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Pitfalls in the Use of Common Luminescent Probes for Oxidative and Nitrosative Stress

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Lucigenin (LC^{2+}, bis-*N*-methylacridinium) and 2′,7′ -dichlorofluorescin ($DCFH_2$) are widely used as chemiluminescent or fluorescent probes for cellular oxidative stress, to reflect levels of superoxide ($O_2^{\cdot -}$) and hydrogen peroxide, respectively. We report mechanistic studies that add to the growing evidence for the unsuitability of either probe except in very well-defined circumstances. The ability for lucigenin to *generate* superoxide via reduction of LC^{2+} to $LC^{\cdot +}$ and redox cycling with oxygen depends on the reduction potential of the $LC^{2+}/LC^{\cdot +}$ couple. Redox equilibrium between $LC^{\cdot +}$ and the redox indicator benzyl viologen is established in microseconds after generation of the radicals by pulse radiolysis and indicated $E(LC^{2+}/LC^{\cdot +}) \sim -0.28$ V vs. NHE. Reaction of $LC^{\cdot +}$ with O_2 to generate $O_2^{\cdot -}$ was also observed directly similarly, occurring in milliseconds, with a rate constant $k \sim 3 \times 10^6\ M^{-1}\ s^{-1}$. Quinones act as redox mediators in $LC^{\cdot +}/O_2$ redox cycling. Oxidation of $DCFH_2$ to fluorescent DCF is not achieved by $O_2^{\cdot -}$ or H_2O_2, but $NO_2^{\cdot}$ reacts rapidly: $k \sim 1 \times 10^7\ M^{-1}\ s^{-1}$. Oxidation by H_2O_2 requires a catalyst: cytochrome *c* (released into the cytosol in apoptosis) is very effective (even 10 nM). Fluorescence reflects catalyst level as much as $O_2^{\cdot -}$ production.

KEY WORDS: Oxidative stress; lucigenin; dichlorofluorescin; superoxide; hydrogen peroxide.

INTRODUCTION

The diverse roles of free radicals in biological processes are of intense current interest. Within this field, cellular oxidative stress has been linked to numerous pathological conditions [1]: the search term "oxidative stress" retrieves currently about 16,000 articles in the PubMed (MEDLINE) database. In addition, the explosion of interest in nitric oxide as a physiologically important molecule [2] has introduced the related term "nitrosative stress." Both stress responses involve excess production of free radicals: superoxide ($O_2^{\cdot -}$, a precursor to hydrogen peroxide), and nitric oxide, which also can act together, yielding peroxynitrite and hence powerful free radical oxidants, nitrogen dioxide ($NO_2^{\cdot}$), hydroxyl ($^{\cdot}OH$), and carbonate ($CO_3^{\cdot -}$) radicals [3].

Despite wide interest, the identification of specific probes for oxidative and nitrosative stress is proving difficult. Lucigenin (LC^{2+}, bis-*N*-methylacridinium) is widely used as a chemiluminescent probe for superoxide radicals. The mechanism of lucigenin chemiluminescence probably involves addition of $O_2^{\cdot -}$ to the radical-cation $LC^{\cdot +}$ and production of *N*-methylacridone in an excited state via a dioxetane [4]. However, the production of $O_2^{\cdot -}$ by "redox cycling" the radical [Eq. (1)] has been demonstrated [5,6]:

$$LC^{\cdot +} + O_2 \rightleftarrows LC^{2+} + O_2^{\cdot -} \qquad (1)$$

The position and kinetics of equilibrium have not been measured. One report indicates an equilibrium constant $K_1 = 10^{-6}$ [7], whereas electrochemical studies [8] sug-

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gest $K_1 \sim 0.3$. Lucigenin is an analogue of methyl viologen, and the redox and kinetic properties of viologen radicals can be easily characterized by pulse radiolysis [9]. We have used this technique to define Eq. (1).

The most common luminescent probe for hydrogen peroxide is 2′,7′-dichlorofluorescin ($DCFH_2$), oxidized to the fluorescent product DCF. However, a catalyst is required [10]. We report the activity of cytochrome *c* in this role, relevant to the putative link between oxidative stress and apoptosis. A preliminary report has been published [11].

EXPERIMENTAL

Pulse radiolysis of aqueous solutions containing formate was used to generate superoxide, viologen, and lucigenin radicals [9] and solutions containing nitrite to generate $NO_2^{\cdot}$ [12]. Superoxide was generated enzymatically using hypoxanthine and xanthine oxidase [11]. Fluorescence was measured using a Perkin-Elmer LS-50B fluorimeter.

RESULTS AND DISCUSSION

The single-electron reduction potential of lucigenin $E(LC^{2+}/LC^{\cdot+})$ was measured by characterizing the redox equilibrium [Eq. (2)] with the known redox indicator benzyl viologen (BV^{2+}), which was established in microseconds [13]:

$$BV^{\cdot+} + LC^{2+} \rightleftarrows BV^{2+} + LC^{\cdot+} \qquad (2)$$

This indicated $E(LC^{2+}/LC^{\cdot+}) = -0.28$ V versus NHE, and hence K_1 is calculated to be of the order of 50 (because $E(O_2[1\ M]/O_2^{\cdot-}) = -0.19$ V [9]). This value is significantly greater than both earlier claims [7,8]. That $K_1 \gg 1$ is not in doubt, however, because by generating $LC^{\cdot+}$ in solutions of varying $[O_2]$, we observed directly reaction with O_2, Eq. (1) occurring in milliseconds to an equilibrium value. Representative transient absorptions are shown in Fig. 1. The exponential decay of $LC^{\cdot+}$ was linearly dependent on $[O_2]$ and indicated $k_1 = 3 \times 10^6\ M^{-1}\ s^{-1}$.

This rate constant is rather lower than those of other viologen radicals in reacting with oxygen [14], as might be expected from the higher reduction potential, but it is still a very fast reaction. If $K_1 \sim 50$, then k_{-1} (rate constant for reaction of $O_2^{\cdot-}$ with $LC^{\cdot+}$) is $\sim 6 \times 10^4\ M^{-1}\ s^{-1}$, a value ~50,000-fold lower than that for reaction of $O_2^{\cdot-}$ with superoxide dismutase. Hence, unless intracellular levels of LC^{2+} approach ~0.1 *M* (far lower extracellular concentrations are commonly used [15]), in the presence of normal levels of SOD, $O_2^{\cdot-}$ cannot be an important route of reduction of LC^{2+} to $LC^{\cdot+}$—the presumed key intermediate in chemiluminescence. Rather, flavoprotein reductases are likely reductants forming $LC^{\cdot+}$. There is then the complication of redox cycling, Eq. (1) occurring to *generate* $O_2^{\cdot-}$ in competition to $LC^{\cdot+}$ reacting with $O_2^{\cdot-}$ to form the dioxetane at a rate not yet characterized.

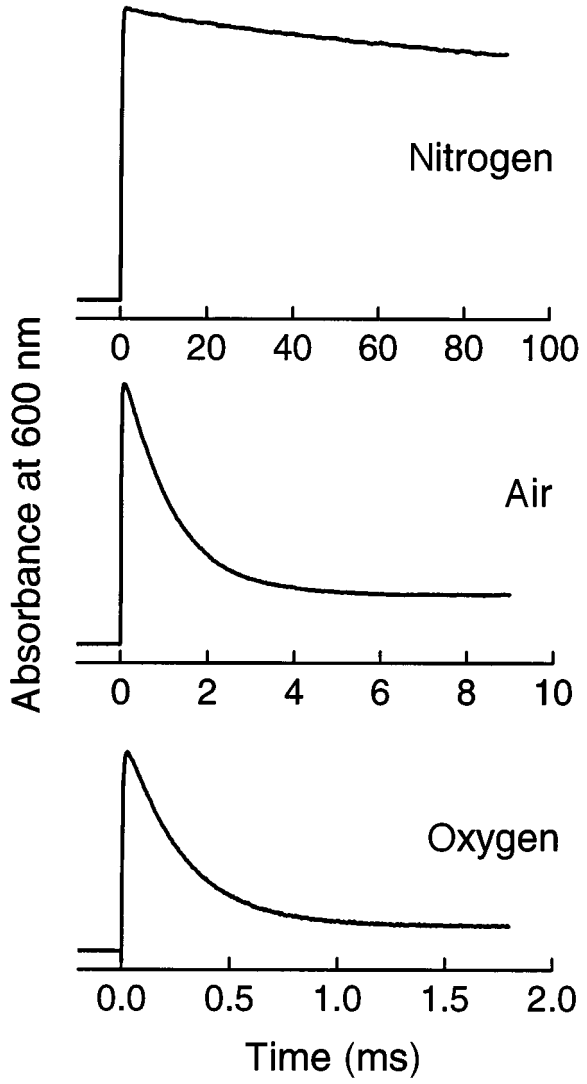

Fig. 1. Stability of the lucigenin radical-cation in nitrogen-, air- or oxygen-saturated aqueous solutions, observed by pulse radiolysis. Note the differing time scales.

The effects of cytochrome *c* on the fluorescence signal generated from $DCFH_2$ in solutions containing hypoxanthine and xanthine oxidase to generate $O_2^{\cdot-}$ at variable, steady rates were investigated [11]. Fig. 2 shows that in the presence of 10 nm cytochrome *c*, fluorescence

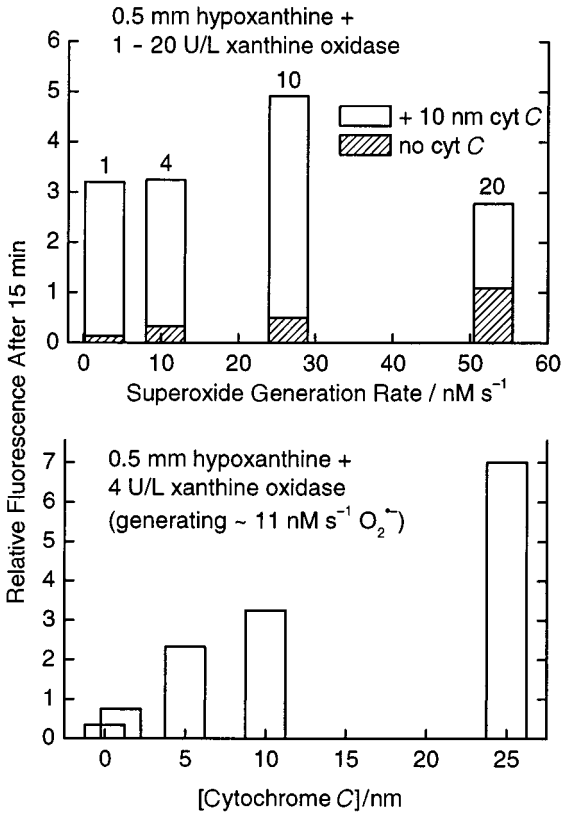

Fig. 2. Fluorescence signal intensities obtained from a solution of $DCFH_2$ (10 μM) with varying rates of superoxide generation with and without cytochrome *c* (10 nm), or at varying cytochrome *c* concentrations at a steady rate of radical generation.

hardly changed over a ~20-fold range of superoxide generation rate, whereas at a constant radical production rate, fluorescence was linearly dependent on cytochrome *c* over the range 1–25 nm. Clearly, DCF fluorescence in stressed cells containing $DCFH_2$ must often reflect the increase in cytosolic cytochrome *c* that occurs by transfer from the mitochondria in apoptotic cells [16,17].

Although even high concentrations of H_2O_2 do not oxidize $DCFH_2$ in the absence of a catalyst, radicals associated with nitrosative stress ($NO_2^{\cdot}$, $CO_3^{\cdot -}$), as well as $^{\cdot}OH$ radicals of course, oxidize $DCFH_2$ very rapidly. Thus pulse radiolysis observations of the formation of DCF via the intermediate radical $DCFH^{\cdot}$ from oxidation of $DCFH_2$ by $NO_2^{\cdot}$ showed radical formation occurred with a rate constant k_3 of $\sim 1 \times 10^7\ M^{-1}\ s^{-1}$:

$$NO_2^{\cdot} + DCFH_2 \rightarrow NO_2^- + DCFH^{\cdot} \qquad (3)$$

Glutathione thiyl radicals ($GS^{\cdot}$) also oxidized $DCFH_2$ at a broadly similar rate, although the position of the equilibrium [Eq. (4)] and its perturbation via the coupled "redox switch" [Eq. (5)] which then leads on to *generate* $O_2^{\cdot -}$ [Eq. (6)] [18] remains to be characterized and is the subject of current work.

$$GS^{\cdot} + DCHF_2 \rightleftarrows GSH + DCFH^{\cdot} \qquad (4)$$

$$GS^{\cdot} + GS^- \rightleftarrows (GSSG)^{\cdot -} \qquad (5)$$

$$(GSSG)^{\cdot -} + O_2 \rightarrow GSSG + O_2^{\cdot -} \qquad (6)$$

CONCLUSIONS

Characterizing the rates of reactions of radical intermediates in the chemistry of luminescent probes for oxidative and nitrosative stress, using kinetic spectrophotometry after generation by pulse radiolysis or trapping radicals and observing formation using EPR spectroscopy [11,19] are essential tools if these probes are to be used with confidence. There is no question that lucigenin has the propensity to *generate* the species to be measured and that the oxidation of leuco dyes such as $DCFH_2$ cannot be used reliably unless it can be shown that catalysis is saturated, that thiol status is not changing, or that nitrosative stress can be decoupled from oxidative stress.

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