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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 $N_3^{\bullet}$ ($^2k = 2.1 \cdot 10^9 \text{ M}^{-1}\text{s}^{-1}$, at pH = 7.2) and $CO_3^{\bullet-}$ ($^2k = 7.6 \cdot 10^8 \text{ M}^{-1}\text{s}^{-1}$, 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.

Graphical Abstract

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Keywords

Amplex[®] Red; hydrogen peroxide; peroxynitrite; pulse radiolysis; coumarin boronic acid

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 ($\text{O}_2^{\bullet-}$), hydrogen peroxide (H_2O_2), peroxynitrite (ONOO^-), hydroxyl radical ($\bullet\text{OH}$), nitrogen dioxide radical ($\bullet\text{NO}_2$), carbonate radical anion ($\text{CO}_3^{\bullet-}$) 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 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 H₂O₂. 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 and ONOO⁻ are formed simultaneously. Moreover, resorufin can undergo H₂O₂ 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 H₂O₂, 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 H₂O₂. 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 H₂O₂/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.

Experimental section

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 ($\epsilon = 1.7 \cdot 10^3 \text{ M}^{-1} \text{ cm}^{-1}$) [17]. The concentration of HRP was determined spectrophotometrically at 403 nm ($\epsilon = 1.02 \cdot 10^5 \text{ M}^{-1} \text{ cm}^{-1}$) [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 (K. D bowska, D. D bski, B. Michałowski, J. Adamus, A. Sikora; manuscript 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.

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. N₂O-saturated aqueous solution of 0.01 M KSCN was irradiated and then the dose was calculated assuming the radiation chemical yield $G[(\text{SCN})_2^{\bullet-}] = 6.2 \cdot 10^{-7} \text{ mol} \cdot \text{J}^{-1}$ and the molar absorption coefficient at 475 nm $\epsilon = 7.6 \cdot 10^3 \text{ M}^{-1} \text{cm}^{-1}$ [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 ($\bullet\text{OH}$) samples were saturated with N₂O prior to irradiation so reaction 2 could occur [24].

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, $\bullet\text{OH}$ radical has been converted into other, more selective oxidants.

We employed azide radical, $\text{N}_3^\bullet$, as a specific one-electron oxidant and $\text{CO}_3^{\bullet-}$, $\bullet\text{NO}_2$ and glutathionyl radicals ($\text{GS}^\bullet$) as examples of biologically-relevant one-electron oxidants. We have chosen $\text{N}_3^\bullet$ as a specific one-electron oxidant because of high reduction potential of the redox couple $\text{N}_3^\bullet/\text{N}_3^-$ (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 N₂O containing a 10% addition of acetonitrile to increase the solubility of Amplex[®] Red. Formation of $\bullet\text{N}_3$ radical in such system is described by reaction 3, which under the conditions used is completed within 10 ns.

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 $\text{CO}_3^{\bullet-}$ radical in such a system is described by reactions 4 and 5. The reduction potential of $\text{CO}_3^{\bullet-}/\text{CO}_3^{2-}$ couple is 1.78 V (vs. NHE) [27, 28].

Nitrogen dioxide was generated by the irradiation of N_2O -saturated aqueous solution of 10 mM NaNO_2 , 50 mM NaNO_3 (containing 5% CH_3CN and 50 mM phosphate buffer, pH 7.5). In such system hydroxyl radicals and hydrated electrons are converted into $\bullet\text{NO}_2$ in reactions 2 and 6–8. $\text{NO}_2^{\bullet}$ has mild oxidizing properties ($E^\circ \text{NO}_2^{\bullet}/\text{NO}_2^-$ couple is 1.04 V vs. NHE), it has an absorption spectrum with weak absorption band in the visible range [29, 30].

Glutathione radical is also a very important oxidizing species since glutathione is one of the most common antioxidants in human body taking part in many cellular redox reactions [32]. Glutathione radical ($\text{GS}^{\bullet}$) was generated by the radiolysis of the aqueous solution containing 2.5 mM glutathione, 1.6 M CH_3OH , 10% CH_3CN and 60 mM phosphate buffer, according to reactions 9–11. Glutathione radical shows mild oxidative properties (E° of $\text{GS}^{\bullet}, \text{H}^+/\text{GSH}$ couple is 0.94 V vs. NHE) [33, 34].

All the pulse radiolysis experiments were carried out at room temperature in a quartz cell with the optical path of 1 cm. For the determination of rate constant of radical disproportionation, the second-order decay kinetics equation (12) was fitted to the kinetic traces of the decay of the intermediate radical.

$$\Delta A t = \frac{B \cdot \Delta A_n \cdot t + 1}{B \cdot t + \frac{1}{\Delta A_0}} \quad (12)$$

where:

$$B = \frac{2 \cdot k_{dis}}{\epsilon_s \cdot l} \quad (13)$$

and t is the time of reaction, k_{dis} is the rate constant of the radical disproportionation, ϵ_s is the molar absorption coefficient of the species observed and l is the optical path length of the cuvette.

Amplex[®] Red oxidation by peroxynitrite

Oxidation of Amplex[®] Red by peroxynitrite was studied spectroscopically by monitoring the formation of resorufin with the use of stopped flow technique. The solution of Amplex[®] Red (200 μM) containing 50 mM phosphate buffer (pH = 7.4), 100 μM dtpa and 10% CH_3CN was mixed dynamically with solution of peroxynitrite (40 μM) in 1 mM NaOH and the time evolution of absorption spectra were recorded. This has been compared with the kinetics of peroxynitrite self-decomposition under the same conditions. Absorption spectra were obtained with Applied Photophysics SX20 Stopped-Flow Spectrometer equipped with 150W air-cooled Xe arc lamp and photodiode array detector. Data were collected using Pro-Data SX software version 2.1.0.

HRP-catalyzed oxidation of Amplex[®] Red by peroxynitrite; the influence of bicarbonate and the presence of boronate

Relationship between the yield of oxidation of Amplex[®] Red by peroxynitrite in the absence and presence of the horseradish peroxidase enzyme and bicarbonate (HCO_3^-) was determined by measuring the fluorescence intensity due to resorufin formed. To the three series of 50 mM phosphate buffer solutions of 20 μM Amplex[®] Red, one containing 500 nM HRP, second containing 25 mM HCO_3^- , third without additives, small amount of peroxynitrite (0.1–1 μM) was added as bolus by rapid mixing. Fluorescence was measured at room temperature in a 1×1 cm quartz cuvette with the Varian Cary Eclipse fluorescence spectrometer; excitation wavelength was set to 573 nm, emission wavelength was monitored at 586 nm, excitation and emission slit were set to 2.5 nm and 2.5 nm respectively.

Fluorescence intensity was measured 2 minutes after the addition of peroxynitrite to the sample.

Kinetics of resorufin formation in the aqueous solutions containing 100 μM Amplex[®] Red, 20 μM H_2O_2 or ONOO^- , 25 mM phosphate buffer, 50 μM dtpa, 5% CH_3CN for different HRP concentrations was determined with the use of stopped-flow technique described in the previous section. The concentration of resorufin formed was calculated assuming the molar absorption coefficient at 571 nm of $6.3 \cdot 10^4 \text{ M}^{-1}\text{cm}^{-1}$ [9].

The influence of boronate (coumarin-7-boronic acid, CBA) on the extent of oxidation of Amplex[®] Red by hydrogen peroxide and peroxynitrite in HRP-catalyzed system was determined by measuring the fluorescence intensity of samples containing 100 μM of Amplex[®] Red, 0 or 100 μM CBA, 500 nM HRP, 50 mM phosphate buffer (pH = 7.4), 100 μM dtpa, 10% CH_3CN after bolus addition of 2–10 μM of H_2O_2 or ONOO^- . For resorufin measurements the excitation wavelength was set to 573 nm and the emission spectra were collected in the range from 578 to 700 nm, excitation and emission slit were both set to 2.5 nm. To measure 7-hydroxycoumarin (COH, product of CBA oxidation by peroxynitrite) excitation wavelength was set to 370 nm, emission spectra were collected in the range from 378 to 570 nm, excitation and emission slits were both set to 2.5 nm. Fluorescence intensity was measured 2 minutes after the addition of oxidant to the sample.

Results and Discussion

Acid-base and redox properties of Amplex[®] Red

To understand the molecular transformations of Amplex[®] Red upon oxidation, its protonation status under the experimental conditions used needs to be established. Therefore, we firstly determined the pK_a values of Amplex[®] Red by spectrophotometric titration, as shown in Fig. S1, Supplemental Data. While there was no significant change in absorption spectra within the pH range of 5.5 to 7, further increase in pH leads to increase in absorption between 240 nm and 340 nm, indicating a change in the protonation status of the probe. The assignment of the species present at pH 5.5 to a neutral (non-deprotonated) molecule has been established by similarity of the observed spectrum with the spectra of Amplex[®] Red measured in ethanol (Fig. S2, Supplemental Data). Amplex[®] Red possesses two phenolic hydroxyl groups, which are expected to undergo deprotonation with increasing pH of the solution. As can be seen in Fig. S1, (Supplemental Data), the titration curve is best fitted with the theoretical titration function assuming the two pK_a values of 8.5 and 9.6. This indicates that at physiological pH (7.4) Amplex[®] Red exists predominantly (~93%) in the neutral form, while at higher pH (> 8.5), the monoanionic and/or dianionic forms will predominate. As non-deprotonated phenolic compounds are typically difficult to oxidize, even low amount of anion may contribute to oxidation reactions in the case of relatively weak oxidants.

Pulse radiolysis - reaction of Amplex[®] Red with $\text{N}_3^\bullet$ radical

To characterize the mechanism of one-electron oxidation of Amplex[®] Red, azidyl radical ($\text{N}_3^\bullet$) was used as a specific one-electron oxidant (see Experimental section). One-electron

oxidation of Amplex[®] Red at pH 7.2 is accompanied by the formation of a species absorbing at 310 nm and 820 nm (Fig. 2A). This species is not stable and upon its decay a new species, absorbing at 630 nm is formed within 100 μs after the pulse. Within another 1 ms this species also decays, and a stable product, characterized by a strong absorption band with maximum at 570 (±5) nm is formed (Fig. 2B). This species has been assigned to resorufin ($\lambda_{\text{max}} = 573$ nm), based on fluorescence measurements performed after the pulse radiolysis experiments. At pH 10.5 the peak at 310 nm is broadened and its maximum is shifted to 370 nm (Fig. 3A). This change in the spectra may be caused by the deprotonation of the generated Amplex[®] Red radical species, hence we have determined the influence of pH on the spectra obtained during the oxidation of 200 μM Amplex[®] Red in the solution containing 0.1 M NaN₃. Dependence of the maximum absorbance measured at 370 nm on the pH of the solution is shown in the Fig. 3B. Theoretical function was fitted to the titration curve yielding the pK_a of Amplex[®] Red radical species equal to 8.0. Therefore, the first species formed during the oxidation of Amplex[®] Red with N₃[•] at pH = 7.2 (Fig. 2) may be ascribed to radical cation or neutral radical, rather than the radical anionic form (See Supplemental Fig. S3 for the structures of possible acid-base forms of Amplex[®] Red and its one-electron oxidation product).

Pulse radiolysis - reaction with carbonate radical anion and nitrogen dioxide radical

One of the aims of this study was to determine if radicals formed from the decomposition of peroxynitrite (reaction 14) or its reaction with CO₂ (reaction 15 and 16) are able to oxidize Amplex[®] Red. Therefore, we tested if carbonate radical anion (CO₃^{•-}) and nitrogen dioxide ([•]NO₂) can produce similar products upon reaction with Amplex[®] Red as observed for azidyl radical. *In vivo*, carbonate radical anion can be formed via the reaction of peroxynitrite (ONOO⁻) with carbon dioxide (CO₂) leading to the formation of transient nitrosoperoxocarbonate (ONOOCO₂⁻), decomposing to carbonate radical anion (CO₃^{•-}) and nitrogen dioxide ([•]NO₂) (equations 15–16) [36, 37].

Published reports indicate that peroxynitrite-derived radical species may be responsible for the oxidation of many fluorescent probes, rather than ONOO⁻ itself [38–40]. Thus we tested the reactivity of both CO₃^{•-} and [•]NO₂ towards Amplex[®] Red.

Due to experimental limitations, pulse radiolysis studies of carbonate radical anion are limited to pH > 10. As pK_a values of Amplex[®] Red are equal to 8.5 and 9.6, it can be

assumed that in the pulse radiolysis experiments with carbonate (pH = 10.3) Amplex[®] Red exists predominantly in the dianionic form. Similarly to the data obtained with azide radical, carbonate radical anion reacted with the probe giving a light-absorbing product (Fig. 4A, solid circles) with two main absorption bands – one narrow peak around 310–430 nm with the maximum at 370 nm, second peak starting from 650 nm, with the maximum at 820 nm. This spectrum can be assigned to the Amplex[®] Red neutral radical or radical anion as the abstraction of one electron by carbonate radical anion is the first reaction occurring. In the longer timescale (ca. 90 μs) a disappearance of those two peaks at 370 and 820 nm with a simultaneous buildup of an absorption band with maximum at 630 nm can be observed (Fig. 4A, open triangles). As this reaction follows second-order kinetics, the most likely reaction is the disproportionation of two radicals to form the parent compound (Amplex[®] Red) by the reduction and a second product by the oxidation of this radical species.

The secondary intermediate absorbing between 550 – 650 nm is also unstable and decays in a subsequent reaction with the formation of the product strongly absorbing at 570 nm (Fig. 4B). We tentatively assigned this reaction to a hydrolysis process that leads to the formation of the final and stable product – resorufin, with the characteristic absorption spectrum, as described in the literature (Fig. 4B, solid squares) [8].

Nitrogen dioxide ($\bullet\text{NO}_2$) is another reactive species that undergoes typical radical reactions including electron transfer, hydrogen abstraction and addition to double bonds [36]. Generation of $\bullet\text{NO}_2$ *in vivo* can be catalyzed by myeloperoxidase, eosinophil peroxidase and other oxidants [41–43]. Nitrogen dioxide was able to oxidize Amplex[®] Red producing the same radical intermediate product $\text{AR}^\bullet$, but the observed yield of that product was very low and the transient absorption spectrum was only slightly above the noise level of the apparatus. We have also observed the formation of secondary transient species absorbing at 630 nm. The decay of that species was observed within 10 ms with concomitant formation of resorufin (Figure S4). All of these data support our conclusion that oxidation of Amplex Red by nitrogen dioxide proceeds via one-electron transfer, but with the formation of low steady-state concentration of Amplex Red radical. Additionally, we have compared the yield of resorufin formed in the reaction of $\text{N}_3^\bullet$ and $\bullet\text{NO}_2$ radicals (data not shown). The observed yield of resorufin was the same for both oxidants, suggesting the occurrence of equilibrium during the first step electron transfer, following by rapid conversion of the Amplex Red radical into secondary intermediate and eventually resorufin:

Glutathione radical (GS[•]) is an important biologically-relevant oxidizing species as glutathione is one of the most common antioxidants in human body taking part in many cellular redox reactions [32]. Although it has been shown that GS[•] radical is capable of oxidizing dichlorodihydrofluorescein (DCFH) probe [44], we did not obtain any results indicating oxidation of Amplex[®] Red (200 μM) by glutathione radical in pulse radiolysis experiments. On the contrary, Amplex[®] Red radical can be reduced by GSH (see below).

Kinetics

Kinetic traces for the three chosen wavelengths (820 nm for the primary radical species, 630 nm for the secondary oxidation product, and 570 nm for resorufin) obtained from the experiment on oxidation of Amplex[®] Red by CO₃^{•-} are a good example of consecutive reactions with spectroscopically and kinetically-resolved intermediates (Fig. 4C).

For carbonate radical anion and azidyl radical a series of the pseudo-first order buildup traces of the primary radical absorption bands in the presence of different (50–250 μM) Amplex[®] Red concentrations were collected. From those data the second order rate constants of oxidation were determined and they equal $(2.1 \pm 0.1) \cdot 10^9 \text{ M}^{-1}\text{s}^{-1}$ for N₃[•] at pH = 7.2 (Fig. 5) and $(7.6 \pm 0.3) \cdot 10^8 \text{ M}^{-1}\text{s}^{-1}$ for CO₃^{•-} at pH = 10.3 (Fig. S5, Supplemental Data).

Determination of Amplex[®] Red redox properties

Redox properties of Amplex[®] Red were determined with the use of cyclic voltammetry (see Supplemental Data for methodological details) and pulse radiolysis techniques.

Voltammogram of Amplex Red oxidation in CH₃CN and consecutive reduction is shown in the Fig. S6 (Supplemental Data). Amplex[®] Red showed oxidation peak at 0.67 V (vs. Fc/Fc⁺) and reduction peak at -0.05 V (vs. Fc/Fc⁺). A significant difference in potentials between oxidation and reduction peaks and the shape of voltammogram suggest that oxidation of Amplex[®] Red is an irreversible process at the scan rate used.

The reduction potential of Amplex[®] Red radical in aqueous solution was determined by pulse radiolysis. The dependence of the pseudo-first order rate constant of the reaction of Amplex[®] Red with azidyl radical on the initial concentration of Amplex[®] Red has a non-zero intercept, indicating a redox equilibrium (reaction 20):

The observed rate constant of approach to that equilibrium can be expressed by equation 21:

$$k_{obs} = k_f AR + k_r N_3^{-} \quad (21)$$

The plot has a slope (k_f) of $2.1 \cdot 10^9 \text{ M}^{-1}\text{s}^{-1}$ and an intercept of $1.2 \cdot 10^5 \text{ s}^{-1}$ (if the reaction is reversible the intercept equals $k_f[N_3^{-}]$, and k_r $1.2 \cdot 10^6 \text{ M}^{-1}\text{s}^{-1}$). The reversibility of reaction 20 was confirmed in another kinetic experiment. For azidyl radical a series of the pseudo-first order buildup traces of Amplex[®] Red radical were collected at constant concentration of

Amplex[®] Red (250 μ M) and different (0.01–0.1 M) concentrations of NaN_3 . From those data the k_r rate constants was determined to be equal $(1.1 \pm 0.3) \cdot 10^6 \text{ M}^{-1}\text{s}^{-1}$ (Fig. S7, Supplemental Data). From those data the equilibrium constant K_{eq} and the reduction potential of Amplex[®] Red radical $E^\circ(\text{AR}^\bullet/\text{AR})$ at pH 7.2 can be calculated and those values are equal $K_{\text{eq}} = (1.9 \pm 0.6) \cdot 10^3$; and $E^\circ(\text{AR}^\bullet/\text{AR}) = 1.14 \text{ V}$.

The rate constant of Amplex[®] Red radical disproportionation

The comparison of the dose dependence of absorbance of $\text{SCN}_2^{\bullet-}$ with the one of Amplex[®] Red-derived radical in the same conditions, enabled us to determine its molar absorption coefficient (Supplemental Figure S8). The calculated molar absorption coefficient of Amplex[®] Red-derived radical at pH 7.2 is equal to $2.8 \cdot 10^3 \text{ M}^{-1}\text{cm}^{-1}$ at 820 nm and by using this value, we determined the rate constant for $\text{AR}^\bullet$ disproportionation equal to $(2.8 \pm 0.1) \cdot 10^8 \text{ M}^{-1}\text{s}^{-1}$ (pH = 7.2).

Amplex[®] Red oxidation by peroxynitrite

As presented above, the results of pulse radiolysis experiments indicate that Amplex[®] Red can be oxidized by relatively strong one-electron oxidants including carbonate radical anion and nitrogen dioxide. Therefore, we also tested if peroxynitrite is able to oxidize Amplex[®] Red under physiological conditions as it yields one-electron oxidants during its decomposition. Addition of 20 μ M peroxynitrite to a buffered solution of 100 μ M Amplex[®] Red led to the formation of resorufin, observable as a build-up of an absorption band with maximum at 573 nm (Fig. 6A). The reaction yielded 2.6 μ M of resorufin. To get insight into mechanism of oxidation of Amplex[®] Red by ONOO^- we investigated the kinetics of the reaction. Oxidation of Amplex[®] Red occurred on the timescale of several seconds what indicated that the process of oxidation is rather slow. Kinetic traces of Amplex[®] Red oxidation by ONOO^- and peroxynitrite self-decomposition at the physiological pH (7.4) are shown in the Fig. 6B. These two processes occur with very similar kinetics ($k_{\text{obs}} = 0.20 \text{ s}^{-1}$ for ONOO^- decomposition vs. $k_{\text{obs}} = 0.21 \text{ s}^{-1}$ for resorufin formation) suggesting that peroxynitrite does not react directly with Amplex[®] Red but the products of peroxynitrite decomposition can transform Amplex[®] Red into resorufin.

Previously we have shown that boronates (boronic acids and esters) react directly with peroxynitrite forming corresponding phenolic products [45]. Therefore, we tested if Amplex[®] Red can affect the yield of boronate oxidation. We reasoned that if Amplex[®] Red reacts directly with ONOO^- , it would compete with boronate probe leading to lower extent of boronate oxidation. However, if Amplex[®] Red does not react directly with ONOO^- , it should not affect the yield of boronate oxidation even at highest concentrations tested. We used fluorescein benzylboronate ester (FBBE) as a boronate probe, as its oxidation product can be easily resolved spectrophotometrically ($\lambda_{\text{max}} = 490 \text{ nm}$) from resorufin ($\lambda_{\text{max}} = 593 \text{ nm}$) and it reacts directly with ONOO^- with the second-order rate constant $^2k = (2.8 \pm 0.1) \cdot 10^5 \text{ M}^{-1}\text{s}^{-1}$ (K. D bowska, D. D bski, B. Michałowski, J. Adamus, A. Sikora; manuscript in preparation). Results indicated that Amplex[®] Red (25–100 μ M) did not affect peroxynitrite-induced oxidation of boronate probe FBBE (25 μ M) (Fig. S9, Supplemental Data). The yield of product formed from FBBE did not change significantly. However, small amounts of resorufin were formed from Amplex[®] Red oxidation, which is expected due to formation of

radical products *via* the minor, radical pathway of FBBE oxidation by peroxynitrite [46]. The data obtained from this competition experiment indicate that Amplex[®] Red does not react directly with peroxynitrite or the rate constant of the reaction is more than one order of magnitude lower than of the reaction of peroxynitrite with FBBE. These results are in agreement with the conclusions of the stopped flow kinetic experiments.

Another biologically-relevant oxidizing species, hypochlorous acid (HOCl), which is a two-electron oxidant, is also able to oxidize Amplex[®] Red at pH 7.4 but under the experimental conditions used the yield of resorufin is relatively low, not exceeding 10% (Fig. S10, Supplemental Data).

Influence of HRP on the AR reaction with peroxynitrite

We also checked if the horseradish peroxidase enzyme used commonly as a catalyst in the Amplex[®] Red/hydrogen peroxide assay may affect the yield of the Amplex[®] Red/ONOO⁻ reaction. Addition of the small amounts of peroxynitrite to the solution of Amplex[®] Red without HRP caused only a slight oxidation of the compound to resorufin (Fig. 7). The addition of decomposed peroxynitrite to the aqueous solution of Amplex[®] Red did not result in probe oxidation indicating that short-lived radicals formed from peroxynitrite are involved in Amplex[®] Red oxidation (data not shown). In the presence of 25 mM bicarbonate (resulting in approximately 1.8 mM CO₂ concentration) the yield of oxidation was slightly higher (1.9-fold increase). Addition of 0.5 μM HRP resulted in a 5.8-fold increase in the yield of resorufin indicating that the oxidation occurs more efficiently in the presence of the enzyme, suggesting that HRP can play a catalytic role in this reaction (Fig. 7). This leads to the conclusion that the Amplex[®] Red/HRP assay is not completely specific for H₂O₂ and other oxidants of peroxidase (e.g., peroxynitrite) may contribute to HRP-catalyzed probe oxidation.

The comparison of the influence of HRP on the rate and yield of Amplex[®] Red oxidation by hydrogen peroxide and peroxynitrite is shown in Fig. 8 and Fig. 9. When HRP was not present addition of hydrogen peroxide to the solution of Amplex[®] Red did not cause its oxidation. Addition of the same amount of peroxynitrite led to resorufin formation with approximately 13% yield (about 2.6 μM of resorufin was formed from 20 μM of ONOO⁻) and with approximately 36% yield (about 7.2 μM of resorufin was formed from 20 μM of ONOO⁻) when 400 nM of HRP was present. Clearly, HRP catalyzes the oxidation of Amplex[®] Red by both oxidants, however the catalytic oxidation by H₂O₂ is both faster and gives a higher yield of resorufin.

The experimental rate constant in such a system can be expressed by equation 22:

$$k_{\text{exp}} = k_0 + k_{\text{cat}} \cdot C_{\text{HRP}} \quad (22)$$

where k_0 is the rate constant of resorufin formation in the absence of peroxidase and k_{cat} is the catalytic rate constant. The determined values of k_{cat} for the HRP-catalyzed oxidation of Amplex[®] Red to resorufin are equal to $7.3 \cdot 10^6 \text{ M}^{-1} \text{ s}^{-1}$ and $1.9 \cdot 10^6 \text{ M}^{-1} \text{ s}^{-1}$ for hydrogen peroxide and peroxynitrite, respectively. Although the catalytic rate constant for hydrogen

peroxide is approximately 4 times higher than for peroxynitrite, these results indicate the lack of selectivity of the assay for H_2O_2 in systems where peroxynitrite may be also formed.

As the value of the catalytic rate constant of HRP-mediated oxidation of Amplex[®] Red by ONOO^- is close to the rate constant of the reaction of boronates with ONOO^- , we tested if addition of the excess of boronates over peroxidase can be used to prevent peroxynitrite-dependent probe oxidation. As a boronate competitor, coumarin-7-boronic acid (CBA) was used due to its high reactivity towards peroxynitrite ($^2k = 1.1 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$) [20], low reactivity towards hydrogen peroxide ($^2k = 1.5 \text{ M}^{-1}\text{s}^{-1}$) [20], and fluorescence properties of the oxidation product – 7-hydroxycoumarin ($\lambda_{\text{ex}} = 370 \text{ nm}$, $\lambda_{\text{em}} = 455 \text{ nm}$). CBA presence (100 μM) in the solution containing Amplex[®] Red (100 μM) and HRP (0.5 μM) did not affect the oxidation of Amplex[®] Red when hydrogen peroxide was used as an oxidant, but significantly reduced the resorufin fluorescence when peroxynitrite was added as bolus (Fig. 10A, B). Small amounts of resorufin formed when peroxynitrite was added may be caused by the formation of radicals *via* the minor pathway of CBA oxidation, similarly as it was in the case of FBBE oxidation described above. Fluorescence properties of 7-hydroxycoumarin allow for simultaneous monitoring of its formation resulting from oxidation by peroxynitrite (Fig. 10C).

Conclusions

Peroxidase-catalyzed oxidation of Amplex[®] Red by hydrogen peroxide is a *gold standard* for measurements of hydrogen peroxide formation in cell-free systems or its release from cells into the extracellular medium. While it has been used as a specific assay for hydrogen peroxide for over a decade, the mechanism of its oxidation has not been reported and the selectivity was typically assumed based on specificity of peroxidase to hydrogen peroxide, rather than determined experimentally.

The data presented in this paper indicate that one-electron oxidation of Amplex[®] Red (**1**) leads to the formation of resorufin (**4**) as a final product, through the disproportionation of Amplex[®] Red-derived radical species (**2**) with subsequent hydrolysis of N-acetylresorufin (**3**), as shown in Figure 11. We assume that one electron oxidation of Amplex[®] Red leads to the formation of neutral, phenoxyl-type radical (**2**). The resonance stabilization is enabled by gradual flattening of the structure during stepwise oxidation, as indicated by the results of quantum chemical-based calculations of geometries of the probe, product and reaction intermediates (Fig. S11). The relative reactivity of different oxidants towards Amplex[®] Red, with $\text{GS}^\bullet$ non-reactive towards the probe, indicates that only strong one-electron oxidants are capable of probe oxidation.

We also show that peroxynitrite-derived strong one-electron oxidant, carbonate radical anion, can efficiently oxidize Amplex[®] Red dianionic form with high second-order rate constant $(7.6 \pm 0.3) \cdot 10^8 \text{ M}^{-1}\text{s}^{-1}$. Although the experimental limitation prevented us from determination of this rate constant at more physiological pH (7.2), this was possible in case of azidyl radical ($\text{N}_3^\bullet$). As the reduction potential of $\text{N}_3^\bullet$ is 0.3 V lower than of $\text{CO}_3^{\bullet-}$, we assume that $\text{CO}_3^{\bullet-}$ can also efficiently oxidize the probe at physiological pH. This is

supported by the observed increase of the yield of resorufin in the presence of bicarbonate during oxidation of Amplex[®] Red by bolus peroxynitrite.

The estimated value of reduction potential of Amplex Red radical $E(\text{AR}^\bullet/\text{AR}) = 1.14 \text{ V}$ suggests that GSH should be able to reduce Amplex Red radical. In fact, we tested the effects of glutathione on the HRP-catalyzed oxidation of Amplex Red to resorufin by H_2O_2 , and observed concentration-dependent inhibition of resorufin formation (Figure S12). Thus, we conclude that intracellularly Amplex Red radical can be reduced by GSH, preventing resorufin formation. As the presence of superoxide dismutase (SOD) in the reaction mixture led to increase in the yield of resorufin (Fig. S12), we also conclude that superoxide (generated from $\text{GSSG}^{\bullet-}$ formed upon the reaction of $\text{GS}^\bullet$ with GS^-) is able to reduce $\text{AR}^\bullet$. Other biologically relevant reductants (e.g., ascorbate) are also expected to reduce $\text{AR}^\bullet$ to AR.

We have also demonstrated that peroxynitrite-dependent oxidation of Amplex[®] Red is due to ONOO^- -derived radical oxidants rather than by ONOO^- itself. Interestingly, the peroxynitrite-induced oxidation of Amplex[®] Red can be catalyzed by horseradish peroxidase leading to a significant increase in the yield of resorufin. This is consistent with the reports showing HRP-catalyzed oxidation of phenolic compounds by peroxynitrite [51,52] and our previous report on oxidation of Amplex[®] Red under the conditions of co-generated fluxes of $\bullet\text{NO}$ and $\text{O}_2^{\bullet-}$ [9]. As a consequence, results obtained with H_2O_2 /HRP assay might overestimate the amount of H_2O_2 if peroxynitrite is present in the investigated system and this fact must be taken into consideration as the catalytic rate constants for both oxidants are within the same order of magnitude. The requirement of catalysis by HRP is clearly not sufficient for identification of the oxidant, and the effects of catalase and boronate on the yield of Amplex[®] Red conversion to resorufin should always be tested to establish the contribution of H_2O_2 and ONOO^- to the total signal detected. The data shown in Figure 10 suggest that the combination of CBA probe with Amplex Red and HRP can be used for simultaneous determination of the amounts of hydrogen peroxide and peroxynitrite.

While Amplex[®] Red remains a valuable tool for detection and quantification of hydrogen peroxide, the limitations indicated here and in previous reports (e.g. probe photo-oxidation, photosensitization by resorufin) should be taken into account during experimental design and data interpretation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AR[•]	Amplex [®] Red radical
CBA	coumarin-7-boronic acid
CH₃CN	acetonitrile
CO₂	carbon dioxide
CO₃^{•-}	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[•]	glutathionyl radical
GSH	glutathione
H₂O₂	hydrogen peroxide
HCO₃⁻	bicarbonate
HRP	horseradish peroxidase
KSCN	potassium thiocyanate
[•]NO₂	nitrogen dioxide radical
N₂O	nitrous oxide
N₃[•]	azide radical
NaN₃	sodium azide
NaOH	sodium hydroxide
[•]OH	hydroxyl radical
O₂^{•-}	superoxide radical anion
ONOO⁻	peroxynitrite
ONOO₂⁻	nitrosoperoxocarbonate
ROS	reactive oxygen species
RNS	reactive nitrogen species

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Highlights

1. Mechanism of 1-electron oxidation of Amplex Red has been studied by pulse radiolysis.
2. One-electron oxidation of Amplex Red leads to the formation of fluorescent resorufin.
3. HRP catalyzes oxidation of Amplex Red by hydrogen peroxide and peroxynitrite.
4. In the absence of HRP peroxynitrite-derived radicals oxidize Amplex Red to resorufin.

Figure 1.
Scheme of oxidative transformation of Amplex[®] Red to resorufin.

Figure 2.

Transient absorption spectra obtained by pulse radiolysis of Amplex[®] Red (200 μM) in a N₂O-saturated aqueous solution containing 0.1 M NaN₃ (pH = 7.2) and 10% CH₃CN **A:** spectra recorded 5 μs (solid circles) and 50 μs (open triangles) after the electron pulse; **B:** spectra recorded 200 μs (open triangles) and 1 ms (solid squares) after the electron pulse. Arrows indicate the direction of spectral evolution over time. Administered radiation dose per pulse was approx. 50 Gy.

Figure 3.

A: Transient absorption spectra obtained by pulse radiolysis of Amplex® Red (200 μ M) in a N_2O -saturated aqueous solution containing 0.1 M NaN_3 and 10% CH_3CN at pH = 7.2 and pH = 10.5. Spectra were collected 5 μ s after the electron pulse. **B:** Determination of pK_a of Amplex® Red radical. Solutions consisted of 200 μ M Amplex® Red, 0.1 M NaN_3 , 10% CH_3CN , 50 mM phosphate buffer of pH in the indicated range. Solutions were saturated with N_2O prior to irradiation with 17 ns pulse (50 Gy dose). Absorbance at different pH values was measured at 370 nm.

Figure 4.

Transient absorption spectra obtained by pulse radiolysis of Amplex[®] Red (200 μM) in a N₂O-saturated aqueous solution containing 0.25 M Na₂CO₃ and 0.25 M NaHCO₃ (pH = 10.3) and 10% CH₃CN **A:** spectra recorded 9 μs (solid circles) and 90 μs (open triangles) after the electron pulse; **B:** spectra recorded 100 μs (open triangles) and 2.5 ms (solid squares) after the electron pulse. Administered radiation dose was approx. 50 Gy. **C:** Kinetic traces recorded at three selected wavelengths, corresponding to absorption maxima of the species detected.

Figure 5.

The dependence of the pseudo-first order rate constant of the reaction of Amplex[®] Red with azidyl radical on the initial concentration of Amplex[®] Red. Incubation mixtures contained 0.1 M NaN₃, 10% CH₃CN, 50–250 μM Amplex[®] Red and were N₂O-saturated prior to irradiation with 7 ns electron pulse (15 Gy dose), and the kinetics was monitored at 370 nm.

Figure 6.

A: UV-vis absorption spectra recorded for the aqueous solution containing 100 μM Amplex[®] Red, 25 mM phosphate buffer (pH 7.4), 50 μM dtpa, 5% CH_3CN , immediately after (solid line), 1 (dashed line), 3.5 (dotted line) and 15 s (dot-dashed line) from the addition of 20 μM peroxynitrite. **B:** Comparison of kinetics of resorufin formation (trace registered at 573 nm) determined under the conditions described in **A** with the kinetics of self-decomposition of 100 μM peroxynitrite (trace registered at 302 nm) determined under the same conditions (25 mM phosphate buffer, 2.5% CH_3CN), but in the absence of Amplex[®] Red.

Figure 7.

The yield of resorufin formed in the reaction of 20 μM Amplex[®] Red, 50 mM phosphate buffer (pH = 7.4) with 0.1–1 μM peroxynitrite (squares). Similar experiment was performed in the presence of 25 mM HCO₃⁻ (triangles) or 500 nM HRP (circles). The yield of resorufin was determined from the measured fluorescence intensity changes (excitation at 573 nm, emission at 586 nm) with the use of appropriate calibration curve (fluorescence intensity was measured 2 minutes after the bolus addition of peroxynitrite to the sample).

Figure 8.

A: The pseudo-first-order rate constant of resorufin formation in the aqueous solutions containing 100 μM AR, 20 μM H₂O₂, 25 mM phosphate buffer (pH = 7.4), 50 μM dtpa, 5% CH₃CN as a function of HRP concentration (0–500 nM). Kinetics was determined with the stopped flow technique by monitoring the changes in absorbance at 573 nm. **B:** Conditions as in panel A, but the final concentration of resorufin is plotted as a function of HRP concentration. **C:** Kinetic traces of resorufin formation in the presence of 0 (a), 100 (b), 200 (c), 300 (d), 400 (e) and 500 nM (f) HRP.

Figure 9.

A: The pseudo-first-order rate constant of resorufin formation in the aqueous solutions containing 100 μM AR, 20 μM ONOO⁻, 25 mM phosphate buffer (pH = 7.4), 50 μM dtpa, 5% CH₃CN as a function of HRP concentration (0–400 nM). Kinetics was determined with the stopped flow technique by monitoring the changes in absorbance at 573 nm. **B:** Same conditions as in panel A, but the final concentration of resorufin is plotted as a function of HRP concentration. **C:** Kinetic traces of resorufin formation in the presence of 0 (a), 100 (b), 200 (c), 300 (d), 400 nM (e) HRP.

Figure 10.

A: Yield of resorufin formed in the HRP-catalyzed reaction with H_2O_2 and ONOO^- , calculated from the absorbance measured at 573 nm after bolus addition of indicated amounts of oxidant to a solution containing 100 μM of Amplex[®] Red, 0 or 100 μM CBA, 500 nM HRP, 50 mM phosphate buffer (pH = 7.4), 100 μM dtpa, 10% CH_3CN . **B:** Resorufin and 7-hydroxycoumarin fluorescence spectra recorded after addition of H_2O_2 or ONOO^- ($c = 0\text{--}10\text{ }\mu\text{M}$). Resorufin fluorescence spectra were recorded with excitation at 573 nm and emission at 586 nm. 7-Hydroxycoumarin: excitation was set at 370 nm and emission at 455 nm. Fluorescence intensity was collected 2 minutes after the addition of oxidant to the sample. Other experimental conditions were the same as described for panel **A**. **C:** 7-hydroxycoumarin fluorescence intensity as a function of oxidant concentration. The experimental setup was the same as described for panel **B**.

Figure 11.
Proposed mechanism of conversion of Amplex[®] Red into resorufin upon one-electron oxidation.