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Invited feature article

Control of singlet oxygen production in experiments performed on single mammalian cells

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ABSTRACT

Reactive Oxygen Species, ROS, are small molecules (*e.g.*, hydroxyl radical, superoxide radical anion, hydrogen peroxide, nitric oxide and singlet oxygen) that play key signaling roles in mammalian cells. They are generated as part of normal cell function, but also play roles in a cell's response to perturbation (*e.g.*, disease) and in many disease treatments (*e.g.*, drugs). Although the importance of ROS is acknowledged, a general understanding of the mechanisms of ROS action and their biological effects is inadequate. Thus, new experimental methods that better facilitate studies of ROS behavior in mammalian cells are highly desired.

In this feature article, we focus on one ROS in particular: singlet oxygen, $O_2(a^1\Delta_g)$. We summarize our recent efforts to selectively produce singlet oxygen in sub-cellular, spatially-resolved experiments performed on single mammalian cells. The topics addressed include (1) two-photon excitation of a photosensitizer using a focused laser to initially create a localized femtoliter volume of singlet oxygen, (2) protein-encapsulated photosensitizers that can be localized in cells using genetic engineering, and (3) direct excitation of dissolved oxygen in sensitizer-free experiments. We also provide a brief overview of our recent efforts to monitor singlet oxygen in cells (*e.g.*, direct time-resolved optical detection, fluorescent probes) and to monitor the cell's response to singlet oxygen (*e.g.*, the use of rapid super-resolution microscopy). In all cases, we discuss the advantages and disadvantages of that particular approach/tool.

1. Introduction

Reactive Oxygen Species, ROS, are important in cell function and signaling [1–5]. In mammalian cells, ROS-induced responses range from the protection and proliferation of cells to events that result in cell death, with a given response depending upon the concentration and cellular location of ROS generation, among other things [4,6–10]. Although studies to elucidate the roles played by ROS in these processes have long been performed, much is still not understood [10].

We have long been interested in one particular ROS: singlet oxygen, $O_2(a^1\Delta_g)$, the lowest excited electronic state of molecular oxygen [11,12]. With its unique and characteristic chemistry that results in the oxygenation of organic molecules [13], proteins [14], lipids [15] and nucleic acid bases [16,17], $O_2(a^1\Delta_g)$ is certainly acknowledged as an important ROS that can initiate a plethora of cell responses [4,18]. However, many reactions of $O_2(a^1\Delta_g)$ generate other ROS (*e.g.*, the hydroxyl and the hydroperoxyl radicals) [19–21] and these, in turn, can likewise initiate a variety of cell responses [10,18,22]. Thus, a focused study of the behavior $O_2(a^1\Delta_g)$ in mammalian cells is, by no means, limited in scope.

Following this journal's format for a Feature Article, we do not present an exhaustive and comprehensive review of the field. Rather, we only summarize some of our recent work, including unpublished data, focusing on the development and implementation of tools that facilitate the study of $O_2(a^1\Delta_g)$ behavior in mammalian cells.

2. General background: Photosensitized $O_2(a^1\Delta_g)$ production

Although $O_2(a^1\Delta_g)$ can be produced in a variety of ways in a cell, both endogenously and exogenously [23–25], we have long

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focused our attention on the photosensitized production of $O_2(a^1\Delta_g)$ [11]. In this 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). This sequence of events is clearly important, for example, in cellular systems exposed to sunlight, and it also has practical ramifications for use as a mechanistic tool in laboratory experiments in which lasers and lamps are used as the light sources. It has also been exploited in the medical procedure commonly known as Photodynamic Therapy (PDT) in which cancer cells, for example, can be destroyed by $O_2(a^1\Delta_g)$-mediated processes [26–28].

Over the years, a wide variety of molecules have been used as $O_2(a^1\Delta_g)$ photosensitizers in biological systems [29]. Indeed, it is fair to say that an appreciable amount of activity over the past ~40 years in this field has been devoted to the design, synthesis and testing of $O_2(a^1\Delta_g)$ photosensitizers with a desired chemical, physical, and biological property. The photosensitizer property that has generally been considered the most important is a high quantum yield of $O_2(a^1\Delta_g)$ production, φ_Δ.

In the photosensitized process, the very fact that the precursor to $O_2(a^1\Delta_g)$ is an excited electronic state of a discrete molecule allows for certain aspects of control in the production of $O_2(a^1\Delta_g)$. For example, the judicious inclusion of molecules that quench the sensitizer excited state before the arrival of $O_2(X^3\Sigma_g^-)$ allows for the development of "switches" that activate the production of $O_2(a^1\Delta_g)$ only under specific physiological conditions [30–35].

The photosensitized process for $O_2(a^1\Delta_g)$ production also has many disadvantages, certainly from the perspective of those who want to use it as a mechanistic tool to better understand the behavior of $O_2(a^1\Delta_g)$ in cells. Perhaps the most significant of these disadvantages 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 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) [36]. Carrying this point further, conditions that stabilize charge-transfer interactions between the excited-state sensitizer and oxygen (*i.e.*, $Sens^{\delta+\bullet}$ $O_2^{\delta-\bullet}$) are also known to adversely affect

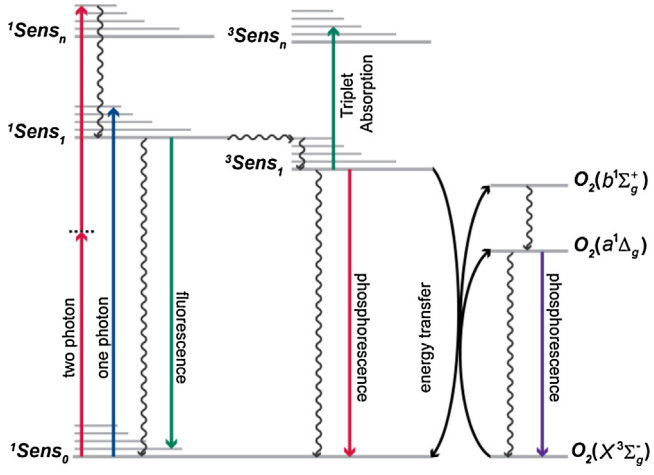

Fig. 1. Diagram that illustrates how one-photon and two-photon transitions can be used to create an excited state of a given sensitizer (Sens). Sensitizers for the production of $O_2(a^1\Delta_g)$ ideally have a large quantum yield of intersystem crossing to produce the longer-lived triplet state that has a higher probability for colliding with ground state oxygen, $O_2(X^3\Sigma_g^-)$. The processes of energy transfer from ^{3}Sens to $O_2(X^3\Sigma_g^-)$ that result in the production of $O_2(a^1\Delta_g)$ and/or $O_2(b^1\Sigma_g^+)$ are shown with curved arrows. Sensitizers can be monitored either by their fluorescence, phosphorescence and/or in a transient absorption experiment. Transitions between the three electronic states of oxygen are discussed later in this article.

$O_2(a^1\Delta_g)$ yields by promoting non-radiative deactivation channels to populate the ground electronic state of both molecules [11,37,38].

Sensitizer incorporation into a given cell has traditionally been achieved by incubating the cell in a medium that contains the sensitizer. As such, sensitizer location and, hence, the site of $O_2(a^1\Delta_g)$ production in the cell, is often not specific and/or well-defined, and this points to yet another disadvantage of the photosensitized production of $O_2(a^1\Delta_g)$, at least as it is currently commonly used. Aspects of these photosensitizer-related issues have been addressed in recent more comprehensive reviews [11,39]. We discuss solutions to some of these problems in the next section.

3. Addressing and solving sensitizer-dependent problems

It is now possible to better address and, in some cases, overcome many of the limitations associated with the photosensitized production of $O_2(a^1\Delta_g)$ for experiments performed in single cells. Pertinent approaches include (1) techniques to control the light that produces the sensitizer excited state such that $O_2(a^1\Delta_g)$ is initially formed in a confined and specifically-localized volume of ~1 femtoliter, (2) the use of genetic engineering to localize a sensitizer-containing protein to a specific cellular domain or compartment, and (3) the direct irradiation of $O_2(X^3\Sigma_g^-)$ in a sensitizer-free system to initially and selectively make $O_2(a^1\Delta_g)$ at the exclusion of other ROS. Moreover, when these approaches are combined with state-of-the-art time- and space-resolved techniques to monitor $O_2(a^1\Delta_g)$ and a cell's response to $O_2(a^1\Delta_g)$, one is indeed better placed to provide new insight into the roles that $O_2(a^1\Delta_g)$ can play in modulating cell behavior. In separate sections below, we briefly summarize some of our contributions to the development and implementation of these tools.

3.1. Control of actinic light

Over the years, the excitation of $O_2(a^1\Delta_g)$ photosensitizers has traditionally been achieved through a one-photon process (Fig. 1). For most sensitizers, this is readily achieved through the use of sunlight, lamps and lasers over the wavelength range of ~300–700 nm. For practical photodynamic therapeutic applications in medicine, a large effort has been devoted to the design and synthesis of sensitizers (*i.e.*, the PDT "drug") that have a one-photon transition at ~700–900 nm to accommodate the fact that tissue is reasonably transparent in this range [26].

The excitation of a $O_2(a^1\Delta_g)$ sensitizer can also occur via a two-photon transition under conditions in which the incident photon flux density (*i.e.*, irradiance) is sufficiently high (Fig. 1) [11]. For most sensitizers, this is likewise readily achieved over the range of ~700–900 nm. Moreover, this is a wavelength domain easily accessible with modern femtosecond pulsed lasers, which are the preferred light source for two-photon experiments; among other things, the peak power is high and the narrow pulse width of ~100 fs minimizes the chance for excited state absorption during the irradiation process.

Although the illustration shown in Fig. 1 shows a two-photon process that populates a different state than the one-photon process, this is not a general rule. Rather, the state initially populated in one- and two-photon processes can also be the same. A key deciding factor in these cases is the symmetry of the molecule that is irradiated [40,41], with particular consideration of the distribution of ground state conformations and their respective symmetries [42,43]. In any event, for our current purpose, the initial state populated is irrelevant as long as Kasha's rule is obeyed