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Review Article

Light-induced oxidant production by fluorescent proteins

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ABSTRACT

Oxidants play an important role in the cell and are involved in many redox processes. Oxidant concentrations are maintained through coordinated production and removal systems. The dysregulation of oxidant homeostasis is a hallmark of many disease pathologies. The local oxidant microdomain is crucial for the initiation of many redox signaling events; however, methods to control oxidant product are limited. Some fluorescent proteins, including GFP, TagRFP, KillerRed, miniSOG, and their derivatives, generate oxidants in response to light. These genetically-encoded photosensitizers produce singlet oxygen and superoxide upon illumination and offer spatial and temporal control over oxidant production. In this review, we will examine the photosensitization properties of fluorescent proteins and their application to redox biology. Emerging concepts of selective oxidant species production via photosensitization and the impact of light on biological systems are discussed.

1. Introduction

Oxidants, such as superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) have a multifaceted role in cells. The endogenous production of oxidants contributes to both destructive pathways and cell signaling [1]. The controlled production of oxidants can activate redox signaling pathways and is important for maintaining homeostasis [1]. However, the overproduction of oxidants, such as during reperfusion injury, leads to toxicity and death of the cell [2]. Dysregulation of oxidant production is associated with numerous pathologies, however, attempts to treat disease with antioxidants such as vitamin E have been unsuccessful and suggest the role of oxidants in the cell is more complex than originally thought [3]. For example, the antioxidant may not selectivity target the oxidant and instead lead to global changes in redox homeostasis [4]. Moreover, antioxidants would have to achieve high concentrations to compete with endogenous target molecules for the oxidant [5,6]. New approaches are focused on combating specific sources of oxidants using targeted redox medicine [4].

Oxidants can react with redox sensitive targets in the cellular environment. These processes can occur through enzyme-mediated processes [7]. Much like other secondary messengers, specificity of a redox signal can be achieved through modulation of the microdomain [8]. For example, an accumulation of H_2O_2 can be restricted to a microdomain via the local inhibition of oxidant scavenging enzymes [8,9]. This allows the local oxidant concentration to rise to a moderate level to elicit a signaling response, yet prevent a toxic accumulation of H_2O_2 [9–12]. Fluorescent biosensors can measure oxidants including peroxynitrite [13] and H_2O_2 [14]. These sensors can be targeted to specific regions, or microdomains, in the cell to detect localized changes in the redox state [15]. Alternatively, the modification of intracellular oxidant levels often relies on global exogenous administration of oxidants, application of toxins or mutations that cause continuous oxidant production.

Advances in photochemistry and optogenetics have led to the development of tools that offer simultaneous spatial and temporal control of oxidant production [16]. Fluorescent proteins are widely used to study redox biology. While most are employed for their fluorescent properties (e.g. oxidant biosensors), some fluorescent proteins produce oxidants in response to light. These proteins are genetically-encoded photosensitizers and can be targeted to distinct cellular compartments to study oxidant microdomains [16]. These tools have been used for a variety of applications, which require spatial and temporal control over oxidant production. Depending on their photochemistry, the photosensitizers can produce singlet oxygen ($^1O_2$) and $O_2^{\bullet-}$, however, little is known about the relative and/or absolute contribution of each species to an observed phenotype. Moreover, each oxidant has unique chemistry that may render particular biological reactions more likely to occur [8,17,18]. While there are many novel approaches to target photosensitizers, this review will examine fluorescent proteins that generate oxidants.

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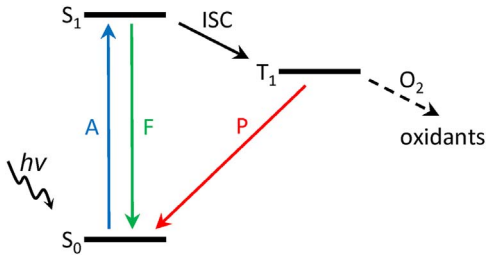

Fig. 1. Jablonski Diagram of the photosensitization processes and generation of oxidants. Upon illumination (hv), a photosensitizer in the ground state (S_0) can absorb light (A, blue) and transition to an excited singlet state (S_1). A photosensitizer in S_1 can return to S_0 energy via energy loss in the form of heat or fluorescence (F, green). From the S_1, a photosensitizer may also progress to a comparatively longer lived excited triplet state (T_1) via intersystem crossing (ISC). A photosensitizer can then return to ground state via phosphorescence (P, red). Alternatively, in the presence of oxygen (O_2) the T_1 can generate oxidants through type I or type II processes. Figure modified from [19].

2. Photosensitization

Oxygen-dependent photosensitization is a process that involves oxygen, light, and a photosensitizer. Photosensitizers are molecules, which, upon absorption of light, initiate the photochemical creation of oxidants. Photosensitizers may be synthesized endogenously or administered exogenously, as is done in medical therapeutic and diagnostic applications. When illuminated at an appropriate wavelength, the absorption of a photon of light results in the population of a short-lived excited singlet state (Fig. 1) [19,20]. Once excited, the photosensitizer electron may return to the ground state by emission of fluorescence. Alternatively, the singlet state may evolve to a relatively longer-lived excited triplet state through the process of intersystem crossing [19,20]. The triplet state may relax to the ground state through non-radiative mechanisms or via phosphorescence. Photosensitization is based on the efficient quenching of the triplet state by molecular oxygen, which is itself a triplet. It is this quenching of the photosensitizer triplet state by oxygen that generates the oxidant. Efficient photosensitizers, therefore, have high rates of intersystem crossing to the triplet state [21]. Oxygen-independent photosensitization can also occur via triplet-triplet energy transfer, whereby the photosensitizer triplet state is quenched by a target molecule [22].

A triplet state photosensitizer can form either $O_2^{\bullet-}$ or $^1O_2$ through a type I or type II mechanism, respectively (Fig. 2). In the type I mechanism, the triplet state photosensitizer obtains an electron from its environment and becomes a radical species. In protein encased photosensitizers, the surrounding amino acids can act as electron donors. The reduced photosensitizer then reacts with oxygen to generate $O_2^{\bullet-}$. The type I mechanism may also include hydrogen abstraction from a target molecule, ultimately resulting in oxidation products [23]. However,

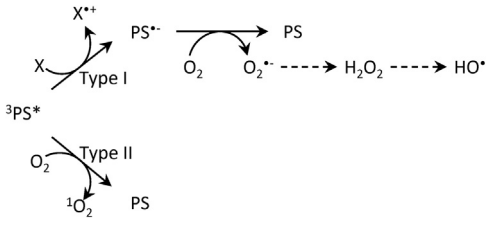

Fig. 2. Photosensitization processes to generate superoxide and singlet oxygen. A photosensitizer in the excited triplet state ($^3PS^*$) can undergo a type I or type II reaction to generate superoxide ($O_2^{\bullet-}$) or singlet oxygen ($^1O_2$), respectively. In a type I process 3PS* interacts with an oxidizable substrate (X) to generate a radical cation ($X^{\bullet+}$) and a photosensitizer radical anion ($PS^{\bullet-}$). The $PS^{\bullet-}$ can interact with oxygen to form $O_2^{\bullet-}$ and the ground state photosensitizer (PS). Depending on the environmental milieu, $O_2^{\bullet-}$ can be dismutated to hydrogen peroxide (H_2O_2) spontaneously or enzymatically and subsequently converted to the hydroxyl radical ($HO^{\bullet}$) via the Fenton reaction. In a type II process, 3PS* returns to PS via energy transfer to ground state oxygen O_2 resulting in the formation of $^1O_2$. Figure modified from [28].

this mechanism requires a close proximity between the triplet state photosensitizer and the target; an interaction which may be hindered by protein encasement of a photosensitizer [23]. In the type II mechanism, the triplet excited state transfers energy directly to oxygen to form $^1O_2$. Both type I and type II processes can occur in all photosensitizers. For example, the commonly used chemical photosensitizers rose bengal and flavin mononucleotide generate both $^1O_2$ and $O_2^{\bullet-}$ [24–26]. However, the relative importance of one or the other process is often influenced by intrinsic properties of the photosensitizer, oxygen concentration, surrounding environment and pH. Encasing the photosensitizer within a protein can confer some control over the local environment as well as provide selective cellular targeting of the photosensitizer [27].

Targeted light-induced production of oxidants is widely used in photodynamic therapy to treat cancer and local infections [20,29–31]. Diseased cells are targeted with a photosensitizer and illuminated, resulting in selective destruction. The photosensitization mechanism to generate $O_2^{\bullet-}$ may compete with the mechanism to produce $^1O_2$. However, the contribution of a particular oxidant species to cell death is usually unclear. Current approaches to optimize the effectiveness of photodynamic therapy include improving selective targeting, intracellular localization of the photosensitizer to an oxidative-vulnerable compartment and designing photosensitizers that generate large yields of oxidants [27,32].

3. Measures of oxidants

The quantum yield of a particular oxidant is defined as the fraction of optical excitations that result in the formation of that oxidant. For example, rose bengal has a $^1O_2$ quantum yield of 0.76 [33]. $^1O_2$ is quantified using a number of methods that take advantage of its physical properties, such as phosphorescence at 1270 nm or its specific reaction products (reviewed in [34,35]). Like most measures of oxidants, each is not without limitations. The most accepted measure of $^1O_2$ is the time-resolved optical detection of phosphorescence. These experiments are relatively challenging due to the extremely weak phosphorescence signal. Other methods use the reactive nature of $^1O_2$ and measure reaction products, which are readily assayed. For example, $^1O_2$ generation is measured via photobleaching of the probe ADPA (anthracene-9,10-dipropionic acid). Alternatively, the reaction of $^1O_2$ and a probe can result in a fluorescent product as seen with singlet oxygen sensor green (SOSG) [36] or fluorescein–anthracene probes such as DMAX or DPAX [37]. However, the selectivity of probes for $^1O_2$ or other oxidants is unclear. For example, $O_2^{\bullet-}$ can interfere with $^1O_2$-mediated SOSG fluorescence [26], and $^1O_2$–independent mechanisms can bleach ADPA [38]. Appreciation of the caveats of these detectors is important given that most photosensitizers generate both $^1O_2$ and $O_2^{\bullet-}$ [24,25]. Moreover, some indicators are light-sensitive, and the indicators can act as photosensitizers themselves to generate oxidants [36,39].

Detection of $O_2^{\bullet-}$ and downstream oxidants such as H_2O_2, as well as associated caveats, are reviewed elsewhere [5,40]. In brief, photosensitization-mediated $O_2^{\bullet-}$ detection has typically relied on dihydroethidium (DHE) bleaching [41] or fluorescence of DHE-oxidation products [42], which lack $O_2^{\bullet-}$-specific product separation [43,44]. The photophysics *in vitro* may differ greatly from that in the cellular milieu. In biological systems researchers often use scavengers or enhancers to determine the oxidant responsible for an observed phenotype. For example, uric acid can scavenge $^1O_2$, while deuterated water (D_2O) can extend $^1O_2$ lifetime [45,46]. $O_2^{\bullet-}$ can be removed with superoxide dismutase (SOD) or SOD mimetics [47]. High concentrations of scavengers are often necessary to compete with the reaction of the oxidant with a biological target. While these concentrations may be suitable for *in vitro* studies, high concentrations of a scavenger may have secondary effects. For example, concentrations of azide that are required to efficiently scavenge $^1O_2$ will also inhibit cellular respiration. Much like the caveats