of using two-photon excitation is the ability to obtain a highly-localized spatial distribution of $O_2(a^1\Delta_g)$ at any chosen point inside or outside a cell [10,33–35]. As described above, one again uses an objective to focus the exciting light down to a diffraction-limited spot in the image plane of the microscope [10,31]. Because longer wavelengths of light are used in these experiments (to avoid exciting one-photon transitions, see Fig. 1), the cross-sectional diameter of this diffraction-limited spot is larger than what would be obtained with the shorter wavelengths used in one-photon experiments. Nevertheless, this phenomenon is somewhat offset by the fact that the probability of light absorption in the two-photon process scales with the square of the incident light intensity and, as such, the "spot" size of excited states produced is smaller than that defined by the diffraction of light [32,36,37]. Most importantly, even though incident light will still be scattered in a two-photon experiment, the irradiance of the scattered light is not sufficiently high to induce a two-photon transition. Rather, the simultaneous absorption of two-photons that results in the population of a sensitizer excited state will only occur in the confined volume of the focused laser beam where the irradiance is sufficiently high [10,31,33] (see Fig. 2).

The transition probability for two-photon absorption (*i.e.*, the so-called two-photon absorption cross section) is quantified in Göppert-Mayer, GM, units ($1\ GM = 10^{-50}\ cm^4\ s$) [37,38]. Although it may be assumed that a sensitizer with an appreciable two-photon absorption cross section is needed for these experiments, sufficient amounts of $O_2(a^1\Delta_g)$ for a variety of cell experiments can nevertheless be made using sensitizers with absorption cross sections as small as ~1 GM [35]. An absorption cross section of ~1 GM at a convenient excitation wavelength is common for many molecules capable of sensitizing the production of $O_2(a^1\Delta_g)$ and, as such, this parameter is generally not a limiting factor in the choice of a two-photon sensitizer. However, if one wants to obtain better selectivity in the production of $O_2(a^1\Delta_g)$, certainly relative to what