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Original Contribution

Mechanism of oxidative conversion of Amplex® Red to resorufin: Pulse radiolysis and enzymatic studies

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ABSTRACT

Amplex® Red (10-acetyl-3,7-dihydroxyphenoxazine) is a fluorogenic probe widely used to detect and quantify hydrogen peroxide in biological systems. Detection of hydrogen peroxide is based on peroxidase-catalyzed oxidation of Amplex® Red to resorufin. In this study we investigated the mechanism of one-electron oxidation of Amplex® Red and we present the spectroscopic characterization of transient species formed upon the oxidation. Oxidation process has been studied by a pulse radiolysis technique with one-electron oxidants ($N_3^{\bullet}$, $CO_3^{\bullet-}$, $^{\bullet}NO_2$ and $GS^{\bullet}$). The rate constants for the Amplex® Red oxidation by $N_3^{\bullet}$ ($^2k = 2.1 \cdot 10^9\ M^{-1}s^{-1}$, at pH = 7.2) and $CO_3^{\bullet-}$ ($^2k = 7.6 \cdot 10^8\ M^{-1}s^{-1}$, at pH = 10.3) were determined. Two intermediates formed during the conversion of Amplex® Red into resorufin have been characterized. Based on the results obtained, the mechanism of transformation of Amplex® Red into resorufin, involving disproportionation of the Amplex® Red-derived radical species, has been proposed. The results indicate that peroxynitrite-derived radicals, but not peroxynitrite itself, are capable to oxidize Amplex® Red to resorufin. We also demonstrate that horseradish peroxidase can catalyze oxidation of Amplex® Red not only by hydrogen peroxide, but also by peroxynitrite, which needs to be considered when employing the probe for hydrogen peroxide detection.

1. Introduction

The generation of reactive oxygen species and reactive nitrogen species (ROS and RNS) is considered as an integral process of cell functioning in every living aerobic organism. ROS and RNS include an array of such chemical entities of different reactivities, as superoxide radical anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$), hydroxyl radical ($^{\bullet}OH$), nitrogen dioxide radical ($^{\bullet}NO_2$), carbonate radical anion ($CO_3^{\bullet-}$) and others. ROS and RNS have been proposed to play an important role in regulatory mechanisms, transmission of biochemical signals and in defense response against microbes, but their excessive production and/or insufficient detoxification can lead to an oxidative/nitrative damage through the ROS and RNS-induced modification of cellular components, including proteins, lipids and DNA [1]. The imbalance in generation and neutralization of ROS and RNS in living organisms, leading to higher steady state concentrations of ROS and RNS, is a widely described phenomenon named as an oxidative stress. This disturbed state of redox homeostasis, common during several diseases (e.g. atherosclerosis, cancer, neurodegenerative diseases, myocardial infarction), can cause irreversible damage and the exacerbation of pathological condition [2]. Development of reliable methods for ROS and RNS detection and quantification is, therefore, a matter of major importance.

Considering many limitations in ROS and RNS detection, caused by the reactivity of these short-lived species, different approaches to ROS and RNS detection were proposed. Among methods being developed recently, one of the most convenient is the ROS and RNS detection with the use of different fluorogenic probes. The main advantage of fluorescence measurements is high sensitivity

Abbreviations: $AR^{\bullet}$, Amplex® Red radical; CBA, coumarin-7-boronic acid; CH_3CN, acetonitrile; CO_2, carbon dioxide; $CO_3^{\bullet-}$, carbonate radical anion; COH, 7-hydroxycoumarin; DCFH, dichlorodihydrofluorescein; dtpa, (carboxymethylimino)bis (ethylenenitrilo)tetraacetic acid; e_{aq}^-, solvated electrons; FBBE, fluorescein benzyl boronate ester; Fc/Fc^+, ferrocene/ferrocenium redox couple; $GS^{\bullet}$, glutathionyl radical; GSH, glutathione; H_2O_2, hydrogen peroxide; HCO_3^-, bicarbonate; HRP, horseradish peroxidase; KSCN, potassium thiocyanate; $^{\bullet}NO_2$, nitrogen dioxide radical; N_2O, nitrous oxide; $N_3^{\bullet}$, azide radical; NaN_3, sodium azide; NaOH, sodium hydroxide; $^{\bullet}OH$, hydroxyl radical; $O_2^{\bullet-}$, superoxide radical anion; $ONOO^-$, peroxynitrite; $ONOOCO_2^-$, nitrosoperoxocarbonate; ROS, reactive oxygen species; RNS, reactive nitrogen species

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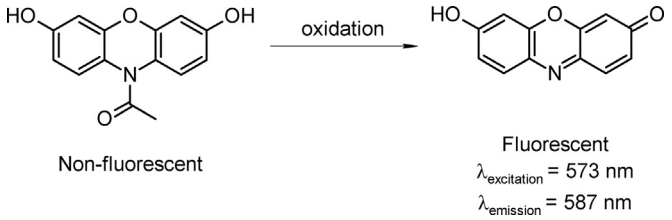

Fig. 1. Scheme of oxidative transformation of Amplex® Red to resorufin.

and the possibility of real-time non-invasive ROS/RNS detection.

Hydrogen peroxide is considered as one of the most important reactive oxygen species due to its particular role in cell functioning including cell signaling [3–6]. Thus, different fluorogenic probes were introduced (i.e. dihydrorhodamine 123, 2,7-dichlorodihydrofluorescein) to determine H_2O_2 concentration [7]. Despite the demonstrated propensity of those probes to artifactual oxidation and self-generation of H_2O_2 by the probes, their use for detection of H_2O_2 or ROS continues. An alternative approach of the use of reduced (dihydro) fluorescent compounds to detect H_2O_2 is to apply peroxidase-catalyzed oxidation of 10-acetyl-3,7-dihydroxyphenoxazine (Amplex® Red). Amplex® Red is a non-fluorescent and colorless compound that upon enzymatic oxidation is transformed into resorufin which is a highly absorbing and fluorescing compound (Fig. 1) [8]. Amplex® Red assay is widely used for specific and quantitative analysis of extracellular H_2O_2. There are, however, a few factors limiting its reliability or applicability. It has been recently shown that Amplex® Red can be oxidized by macrophages activated to produce peroxynitrite ($ONOO^-$) [9]. This should be taken into consideration when planning an experiment in which both H_2O_2 and $ONOO^-$ are formed simultaneously. Moreover, resorufin can undergo photosensitized reduction in the presence of electron donors (NADH or even Amplex® Red itself), and it can undergo oxidation in the presence of peroxidases what complicates data interpretation and quantitative analyses [10,11]. In the presence of suitable electron donors resorufin may undergo redox cycling (e.g. diaphorase-catalysed NAD(P)H oxidation) producing H_2O_2, leading to overestimation of the amount of this oxidant [12,13]. Despite those limitations, Amplex® Red-based assays remain a gold standard for detection and quantification of hydrogen peroxide in cell-free systems and in cellular systems, where the probe is used for determination of extracellularly released H_2O_2. Therefore, understanding the reactivity of Amplex® Red and the mechanism of its transformation into resorufin is crucial. While it has been proposed that oxidative conversion of Amplex® Red into resorufin by H_2O_2/HRP proceeds through one-electron oxidation of Amplex® Red to its radical followed by radical disproportionation to form resorufin [14], no studies aimed at understanding of the actual mechanism of this transformation have been published.

In this paper we present kinetic and spectroscopic characterization of transient species formed upon one-electron oxidation of Amplex® Red using pulse radiolysis technique. This is accompanied by the studies on peroxidase-catalyzed oxidation of Amplex® Red, indicating that in addition to hydrogen peroxide, peroxynitrite is also an oxidizing substrate in the HRP-catalyzed conversion of Amplex® Red to resorufin.

2. Experimental section

2.1. Materials

Amplex® Red was synthesized following the procedure described elsewhere [15]. Peroxynitrite was synthesized according to literature [16]. Peroxynitrite concentration was determined spectrophotometrically at 302 nm ($\varepsilon = 1.7 \cdot 10^3\ M^{-1}cm^{-1}$) [17]. The concentration of HRP was determined spectrophotometrically at 403 nm ($\varepsilon = 1.02 \cdot 10^5\ M^{-1}cm^{-1}$) [18]. All chemicals for synthesis and further experiments were obtained from Sigma-Aldrich and were of the highest grade available. Chloroform was purchased from Chempur Company (Poland). Coumarin-7-boronic acid (CBA) was synthesized as described previously [19,20]. Fluorescein benzyl boronate ester (FBBE) was synthesized by benzylation of fluorescein methyl ester with the use of 4-(iodomethyl)phenylboronic acid pinacol ester (Dębowska et al., in preparation). All aqueous solutions were prepared using water deionized by a Millipore Milli-Q system. Amplex® Red solutions were prepared immediately before each measurement and were kept in the dark to prevent it from oxidation caused by other than desired factors.

2.2. Pulse radiolysis

Pulse radiolysis experiments were performed using ELU-6E linear electron accelerator producing pulses of electrons of 2–17 ns duration. The actual dose absorbed by the sample with each pulse was determined using potassium thiocyanate (KSCN) dosimetry. N_2O-saturated aqueous solution of 0.01 M KSCN was irradiated and then the dose was calculated assuming the radiation chemical yield $G[(SCN)_2^{\bullet-}] = 6.2 \cdot 10^{-7}\ molJ^{-1}$ and the molar absorption coefficient at 475 nm $\varepsilon = 7.6 \cdot 10^3\ M^{-1}\ cm^{-1}$ [21]. Time-resolved absorption spectra were collected using system consisting of Osram XBO150W/OFR xenon lamp, Acton Research SpectraPro 275 monochromator, Hamamatsu Photonics R928 photomultiplier and Tektronix TDS540 oscilloscope. Detailed description of the whole data acquisition system can be found elsewhere [22,23].

Water radiolysis leads to the formation of several primary products (Reaction (1)). To convert solvated electrons (e_{aq}^-) to hydroxyl radicals ($^\bullet OH$) samples were saturated with N_2O prior to irradiation so Reaction (2) could occur [24,25].

$$H_2O \xrightarrow[\text{irradiation}]{} e_{aq}^-,\ H^\bullet,\ {}^\bullet OH,\ H_2,\ H_2O_2,\ HO_2^\bullet \qquad (1)$$

$$e_{aq}^- + N_2O \xrightarrow[H_2O]{} {}^\bullet OH + OH^- + N_2 \qquad k = 9.1 \cdot 10^9\ M^{-1}\ s^{-1} \qquad (2)$$

Hydroxyl radical is a very powerful oxidant with a redox potential of 2.31 V (vs. NHE) that can react through electron transfer, hydrogen abstraction or addition to unsaturated systems [26]. However, its high reactivity leads to unspecific oxidation processes. Therefore, for the purposes of the current study, $^\bullet OH$ radical has been converted into other, more selective oxidants.

We employed azide radical, $N_3^\bullet$, as a specific one-electron oxidant and $CO_3^{\bullet-}$, $^\bullet NO_2$ and glutathionyl radicals ($GS^\bullet$) as examples of biologically-relevant one-electron oxidants. We have chosen $N_3^\bullet$ as a specific one-electron oxidant because of high reduction potential of the redox couple $N_3^\bullet/N_3^-$ (1.33 V vs. NHE) and because it does not show any significant absorption above 300 nm enabling relatively easy spectroscopic characterization of transient species formed upon one-electron oxidation of compounds of interest. The azidyl radical was generated by radiolysis of 0.1 M sodium azide (NaN_3) aqueous solution saturated with N_2O containing a 10% addition of acetonitrile to increase the solubility of Amplex® Red. Formation of $^\bullet N_3$ radical in such system is described by Reaction (3) [25], which under the conditions used is completed within 10 ns.

$$^\bullet OH + N_3^- \rightarrow OH^- + {}^\bullet N_3 \qquad k = 1.2 \cdot 10^{10}\ M^{-1}\ s^{-1} \qquad (3)$$

The carbonate radical anion was generated by a radiolysis of aqueous solution containing 0.25 M CO_3^{2-}, 0.25 M HCO_3^- and 10% (by vol.) acetonitrile and saturated with N_2O. Generation of $CO_3^{\bullet-}$