Published in final edited form as:

Free Radic Biol Med. 2011 July 1; 51(1): 153–159. doi:10.1016/j.freeradbiomed.2011.03.016.

Studies on the photosensitized reduction of resorufin and implications for the detection of oxidative stress with Amplex Red

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Abstract

The photosensitized reduction of resorufin (RSF), the fluorescent product of Amplex Red, was investigated using electron spin resonance (ESR), optical absorption/fluorescence, and oxygen consumption measurements. Anaerobic reaction of RSF in the presence of the electron donor reduced nicotinamide adenine dinucleotide (NADH) demonstrated that during visible light irradiation ($\lambda > 300$ nm), RSF underwent one-electron reduction to produce a semiquinoneimine-type anion radical ($\text{RSF}^{\bullet-}$) as demonstrated by direct ESR. Spin-trapping studies of incubations containing RSF, 5,5-dimethyl-1-pyrroline N-oxide (DMPO) and NADH demonstrate, under irradiation with visible light, the production of the superoxide dismutase (SOD)-sensitive DMPO/ $\bullet\text{OOH}$ adduct. Both absorption and fluorescence spectra of RSF in the presence of NADH demonstrated that the $\text{RSF}^{\bullet-}$ was further reduced during irradiation with formation of its colorless dihydroquinoneimine form, dihydroresorufin (RSFH_2). Both $\text{RSF}^{\bullet-}$ and RSFH_2 , when formed in an aerobic system, are immediately oxidized by oxygen, which regenerates the dye and forms superoxide. Oxygen consumption measurements with a Clark-type oxygen electrode show that molecular oxygen is consumed in a light-dependent process. The suppression of oxygen consumption by addition of SOD or catalase further confirms the production of superoxide and hydrogen peroxide.

Keywords

Amplex Red; Resorufin; Photo-reduction; Free radical; NADH; superoxide; Hydrogen peroxide

Introduction

Fluorogenic probes provide a convenient, sensitive, and versatile means to detect oxidative activity in cells. Usually these compounds are non-fluorescent (or weakly fluorescent), but yield highly fluorescent products upon reaction with reactive oxygen species (ROS) [1, 2]. Fluorescence can be measured or observed with a spectrofluorometer, microtiter plate reader, microscope, or cytometer. Confocal microscopy offers the possibility of additionally observing cellular distribution of ROS production and may provide some degree of specificity through use of various fluorescent probes. Dihydro analogues of fluorescent dyes

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are the most commonly used probes, including 2',7'-dichlorofluorescein (DCFH), dihydrorhodamine 123, and Amplex Red [3–5].

Although these assays are commonly used, there are many controversies regarding their validity [6]. A major concern is generation (or photo-generation) of ROS by the probes themselves and/or their reaction products, which may result in artifactual detection of ROS and/or higher background. We have previously demonstrated that DCFH, upon reacting with horseradish peroxidase (HRP) compound I or compound II, was oxidized to the semiquinone free radical ($\text{DCF}^{\bullet-}$) of 2',7'-dichlorofluorescein (DCF, the oxidation fluorescent product of DCFH), which was subsequently oxygen-oxidized to DCF with the concurrent generation of superoxide radical [7, 8]. The disproportionation of superoxide forms hydrogen peroxide, which, in the presence of peroxidase activity, will oxidize more DCFH to DCF with self-amplification of fluorescence. DCF itself can be further oxidized by HRP compound I or compound II with the formation of the phenoxyl free radical $\text{DCF}^{\bullet}$. In the presence of a reducing agent such as GSH or NADH, $\text{DCF}^{\bullet}$ is then reduced back to DCF with the formation of $\text{GS}^{\bullet}$ or $\text{NAD}^{\bullet}$, respectively, and the subsequent generation of superoxide [9].

Amplex Red is a colorless and non-fluorescent derivative of resorufin (RSF) that is oxidized to the highly fluorescent RSF by H_2O_2 /peroxidase (Scheme 1). The highly sensitive assay based on this reaction can detect as little as 50 nM H_2O_2 or 1×10^{-5} U/ml of HRP [5]. However, several complications in using this assay have been reported. A general problem reported for this assay is that RSF, the final fluorescent product of oxidation, is itself a substrate for peroxidases and, under certain conditions, can be oxidized to a colorless/non-fluorescent compound(s) [5, 10–12]. Some exogenous peroxidase substrates such as p-hydroquinone, acetaminophen, and the anticancer drugs mitoxantrone and ametantrone can inhibit Amplex Red oxidation by consuming H_2O_2 in a competitive process [12]. In these cases the actual peroxide level may be substantially underestimated in a biological system containing Amplex Red. On the other hand, it has been reported that reducing substances such as NADH and GSH interact with HRP to produce H_2O_2 via a superoxide, which subsequently oxidizes Amplex Red without the need for the presence of exogenous H_2O_2 [13]. In addition, RSF can also be reduced by NADPH-cytochrome P450 reductase with the production of superoxide-mediated H_2O_2 [14, 15]. These undesired reactions may result in artifactual detection of ROS and/or a higher background.

It has been reported that xanthene dyes (fluorescein, eosin Y, and rose bengal) can be photo-reduced to their corresponding semi-reduced forms by visible light and, in several cases, the ESR spectrum of the resultant radical has been observed [16, 17]. We have previously demonstrated that DCF undergoes photo-reduction upon visible light irradiation to form the DCF semiquinone free radical ($\text{DCF}^{\bullet-}$), ultimately leading to superoxide generation [18]. DCF is also a moderate photosensitizer able to generate singlet oxygen [19]. RSF is a phenoxazine dye that is structurally similar to fluorescein and DCF. Similar photochemical reactions may take place with RSF and light irradiation, generating superoxide and leading to the production of H_2O_2 upon dismutation. Therefore, in the present work, we have studied the photo-reduction of RSF using electron spin resonance (ESR), optical absorption/fluorescence, and oxygen consumption measurements. Our results clearly indicate that RSF does undergo photo-reduction in the presence of NADH and, that under aerobic conditions, superoxide and subsequently hydrogen peroxide are generated.

Materials and methods

Chemicals

RSF was purchased from Invitrogen Co. (Carlsbad, CA). Diethylenetriaminepentaacetic acid (DTPA), reduced nicotinamide adenine dinucleotide (NADH) and catalase (from bovine

liver, aqueous solution, 40,200 units/mg protein) were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). Superoxide dismutase (from bovine kidney, 11,000 units/mg) was purchased from Calzyme Laboratories, Inc. (San Luis Obispo, CA). The spin trap dimethyl-1-pyrroline *N*-oxide (DMPO) was purchased from Dojindo Molecular Technologies, Inc. (Japan) and was used as received. Chelex-100 resin was purchased from Bio-Rad Laboratories (Hercules, CA). All the samples were freshly prepared in 100 mM phosphate buffer at pH 7.4, except for the samples for ESR measurements of the RSF radical, which are at a higher pH. The buffer was treated with Chelex-100 resin for 24 h to remove traces of transition metal ions. After filtration, 25 μ M DTPA was added to minimize the possibility of trace metal interference.

ESR spectroscopy

All EPR spectra were recorded at room temperature in a quartz flat cell on a Bruker EMX EPR spectrometer equipped with a super high-Q cavity (Bruker, Billerica, MA). Spectra were recorded using an IBM-compatible computer interfaced to the spectrometer. The instrument settings and conditions were as described in the figure legends. The computer simulation was performed using WINSIM, a computer program developed in this laboratory [20]. All the samples analyzed by the use of direct ESR in near anaerobic conditions were prepared and deoxygenated in a quartz flat cell by bubbling nitrogen in the dark for 5 min. Illumination of the sample was carried out directly inside the microwave cavity of the spectrometer using a 340-W slide projector lamp. The fluence rate was 13 mW/cm² as measured with an SPR-4001 Spectroradiometer (Luzchem Research Inc., Ottawa, Ontario, Canada).

Photobleaching measurements

Absorption measurements—Aqueous solutions containing 20 μ M RSF and 100 μ M NADH were prepared. The samples in aerobic or near anaerobic conditions were prepared by bubbling with oxygen or nitrogen in the dark for 5 min, respectively. The solution was irradiated in a sealed quartz cuvette with continuous stirring. The absorbance measurements followed by irradiation were carried out on an Agilent 8453 UV-visible spectrophotometer (Agilent Technologies, Inc., Santa Clara, CA) every 30 s for aerobic samples and every 3 s for anaerobic samples. A 450 W xenon-mercury lamp was used as the light source. A glass filter was used to cut off wavelengths below 300 nm.

Fluorescence measurements—Aqueous solutions of 10 μ M RSF in the presence and absence of NADH (100 μ M) were prepared. The samples were bubbled with nitrogen in the dark for 5 min. The fluorescence emission at 585 nm was continuously recorded with excitation wavelength at 520 nm in a sealed quartz cuvette on an FL3-22 spectrofluorometer (Edison, NJ). For the sample containing NADH, the measurement was stopped after 2 min, the solution was bubbled with oxygen for 1 min, and the measurement continued.

Oxygen consumption experiments

Oxygen consumption measurements were made using a Clark-type oxygen electrode fitted to a 1.8 ml Gilson sample cell with a stirrer and monitored by a Yellow Springs Instrument Co. oxygen monitor (Model 53). Oxygen concentration in the samples as a function of time was recorded by a PC interfaced to the oxygen monitor with a Data Translation DT2801 data acquisition board. All experiments were performed at room temperature, and the incubation conditions were as described in the figure legends.

The computer simulation of kinetic curves was performed using Gepasi (version 3.30) software, which is available to the public over the Internet (<http://www.gepasi.org/>) [21, 22].

Results

Photo-induced production of semiquinoneimine radical of RSF

A well-resolved free radical signal was observed when RSF aqueous solution was irradiated under anaerobic conditions at pH 11.5 in the presence of the reducing agent NADH (Figure 1B). The ESR spectrum is dominated by a 1:1:1 triplet, demonstrating significant spin density on the nitrogen atom of the RSF radical with each group of the triplet comprising 19 lines. This free radical formation depended on light irradiation (Figure 1A). Control experiments showed that the free radical was very weakly formed during irradiation in the absence of the reducing agent NADH (Figure 1C). As the pH decreased, the ESR signals dramatically decreased (data not shown), and no ESR signal was obtained at pH 7.4 (Figure 1D), suggesting this free radical is not stable at neutral pH. However, further experiments revealed that the RSF radical is formed at neutral pH (*vide infra*).

A better resolution of the free radical signal was obtained with a longer scan time (Figure 2A). The 1:1:1 triplet was analyzed and assigned to the splitting of a nitrogen atom, and 19 lines of each group of the triplet could be assigned to the hyperfine splittings of three pairs of equivalent protons (each forms a 1:2:1 splitting pattern) from the phenyl rings. The spectrum simulation (Figure 2B) was quite consistent with our assignment when the following hyperfine coupling constants were used: $a^N = 7.81$ G, $a_{1,8}^H = 2.36$ G, $a_{2,7}^H = 0.73$ G, and $a_{4,5}^H = 0.41$ G. This free radical is identified as the semiquinoneimine type proposed in previous work on redox cycling of RSF by NADPH-cytochrome P450 reductase [14, 15]. Our ESR spectrum confirms that the unpaired electron is largely localized on the nitrogen atom instead of the phenyl-O atom. Since the sample was analyzed at higher pH (11.5), this free radical probably exists as the dianion (deprotonated phenyl-O⁻) form. These radicals are often more stable due to the increased negative charge of the radical resulting in coulombic repulsion, which slows disproportionation. In addition, dianion radicals lack oxidizing properties, which inhibits the reaction.

Photo-induced production of superoxide

When DMPO was used as a spin trap, irradiation of an aerobic system containing RSF and NADH at pH 7.4 resulted in the appearance of a light-dependent composite ESR spectrum containing two major components (Figure 3A and B). The major part of this ESR spectrum is SOD-sensitive (Figure 3F) and exhibits a pattern characteristic of the DMPO/•OOH radical adduct. Computer analysis and simulation of the ESR spectrum (Figure 3G) showed that DMPO/•OOH accounts for 91.2 % of the composite spectrum with hyperfine coupling constant values $a^N = 14.1$ G, $a_\beta^H = 11.2$ G, and $a_\gamma^H = 1.2$ G, which are similar to those previously published [23–25]. Another minor radical species (8.8 %) was attributed to the DMPO/•OH adduct, with hyperfine coupling constants of $a^N = a_\beta^H = 14.9$ G. In addition, the unstable DMPO/•OOH signal decreased rapidly after the light was turned off (Figure 3C). When RSF (Figure 3D) or NADH (Figure 3E) was omitted from the reaction mixture, no obvious DMPO/•OOH signal was detected. It is noted that the DMPO/•OH signal was detected (Figure 3E) when NADH was absent. This small signal in the control experiment was the result of the oxidation of DMPO by singlet oxygen in the presence of phenols (i.e., RSF), which has been previously reported in the literature [26, 27]. In unpublished data we have shown that RSF can generate singlet oxygen through a Type II photochemical pathway. The formation of DMPO/•OOH was inhibited by SOD (Figure 3F) but was not affected by catalase (data not shown), which indicates that superoxide, not hydrogen peroxide, is required for the production of the DMPO/•OOH radical adduct.

Photobleaching of RSF in the presence of NADH

To investigate the intermediate products and mechanism of the photo-reduction of RSF, photobleaching experiments were carried out by both UV-vis absorption and fluorescence measurements. As shown in Figure 4, the absorption spectrum of a solution containing RSF and NADH shows the characteristic peaks of both RSF (571 nm) and NADH (339 nm) before irradiation. In spite of the absence of significant reduction of RSF under aerobic conditions, the absorption peak of NADH at 339 nm dramatically decreased during the irradiation (Figure 4A). Meanwhile, the absorption peak at 259 nm increased, suggesting the conversion of NADH to NAD^+ [28].

In light of the combination of these photobleaching experiments with the results of our ESR studies, we propose the following photochemical free radical chain reaction. First, the RSF molecule is excited by light irradiation to form an excited singlet [$^1(\text{RSF})^*$] and then triplet [$^3(\text{RSF})^*$] state through intersystem crossing, which initiates the reaction by reacting with NADH to produce $\text{NAD}^\bullet$ and the semiquinoneimine radical of RSF ($\text{RSF}^{\bullet-}$). Then $\text{NAD}^\bullet$ reacts with oxygen to produce superoxide anion and NAD^+ :

Superoxide anion could also be produced by direct oxidation of the semi-reduced $\text{RSF}^{\bullet-}$ free radical:

In aqueous solutions, superoxide is always in equilibrium with its conjugated acid, the perhydroxyl radical ($\text{HOO}^\bullet$), with an equilibrium constant of $K_{\text{HOO}^\bullet} = 1.6 \times 10^5 \text{ M}$ [29] and a rate constant of k (reverse direction) = $4.8 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$ [30]. At pH 7.4, the hydroperoxyl radical can oxidize an additional NADH molecule with a second-order rate constant of $k = 9.3 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$, producing $\text{NAD}^\bullet$ and H_2O_2 and continuing the chain reaction [31]:

In the absence of oxygen, the absorption peak of RSF rapidly decreased during irradiation of an RSF solution containing NADH (Figure 4B), suggesting that the $\text{RSF}^{\bullet-}$ was further reduced with formation of its colorless dihydroquinoneimine form, dihydroresorufin (RSFH_2):

Photobleaching of the RSF anaerobic solution measured by fluorescence further confirmed this redox cycling. In this experiment the excitation light of the fluorescence equipment also functions as the irradiation light source (excitation: 520 nm, molar absorption coefficient of RSF: $\epsilon_{520\text{ nm}} = 1.4 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$). As can be seen from Figure 5, in the absence of NADH, no obvious decrease in RSF fluorescence intensity was observed, which is consistent with the ESR results. By contrast, in the presence of NADH, the fluorescence intensity of RSF at 585 nm decreased significantly in a time-dependent manner. After the addition of oxygen, the fluorescence intensity was fully recovered and there was no further decrease with continued irradiation. These results confirm that the colorless RSFH₂ is unstable and, in the presence of oxygen and/or hydroperoxyl radical, can be oxidized to highly fluorescent RSF with formation of more superoxide and H₂O₂ [14, 15, 32].

Oxygen consumption by RSF during irradiation

To further provide a more complete understanding of the mechanism of photo-reduction of RSF, we studied the oxygen consumption of RSF in the absence and presence of NADH as well as the quenching effects of added catalase and SOD during illumination. As shown in Figures 6B and 7B, oxygen was consumed in a reaction mixture containing RSF and NADH when the system was irradiated with either a slide projector lamp or room light. The rate of oxygen consumption was strongly dependent on the intensity of the light. When the light was turned off, no more oxygen was consumed. There was no obvious oxygen consumption when RSF was irradiated alone (Figures 6A and 7A), which is consistent with the ESR and photobleaching observations that NADH greatly stimulated the production of superoxide in the reaction system.

In the case of RSF in the presence of NADH, oxygen evolution was detected right after the addition of 6,030 units/mL catalase (Figure 6C and 7C). The amount of oxygen evolution was about 50% of the oxygen consumed, and the rate of oxygen consumption decreased by half with further irradiation. All these results indicated that oxygen was being converted to hydrogen peroxide during the photo-reduction of RSF in the presence of NADH. In the presence of SOD (1,100 units/ml), the rate of oxygen consumption was significantly inhibited (Figure 6D and 7D), demonstrating that superoxide is an intermediate of oxygen consumption. In addition, oxygen consumption was totally inhibited by SOD when room light was used as the light source of irradiation (Figure 7D). This implies that the production of superoxide by free radical chain reaction chemistry (Eqs. 3, 5 and 6), which is completely superoxide-dependent, was the primary chemical pathway when the photo-reduction was initialized by a lower energy light source.

To provide a better understanding of the mechanism discussed above, the oxygen and hydrogen peroxide changes were computationally simulated using Gepasi software. The initial concentration of oxygen determined in phosphate buffer, pH 7.4, was 240 μM [33], and this value was used in the simulation program (see Materials and methods). Figure 8 shows the simulations of oxygen consumption, hydrogen peroxide production and the quenching effect of added SOD during illumination of a solution containing RSF and NADH with room light. The initial concentrations of the reactants and experimental conditions used are the same as shown in Figure 7 (solid line, 7B; dotted line, 7D). The reaction equations and rate constants are listed in Table 1. The rate constant for reaction of RSF with NADH was set to match the experimental curves and was determined to be 0.12 $\text{M}^{-1} \text{ s}^{-1}$ when the room light was on and 0 when the room light was off.

As expected, oxygen consumption was strongly dependent on light irradiation, and the same amount of hydrogen peroxide produced was stoichiometric with the consumption of oxygen. When SOD was added, the oxygen consumption and hydrogen peroxide production were nearly stopped, which is in agreement with the mechanism discussed above. Also as shown in Figure 8, although oxygen consumption was stopped by SOD and therefore due to a superoxide-dependent chain reaction, the chain reaction can not initiate itself in the absence of light. To simulate the kinetic changes under a projector lamp (Figure 6), the rate constant for the reaction of RSF with NADH was set to be $140\text{ M}^{-1}\text{ s}^{-1}$. Simulations similar to those described above were obtained (data not shown). In this case, however, addition of SOD only suppressed part of the oxygen consumption, which was similar to our experimental results (Figure 6D), demonstrating that superoxide production was mainly from the photosensitized RSF (Eqs. 1, 2 and 3) when the photo-reduction was initialized by a stronger light energy source than room light.

Discussion

In this study, we investigated the photosensitized reduction of RSF by visible light for its implications on the use of Amplex Red in biological systems. The photochemical pathways of the RSF reported in this paper are summarized in Scheme 1. The RSF is photo-reduced by visible light in the presence of the reducing agent NADH. Under anaerobic conditions, the corresponding one-electron-reduced free radical of RSF can be directly detected by ESR spectroscopy at higher pH. To the best of our knowledge, this is the first report of the ESR hyperfine splitting signal and coupling constants of the RSF radical. In the meantime, photobleaching is observed in both absorption and fluorescence spectroscopy, suggesting the production of the further reduced, colorless RSFH₂. On the other hand, in the presence of air, superoxide is generated, setting off a chain reaction in which superoxide is continuously generated provided there is sufficient reductant present. Dismutation of superoxide, either spontaneously or by the enzyme superoxide dismutase, will generate hydrogen peroxide, which further causes Amplex Red oxidation via peroxidase catalysis. The photo-reduction of RSF can be induced by low intensity light. As our results demonstrated, even room light and the excitation light of spectrometers can initiate the chain reaction.

In summary, we have shown for the first time that when RSF is irradiated with visible light in the presence of the reducing agent NADH, the dye is photoreduced with formation of ROS. Our findings have several important implications concerning the use of Amplex Red to detect oxidative stress in biological systems. First, continuous visible irradiation of cells, either from room light exposure or the excitation light of instruments, will result in self-amplification of the RSF fluorescence signal. The time-dependence of RSF fluorescence may also depend on the visible irradiation. Second, our results of superoxide generation during RSF irradiation in the presence of a reducing agent demonstrate that Amplex Red may not be a reliable indicator of oxidative stress in cell systems. Finally, certain precautions should be taken to minimize the light irradiation during measurements. Samples containing Amplex Red should not be exposed to room light before or during measurements. The excitation light intensity must be kept to an absolute minimum during RSF fluorescence measurement.

Acknowledgments

This research was supported by the Intramural Research Program of the NIH, National Institute of Environmental Health Sciences. The authors are indebted to Mrs. Mary Mason and Dr. Ann Motten for their critical reading of the manuscript.

Abbreviations

DMPO	5,5-dimethyl-1-pyrroline <i>N</i> -oxide
ESR	electron spin resonance
ROS	reactive oxygen species
RSF	resorufin
SOD	superoxide dismutase

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Figure 1.

ESR spectra of the semiquinoneimine radical of resorufin in anaerobic solution at pH 11.5 containing NADH. (A and B) The ESR spectra obtained from a solution containing 1 mM RSF, 500 μ M NADH before (dark) and during the irradiation, respectively. (C) Same as B, but with the omission of NADH. (D) Same as B, but at pH 7.4. The instrument settings were as follows: microwave power, 0.5 mW; modulation amplitude, 0.1 G; time constant, 82 ms; scan time, 168 s; scan range, 30 G; and receiver gain, 1×10^5 .

Free Radic Biol Med. Author manuscript; available in PMC 2012 July 1.

Figure 3.

ESR spectra of the DMPO-superoxide adduct detected during the irradiation of an aerobic solution containing RSF and NADH. (A–C) The ESR spectra obtained from a solution containing 100 μ M RSF, 500 μ M NADH and 100 mM DMPO before (dark), during, and after the irradiation (dark), respectively. (D) Same as B, but with the omission of RSF. (E) Same as B, but with the omission of NADH. (F) Same as B, but with the addition of 100 μ g/ml SOD. (G) Simulation of (B) with the following parameters: DMPO/ $\bullet$ OOH ($a^N = 14.1$ G, $a_\beta^H = 11.2$ G, and $a_\gamma^H = 1.2$ G; 91.2%) and DMPO/ $\bullet$ OH ($a^N = 14.9$ G and $a_\beta^H = 14.9$ G; 8.8%). The instrument settings were as follows: microwave power, 20 mW; modulation amplitude, 0.5 G; time constant, 164 ms; scan time, 168 s; scan range, 100 G; and receiver gain, 1×10^5 .

Figure 4.

Photobleaching of RSF in the presence of NADH in aerobic and anaerobic solutions at pH 7.4 during irradiation as measured by UV-vis absorption. (A) Absorption spectra of 20 μ M RSF and 100 μ M NADH after irradiation for 0, 30, 60, 90, 120, and 150 s in aerobic solution. (B) Same as A, except that irradiation was for 0, 3, 6, 9, 12, and 15 s in anaerobic solution. Arrows indicate direction of the changes.

Figure 5. Photobleaching of RSF (10 μ M) in the presence (solid line) and absence (dotted line) of NADH (100 μ M) in anaerobic solution at pH 7.4 measured by fluorescence. The fluorescence emission at 585 nm was continuously recorded with the excitation wavelength at 520 nm. Arrow indicates when the sample was bubbled with oxygen for the experiment in the presence of NADH (solid line).

Figure 6.

Oxygen consumption by RSF in the absence and presence of NADH and the quenching effects of added catalase and SOD during illumination with a 340-W slide projector lamp. The two vertical bars mark the time of addition of catalase or SOD. The arrows indicate the time at which the light was turned off and on (every 2 min). (A) 20 μ M RSF in 100 mM phosphate buffer at pH 7.4. (B) Same as A, except for the addition of 500 μ M NADH. (C) Same as B except the addition of 150 μ g/ml catalase (6,030 units/ml) added after 5 min. (D) Same as B, except for the addition of 100 μ g/ml SOD (1,100 units/ml) added after 5 min.

Figure 7.

Oxygen consumption by RSF in the absence and presence of NADH and the quenching effects of added catalase and SOD during illumination with room light. The two vertical bars mark the time of addition of catalase or SOD. The arrows indicate the time at which the light was turned off and on (cycles of 2 min off and 4 min on). (A) 100 μ M RSF in 100 mM phosphate buffer at pH 7.4. (B) Same as A, except for the addition of 2 mM NADH. (C) Same as B, except for the addition of 150 μ g/ml catalase (6,030 units/ml) added after 7 min. (D) Same as B, except for the addition of 100 μ g/ml SOD (1,100 units/ml) added after 7 min.

Figure 8.

Gepasi simulations of the oxygen and H_2O_2 concentration changes and the quenching effect of added SOD during illumination of a solution containing RSF and NADH with room light. The initial concentrations of the reactants and experimental conditions used are the same as shown in Figure 7 (Solid line, 7B; dotted line, 7D). The initial oxygen concentration was assumed to be 240 μM [33]. Reactions and rate constants are shown in Table 1. The rectangles indicate the time at which the light was turned on (cycles of 2 min off and 4 min on). The vertical bar marks the time of SOD addition.

Scheme 1.

Table 1

Reactions and rate constants used to simulate oxygen consumption in mixtures of RSF and NADH under the conditions used for the experiment shown in Figure 7B and 7D.

Reaction	Constant	Reference
$\text{RSF} + \text{NADH} \rightarrow \text{RSF}^{\bullet-} + \text{NAD}^{\bullet+} + \text{H}^+$	$k_{\text{light on}} = 0.12 \text{ M}^{-1} \text{ s}^{-1}$, $k_{\text{light off}} = 0$ ^a	This work
$\text{RSF}^{\bullet-} + \text{O}_2 \rightarrow \text{RSF} + \text{O}_2^{\bullet-}$	$k = 1.0 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ ^b	This work
$\text{NAD}^{\bullet+} + \text{O}_2 \rightarrow \text{NAD}^+ + \text{O}_2^{\bullet-}$	$k = 1.9 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$	[34]
$\text{HOO}^{\bullet} \leftrightarrow \text{H}^+ + \text{O}_2^{\bullet-}$	$k_+ = 7.7 \times 10^5 \text{ s}^{-1}$ ^c ; $k_- = 4.8 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$	[29, 30]
$\text{HOO}^{\bullet} + \text{HO}_2^{\bullet} \rightarrow \text{H}_2\text{O}_2 + \text{O}_2$	$k = 8.3 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$	[29]
$\text{HOO}^{\bullet} + \text{O}_2^{\bullet-} + \text{H}_2\text{O} \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 + \text{OH}^-$	$k = 9.7 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$	[29]
$\text{HOO}^{\bullet} + \text{NADH} \rightarrow \text{H}_2\text{O}_2 + \text{NAD}^{\bullet}$	$k = 9.3 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$	[31]
$\text{SOD-Cu(II)} + \text{O}_2^{\bullet-} \rightarrow \text{SOD-Cu(I)} + \text{O}_2$	$k = 1.2 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$	[35]
$\text{SOD-Cu(I)} + \text{O}_2^{\bullet-} + 2\text{H}^+ \rightarrow \text{SOD-Cu(II)} + \text{H}_2\text{O}_2$	$k = 2.2 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$	[35]

^aThis rate constant was determined by computer simulation to be $0.12 \text{ M}^{-1} \text{ s}^{-1}$ when the room light is on and 0 when the room light is off.

^bThis rate constant was estimated from a simulation of the oxygen consumption initiated by a projector lamp (Figure 6), but this reaction is not necessary to simulate the oxygen consumption initiated by room light (Figure 7).

^c k_- (reverse direction) found in Ref. 30; k_+ (forward direction) was calculated from the equilibrium constant ($K_{\text{HOO}^{\bullet}} = 1.6 \times 10^5 \text{ M}$) found in Ref. 29.