Published in final edited form as:
Biochemistry. 2012 January 10; 51(1): 265–272. doi:10.1021/bi201672h.

Flavin-linked Erv-family sulfhydryl oxidases release superoxide anion during catalytic turnover†

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Abstract

Typically, simple flavoprotein oxidases couple the oxidation of their substrates with the formation of hydrogen peroxide without release of significant levels of the superoxide ion. However, two evolutionarily-related single-domain sulfhydryl oxidases (Erv2p; a yeast endoplasmic reticulum resident protein and augmenter of liver regeneration, ALR, an enzyme predominantly found in the mitochondrial intermembrane) release up to ~30% of the oxygen they reduce as the superoxide ion. Both enzymes oxidize dithiol substrates via a redox-active disulfide adjacent to the flavin cofactor within the helix-rich Erv domain. Subsequent reduction of the flavin is followed by transfer of reducing equivalents to molecular oxygen. Superoxide release was initially detected using tris(3-hydroxypropyl)phosphine (THP) as an alternative reducing substrate to dithiothreitol (DTT). THP, and other phosphines, showed anomalously high turnover numbers with Erv2p and ALR in the oxygen electrode but oxygen consumption was drastically suppressed upon the addition of superoxide dismutase. The superoxide ion initiates a radical chain reaction promoting the aerobic oxidation of phosphines with the formation of hydrogen peroxide. Use of a known flux of superoxide generated by the xanthine/xanthine oxidase system showed that one superoxide ion stimulates the reduction of 27 and 4.5 molecules of oxygen using THP and tris(2-carboxyethyl)phosphine (TCEP) respectively. This superoxide-dependent amplification of oxygen consumption by phosphines provides a new kinetic method for the detection of superoxide. Superoxide release was also observed by a standard chemiluminescence method using a luciferin analog (MCLA) when 2 mM DTT was employed as a substrate of Erv2p and ALR. The percentage of superoxide released from Erv2p increased to ~65% when monomeric mutants of the normally homodimeric enzyme were used. In contrast, monomeric multi-domain Quiescin-sulfhydryl oxidase enzymes that also contain an Erv FAD-binding fold release only 1-5% of their total reduced oxygen species as the superoxide ion. Aspects of the mechanism and possible physiological significance of superoxide release from these Erv-domain flavoproteins are discussed.

Flavoproteins of the multidomain Quiescin sulfhydryl oxidase (QSOX[1]) family catalyze the generation of disulfides with the reduction of oxygen to hydrogen peroxide (1-4):

†**Funding**: This work was supported in part by National Institutes of Health Grant (GM26643) and USPHS Training Grant 1-T32-GM08550 (JRT).

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Supporting Information
Tables S1 and S2 provide supplementary tables of kinetic parameters. Figures S1-S5 represent kinetic data and SDS-PAGE analysis of cross-linking experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

[1]Abbreviations: ALR, augmenter of liver regeneration; DTT, dithiothreitol; Erv, protein essential for respiration and viability in yeast; MCLA, 2-methyl-6-(p-methoxyphenyl)-3,7-dihydroimidazo[1,2-α]pyrazine-3-one; PDI, protein disulfide isomerase; QSOX, Quiescin-sulfhydryl oxidase (*Hs*QSOX1, recombinant human QSOX1; *Bt*QSOX1, bovine milk enzyme; *Tb*QSOX, *Trypanosoma brucei* QSOX); SOD, superoxide dismutase; sulfo-EGS, ethylene glycol bis(sulfosuccinimidylsuccinate); TCEP, tris(2-carboxyethyl)phosphine; THP, tris(3-hydroxypropyl)phosphine; WT, wild-type; XO, xanthine oxidase.

$$2P-SH+O_2 \rightarrow P-S-S-P+H_2O_2$$

The flow of reducing equivalents during QSOX catalysis is depicted in Figure 1. Protein and peptide thiol substrates reduce a “distal” CxxC motif in a PDI-like thioredoxin domain (Trx1 in Figure 1A, (2-4)). Reducing equivalents are then passed to a “proximal” disulfide in the Erv domain via an interdomain disulfide exchange reaction (3-6). Catalysis is completed by reduction of the bound FAD in the Erv domain and the eventual transfer of reducing equivalents to molecular oxygen. An Erv domain is also found in a number of smaller single-domain FAD-dependent sulfhydryl oxidases with a range of functions and cellular locations (reviewed in (7)). The thioredoxin domain in QSOX is here replaced by mobile peptide segments added to the core Erv domain. In yeast Erv2p, the “shuttle” CxC disulfide in this extension is placed at the C-terminus and receives reducing equivalents from reduced protein disulfide isomerase (PDI; Figure 1B). In contrast, the shuttle CxxC disulfide in the long-form of mammalian augmenter of liver regeneration (lfALR) lies within an 80-residue N-terminal segment (Figure 1C). lfALR is reduced by MIA40 during the generation of disulfide bonds within the mitochondrial intermembrane space. Again an internal disulfide exchange reaction brings reducing equivalents to the Erv domain.

This paper concerns the oxidative half-reaction of these enzymes. The dihydroflavin forms of simple flavoprotein oxidases are believed to react with molecular oxygen via the rate-limiting formation of a caged superoxide-flavosemiquinone radical pair ((8-12) Figure 2). This species decomposes rapidly, either via the intermediacy of a C4a-flavin adduct (path A in Figure 2), or in a second 1-electron transfer to generate oxidized flavin and hydrogen peroxide directly via path B (8-11). For simple flavin dependent oxidases the efficiency of conversion of the radical pair to products is believed to ensure that superoxide does not escape into bulk solution (8, 13, 14). For example, glucose oxidase, D-amino acid oxidase, lactate oxidase and glycolate oxidase release undetectable levels of superoxide during turnover (13, 15). In contrast, a number of flavin dependent dehydrogenases including lipoyl dehydrogenase and glutathione reductase (13) and the flavin binding domain of flavocytochrome b_2 (16) generate significant levels of superoxide aerobically. While the expectation that flavoprotein oxidases do not release significant levels of superoxide is well established, this work shows that both wild-type (WT) forms of both Erv2p and ALR can release up to about one third of their reduced oxygen species as the superoxide ion. Path C in Figure 2 depicts superoxide escape from the radical pair and the formation of a second superoxide ion with the oxidation of the flavosemiquinone.

These surprising observations were made during the investigation of phosphines as alternate model substrates for QSOX, Erv2p and ALR (17, 18). Water-soluble phosphines are now widely used for the reduction of disulfide bonds in a variety of biochemical applications (19-21). Hydration of the thiophosphonium ion intermediate leads to the irreversible formation of phosphine oxide and the liberation of the second sulfhydryl group ((22-24) Figure 3A). We found that tris(2-carboxyethyl)phosphine (TCEP), its methyl analogs, and tris(3-hydroxypropyl)phosphine (THP) proved strikingly proficient substrates for a number of these enzymes (Figure 3 (17, 18)). This was somewhat surprising because, compared to DTT, these phosphines are generally poorer kinetic reductants of a range of disulfides found in small molecules, peptides and proteins (17). In the present study we show that the superoxide ion released from these oxidases initiates an off-enzyme radical-chain reaction that consumes phosphine and can greatly stimulate oxygen uptake. While the superoxide anion is also released from these enzymes using thiol substrates, such thiols are less prone to these radical chain-reactions (25), and so this phenomenon has previously escaped detection. We also consider the mechanism and possible significance of superoxide leakage from the

sulfhydryl oxidases. Finally this work suggests that the increasing use of water-soluble phosphines in biochemistry and biology may lead to the unexpected: a stimulation of reactive oxygen species catalyzed by the superoxide ion.

MATERIALS AND METHODS

Materials and Reagents

DTT, DTNB, α-D-glucose, xanthine, bovine liver catalase, *Aspergillus niger* glucose oxidase, bovine erythrocyte SOD, and bovine milk XO were obtained from Sigma Chemicals. THP was from Calbiochem. TCEP and Sulfo-EGS were from Thermo Scientific. Stock solutions of THP and TCEP were standardized using DTNB (17). When high concentrations of TCEP were to be used, stock solutions were brought to pH 7 by the addition of small volumes of concentrated KOH. Sulfo-EGS stock solutions were freshly prepared in water prior to use.

Protein Expression and Purification

The following proteins were expressed, purified, and quantitated as described earlier: Erv2p (18), sf- and lfALR (26, 27), human QSOX1 (6), bovine QSOX1 (28), and *Trypanosoma brucei* QSOX (29).

Enzyme Assays

Oxygen consumption was measured at 25 °C in a Clark-type oxygen electrode using 2 mL of 50 mM Tris/HCl buffer, pH 7.5, containing 0.3 mM EDTA and 5 mM DTT, THP or TCEP unless otherwise specified. Assays were started by the addition of enzyme and the initial rate of oxygen consumption was used to calculate turnover numbers. The flux of superoxide using the xanthine/xanthine oxidase (XO) system was calculated following the reduction of cytochrome *c* (13, 30, 31). Here, 39 nM of XO was added to 1 mL of 50 mM Tris/HCl buffer, pH 7.5, containing 0.3 mM EDTA, 0.5 mM xanthine and 50 μM cytochrome *c* and the reaction was followed at 550 nm in an Agilent 8453 spectrophotometer. Exactly the same conditions of buffer, temperature and concentrations of xanthine/XO were used in the oxygen electrode to measure the stimulation of oxygen consumption in the presence of 5 mM phosphine. These data allowed calculation of the amplification factor (**F**) for each phosphine: the ratio F = oxygen molecules consumed/ superoxide ion released in the presence of 5 mM phosphine (see the Text).

Calculation of Percentage of Oxygen Molecules Reduced by Sulfhydryl Oxidases that are Released as the Superoxide Ion

Assume that an enzyme simultaneously releases both hydrogen peroxide and superoxide with individual turnover numbers/min of TN_{perox} and $TN_{superox}$. If superoxide undergoes dismutation, without interacting with phosphines (see the text), the turnover number for net oxygen consumption is:

$$TN_{oxygen} = \left[TN_{perox} + 0.5 \times \left(TN_{superox} \right) \right].$$

When phosphines stimulate oxygen consumption (in the absence of superoxide dismutase) the aggregate oxygen consumption turnover number will become: [TN_{perox} + (F × $TN_{superox}$)]. The difference between apparent turnover numbers in the absence and presence of superoxide dismutase (ΔTN/min) then allows calculation of the following:

$$TN_{superox} = \Delta TN / (F - 0.5)$$

$$TN_{perox} = TN_{oxygen} - [\Delta TN / (2F - 1)]$$

The percentage of oxygen molecules that are reduced to directly yield the superoxide ion is then:

$$\text{Percent superoxide of total} = \left(100 \times TN_{superox} / \left[TN_{superox} + TN_{perox}\right]\right)$$

Detection of Superoxide Using MCLA Luminescence

Luminescence experiments with the luciferin analog MCLA (32, 33) were done using 1 μM Erv2p in 0.2 mL of 50 mM phosphate buffer, pH 7.5, containing 0.3 mM EDTA with 2 mM DTT and 50 μM MCLA in the presence or absence of 75 units/mL of SOD. Luminescence was recorded at 465 nm using a Perkin Elmer Fusion plate reader.

Preparation of Apoprotein

ALR apoprotein was prepared by removing FAD under denaturing conditions using guanidine hydrochloride as described earlier (34). The apoprotein was reconstituted by the addition of 0.2 to 1.0 equivalents of FAD per dimer (corresponding to a maximum site occupancy of 50%), incubated for 16 h at 4 °C, and assayed in the oxygen electrode as before at 25 °C.

Cross-Linking Experiments with Sulfo-EGS

These experiments were conducted with WT Erv2p, a mutant lacking its distal disulfide (C176A/C178A), and with a series of quadruple mutants that all lack the distal disulfide with two additional mutations (F111S/L114S, F111R/L114R, F111D/L114S, or F111R/L114S) to induce monomerization. Each protein (1 μM in 1 mL of 50 mM phosphate buffer, pH 7.5) was incubated at room temperature for 30 min in the presence or absence of 300 μM sulfo-EGS cross-linker. Reactions were then quenched by the addition of 50 mM Tris buffer, pH 7.6, for 30 min at room temperature. Samples were concentrated by centrifuge ultrafiltration (Amicon Ultra 0.5 mL, 10kD cut-off) and examined using 12% reducing SDS-PAGE gels.

Mass Spectrometry

Erv2p WT and monomeric mutants (80 μM) were subjected to LC-MS using a Shimadzu LC-20AD and a Micromass Q-Tof Ultima. Analyses were performed using MassLynx software version 3.5.

RESULTS AND DISCUSSION

Erv2p Releases Superoxide Ion during Aerobic Turnover

The initial experiments for this work were performed with yeast Erv2p (Figure 1B). As shown earlier, DTT is a useful model substrate of Erv2p with k_{cat} and K_m values of 230 ± 8 min^{-1} and 7 ± 1 mM determined in the oxygen electrode (Figure S1), in good agreement with values determined earlier (18, 35). However, when DTT was replaced by THP, under otherwise identical conditions, much higher turnover numbers were observed in the oxygen

electrode. Figure 4 shows that a turnover number of 1600 min^{-1} was attained with 5 mM phosphine (compared to 90 min^{-1} for 5 mM DTT in Figure S1). Only modest downward curvature is evident up to 10 mM THP in Figure 4 (closed circles) suggesting that apparent turnover numbers of >5000 min^{-1} would be attained at high THP concentrations. These values are far in excess of any other substrate type for Erv2p. Importantly, these anomalously high turnover numbers were abolished in the presence of superoxide dismutase (SOD; open circles). Further, when SOD was added after the reaction was initiated the rapid rate of oxygen consumption was immediately suppressed (data not shown; see later). Analysis of steady state parameters in the presence of SOD (Figure 4) gave a k_{cat} of 133 ± 1 /min with a K_m of 0.4 ± 0.01 mM). Although the majority of experiments in this work used the neutral THP derivative, turnover of the highly polar and anionic phosphine TCEP (Figure 3B) by Erv2p is also strongly depressed by superoxide dismutase (Figure S2).

Phosphines Accelerate Oxygen Consumption by the Xanthine/Xanthine Oxidase System

The data above strongly suggested that the superoxide ion released from Erv2p stimulated THP-driven reduction of molecular oxygen. A quantitation of the release of superoxide cannot simply use the widely-employed superoxide-mediated reduction of cytochrome *c* (30) because phosphines reduce the hemoprotein directly (data not shown). As an alternative, this section describes a new method for superoxide estimation based on the stimulation of oxygen uptake observed above.

To generate a flux of superoxide ion that was independent of Erv2p, we utilized the well-established xanthine/XO system (30, 36, 37). Figure 5 shows that mixing XO with xanthine leads to a low rate of oxygen consumption that is dramatically accelerated upon the addition of 5 mM THP. The enhanced rate is immediately suppressed by SOD (black line, Figure 5). In the absence of XO a slow consumption of oxygen occurs, consistent with the autoxidation rate of THP under these conditions (grey line, Figure 5). Clearly the superoxide ion stimulates oxygen consumption from aerobic solutions of THP.

To characterize this reaction further we needed to measure the flux of superoxide ion formed from xanthine/XO under the conditions of Figure 5. Hence we used the cytochrome *c* reduction spectrophotometric assay (13, 30, 31) in the absence of THP under otherwise identical conditions of enzyme, substrates and buffer (see Materials and Methods). Here a flux amounting to 2.7 μM min^{-1} superoxide (based on cytochrome *c* reduction, data not shown) stimulates consumption of an additional 72 μM min^{-1} oxygen in the oxygen electrode assay (Figure 5). Hence we conclude that one superoxide ion stimulates the consumption of 27 molecules of oxygen by THP. Using the same superoxide-generating system we compared several other water-soluble phosphine substrates of the sulfhydryl oxidases for their ability to promote oxygen consumption. TCEP and its mono-, di-, and tri-methyl esters (Figure 3B) show stimulations of 4.5, 12.6, 13.2 and 17.3-fold respectively (at 5 mM, data not shown). Clearly this reaction is common to all the water-soluble phosphines examined here, with the most polar and anionic phosphine, TCEP, showing the smallest stimulation.

Mechanistic Outlines of Superoxide Phosphine Radical Chain Reaction

While a characterization of the chemical mechanism for the interaction between phosphines and the superoxide radical is not the aim of this contribution, there are several precedents for radical chain reactions involving phosphine derivatives (38-43). Using these earlier studies, a reasonable series of reactions consistent with the work described here is shown in Figure 6 (38-43). Superoxide ion is the initiating radical species generating a phosphinium radical cation. The consumption of oxygen and phosphine is propagated by several phosphinium radical species (Figure 6, propagation). Termination steps include reduction of superoxide

by a phosphinium radical species and the dismutation of superoxide itself (38-41). Overall, these reactions would lead to the reduction of oxygen to hydrogen peroxide and the formation of stoichiometric levels of phosphine oxide with respect to molecular oxygen (Figure 6, sum). These products were confirmed in the reaction between THP and Erv2p as described below.

Products of the Aerobic Incubation of Erv2p with THP

Formation of the phosphine oxide, the expected product formed during the reduction of disulfide bonds (17, 19, 44), was confirmed by ^{31}P NMR (data not shown). Evidence for the formation of significant levels of hydrogen peroxide during this off-enzyme reaction is shown in Figure 7. Limiting levels of THP were added to an air-saturated solution of buffer in the oxygen electrode promoting the consumption of 155 μM oxygen in the presence of 2 μM Erv2p. Catalase restored 65 μM oxygen to the solution (Figure 7) compared to a theoretical value of 78 μM. This deficit is unsurprising given the observations that hydrogen peroxide is directly reduced by phosphines in aqueous solution (45).

Superoxide is also Released by Erv2 using a Thiol Substrate

The data described above shows that SOD drastically suppresses oxygen consumption when THP is used as a substrate of Erv2p. With DTT we observed only a very small decrease in reaction rate (amounting to 8-13% of the observed slope for 6 constructs shown in Table S2). However this result cannot be taken as evidence that Erv2p fails to release the superoxide anion in the presence of thiol substrates. This is because thiols are much less prone than the phosphines to participate in superoxide-initiated radical chain reactions that stimulate oxygen consumption (25). We thus wanted more direct evidence for the release of superoxide by Erv2 in the presence of DTT. One obvious strategy would be to follow the superoxide-driven reduction of cytochrome *c*. However this proved unworkable, because DTT is a relatively rapid non-enzymatic reductant of cytochrome *c* ((46, 47) and unpublished observations). Hence we followed superoxide release via the chemiluminescence generated in the presence of the luciferin analog MCLA (32, 33) (see Methods). The signal is completely suppressed by SOD (grey circles Figure 8). It should be noted that in these experiments we used 2 mM DTT because higher thiol concentrations severely depressed the chemiluminescence reaction (not shown). Further, MCLA cannot be employed to detect superoxide in the presence of THP; even 0.2 mM of the phosphine completely suppresses the chemiluminescence of MCLA in the presence of the xanthine/XO system (Figure S3).

Superoxide Release from Other Sulfhydryl Oxidases of the Erv Family

Since we earlier determined the stimulation of oxygen consumption in the presence of 5 mM THP we could then calculate the percentage of oxygen reduced that is released as the superoxide anion by other Erv-family sulfhydryl oxidases (see Materials and Methods). Table 1 shows that Erv2p and lfALR released 23% and 33 % of the oxygen they consume as the superoxide ion with the balance being hydrogen peroxide. While superoxide release is conveniently expressed by this metric, the percentage of the electron flux leaving Erv2p and ALR as the superoxide ion will be one half of these values (here ~12 and ~17% respectively). lfALR has a shorter cytokine-like form, sfALR, that lacks the N-terminal extension shown in Figure 1C. This protein is also active with the model substrates DTT and THP because both can access the proximal disulfide directly. sfALR also generates significant levels of superoxide (21% Table 1). In contrast 3 QSOXs (*Hs*QSOX1, *Bt*QSOX1, and *Tb*QSOX) show only 1-5% superoxide formation. As mentioned earlier, a number of flavin-dependent oxidases have previously been previously shown to release undetectable levels of superoxide during turnover with their normal substrates (13, 15). We chose one of them, *Aspergillus niger* glucose oxidase, as a negative control: as expected, we saw no

detectable stimulation of the rate of glucose-driven oxygen uptake when the assay is supplemented by 5 mM THP (Table 1).

On the Mechanism of Superoxide Formation by Sulfhydryl Oxidases

We were interested in the considerable variation in superoxide flux between different sulfhydryl oxidases that share the same Erv flavin binding domain (Table 1). We first considered the rather unlikely possibility that the multidomain structures of the larger QSOXs would, in some way, suppress superoxide release. However removal of the thioredoxin domain in TbQSOX generating an HRR-Erv fragment (Figure 1A) led to no significant increase in superoxide flux (1.2 to 1.5% respectively; Table S1). Further, the larger lfALR (with an additional 80-residue N-terminal extension over that of sfALR; Figure 1C) generates more superoxide than the short form of the oxidase (33% vs. 21 %; Table 1). A further consideration is that the prolific superoxide-formers are small homodimers (Table 1) with comparatively short inter-flavin distances (with closest approach between isoalloxazine rings of 17 and 26 Å for Erv2p and ALR respectively). Hence we considered whether intersubunit electron tunneling (48) could lead to superoxide release as depicted in Figure 9A. If this were the case, dimeric enzymes carrying a single FAD per dimer, or monomeric forms of the flavoenzyme, should show greatly attenuated superoxide formation. For the first approach we used sfALR because it is a disulfide-bridged homodimer (26) and remains dimeric when treated with the 6 M guanidine HCl used for FAD removal (see Methods). Following reconstitution with FAD (corresponding from 10-50% of the total flavin binding sites in dimeric ALR) the enzyme was assayed with THP in the presence or absence of SOD. When the rates of oxygen consumption with THP are normalized for flavin content, the superoxide generation rate at the lowest FAD occupancy is actually marginally higher than that at the highest loading used (Figure S4). Unless flavin binding to the ALR dimeric apoenzyme is strongly cooperative, these results do not support the obligatory involvement of interflavin electron tunneling in superoxide generation.

To complement these experiments, we introduced mutations to the subunit interface of Erv2p that are known to induce monomerization of this non-covalent dimeric sulfhydryl oxidase (49). We could then compare the phosphine-driven oxygen consumption catalyzed by dimeric and monomeric forms of Erv2p. In what follows we found that monomeric Erv2p is an efficient generator of superoxide ion. However, in the design of these experiments we needed to remove the shuttle disulfide in Erv2p to address a potential complication of interpretation: the depiction of the transfer of reducing equivalents in Erv2p in Figure 1C is an oversimplification because the CxC shuttle dithiol reduces the proximal CxxC disulfide of the other subunit (49, 50). While THP can reduce the proximal disulfide directly, it is some 20-fold less efficient than the intersubunit pathway involving shuttle to proximal disulfide exchanges (Table S2). We therefore mutated the shuttle CxC motif of the dimeric wild-type Erv2p to AxA so that reducing equivalents supplied by THP are now forced to adopt the same route as those in a monomeric version of the enzyme.

Vala et al. have already demonstrated that mutations within the hydrophobic dimeric interface in Erv2p can induce monomerization of Erv2p (50). Thus F111S and L114S mutants were both monomeric as judged by analytical ultracentrifugation (50). Building on this insight, we constructed a double serine mutation at these positions (F111S/L114S) together with three additional constructs that would be expected to be even more disruptive of dimerization (F111R/ L114R; F111D/L114S; and F111R/ L114S). Unlike the dimeric WT Erv2p, all of these mutants (in the background of the AxA double mutant; see above) were exclusively monomeric at 1 μM as judged by crosslinking experiments using Sulfo-EGS (see Methods; Figure S5). Further, while WT Erv2p was found to be a dimer on LC-MS the quadruple mutants were clearly monomeric under the same experimental conditions (see Methods; data not shown).

In agreement with the results of Vala et al. (50) the 4 monomeric Erv2p mutants were active with DTT (with turnover numbers between 56 and 70/min - comparable to those of the corresponding dimeric shuttle disulfide mutant; Table S2). However analysis of the oxygen consumption with 5 mM THP in the absence or presence of SOD showed that the monomeric mutants generate a significantly higher percentage of oxygen reduced as superoxide (67 ± 12 % of total oxygen reduced Table S2). Clearly the evidence presented here show that superoxide is released at the level of Erv/ALR monomers (Figure 9B) and does not require the obligatory involvement of dimeric forms of these proteins.

Although superoxide release is clearly enhanced by monomerization of Erv2p we do not yet understand the molecular basis for the behavior of these Erv-fold enzymes. More generally the factors governing the overall reactivity of flavoproteins towards molecular oxygen are still not fully delineated. In terms of access, some rapidly-reacting flavoenzymes have well-defined channels leading to the flavin cofactor (51-54). For others, computational approaches reveal multiple paths that oxygen can take towards the prosthetic group (55). Some flavoproteins react relatively slowly with oxygen despite notable solvent-accessibility of the cofactor (10, 16). In addition to issues of access, the microenvironment around the cofactor (e.g. steric factors, H-bonding, polarity and electrostatics (10, 56-62)) can profoundly modulate the oxygen-reactivity of flavoproteins. In terms of the Erv-family flavoproteins studied here, there is a channel from the surface of Erv2p to the si-face of the flavin (7, 35). However this feature is noticeably absent in ALR (7) although both oxidases generate roughly the same amount of superoxide. The same Erv fold in QSOX releases a 6- to 25-fold smaller percentage of superoxide ion but the crystal structure of the HRR-Erv fragment of QSOX shows a clear path from solvent to the si-face of the flavin ring (63). Clearly consideration of these static structures cannot explain the marked differences in the disposition of reducing equivalents between Erv-family enzymes. Future work will require the consideration of dynamic approaches (10, 55) and the application of site-directed mutagenesis to uncover factors promoting superoxide release in these oxidases.

In summary, this work has uncovered another potential source of superoxide in the cell. While superoxide released by the long form of augmenter of liver regeneration in the mitochondrial intermembrane space would likely be scavenged by superoxide dismutase or by oxidized cytochrome *c*, ALR has additional cytosolic, nuclear and extracellular locations. Perhaps ALR-generated superoxide at these extra-mitochondrial locations might serve signaling functions. Finally we note that the increasing use of phosphines in biochemistry and cell biology as protective agents and antioxidants might have the unintended consequence of promoting hydrogen peroxide formation in systems where superoxide release occurs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Pavan Kumar Mantravadi for his help with the NMR experiments and Thorpe lab members for providing various constructs of QSOX used in this study.

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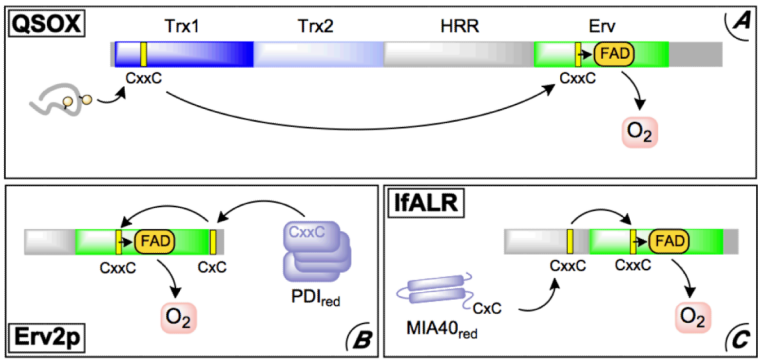

Figure 1.

Domain structure of three Erv-domain containing sulfhydryl oxidases. Panels A-C show the flow of reducing equivalents between redox centers in mammalian QSOX, yeast Erv2p, and the long form of augmenter of liver regeneration (lfALR). Cytochrome *c* (not shown) has been demonstrated to be an alternative electron acceptor for lfALR and its yeast ortholog, Erv1p (see text).

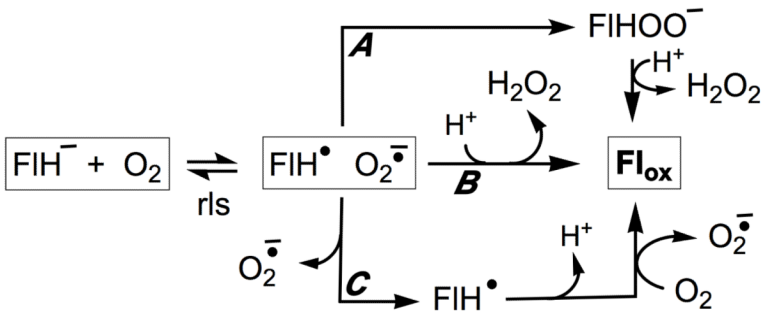

Figure 2.

Depiction of the reaction of dihydroflavin with molecular oxygen. Two routes to the release of hydrogen peroxide are shown as A and B. The pathway depicted in C involves superoxide escape from the caged radical pair followed by reoxidation of the flavin semiquinone by a second molecule of oxygen.

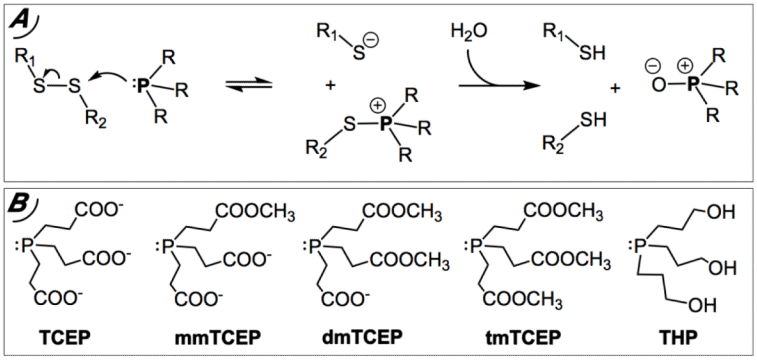

Figure 3.

The reduction of disulfides by phosphines in aqueous solution, and structure of the water-soluble phosphines used in this work. Panel A: Reduction of disulfides proceeds via a thiophosphonium species. Panel B: TCEP, tris(2-carboxyethyl)phosphine; its methyl esters; and THP, tris(3-hydroxypropyl)phosphine.

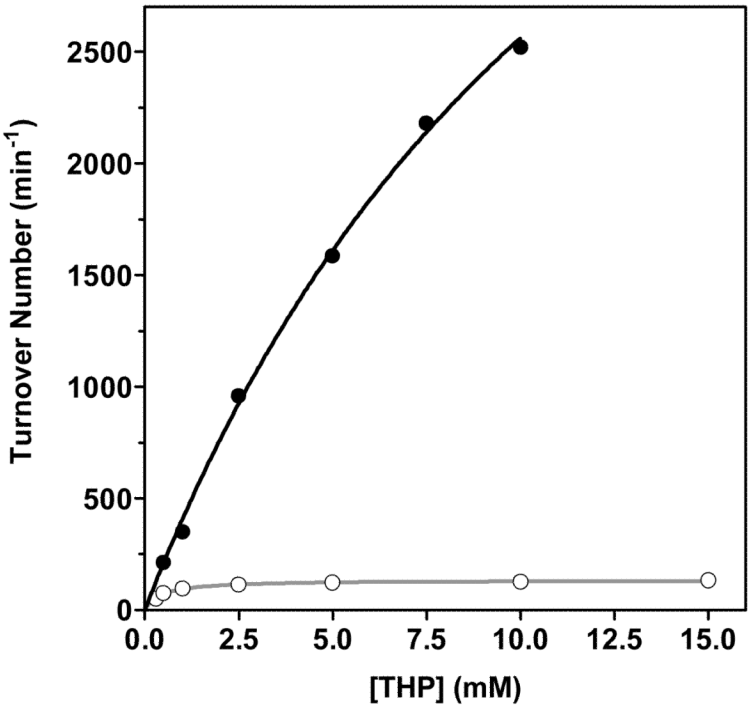

Figure 4.

THP as a substrate of Erv2p in the oxygen electrode. The apparent turnover numbers were calculated using 100 nM Erv2p adding the indicated concentration of THP to 2 mL of 50 mM Tris/HCl buffer (pH 7.5, 25 °C) containing 0.3 mM EDTA in the absence or presence of 80 units/mL of SOD (closed and open circles respectively). Data points (in duplicate) were fit to Michaelis Menten kinetics yielding the following parameters: k_{cat} 6200 ± 570 min^{-1}, K_m 14 ± 2 mM in the absence of SOD; and k_{cat} 133 ± 1 min^{-1}, K_m 0.4 ± 0.01 mM with SOD. As mentioned in the text, the kinetic complexity of the reaction in the absence of SOD (closed circles) precludes simple conclusions concerning the significance of these steady state parameters.

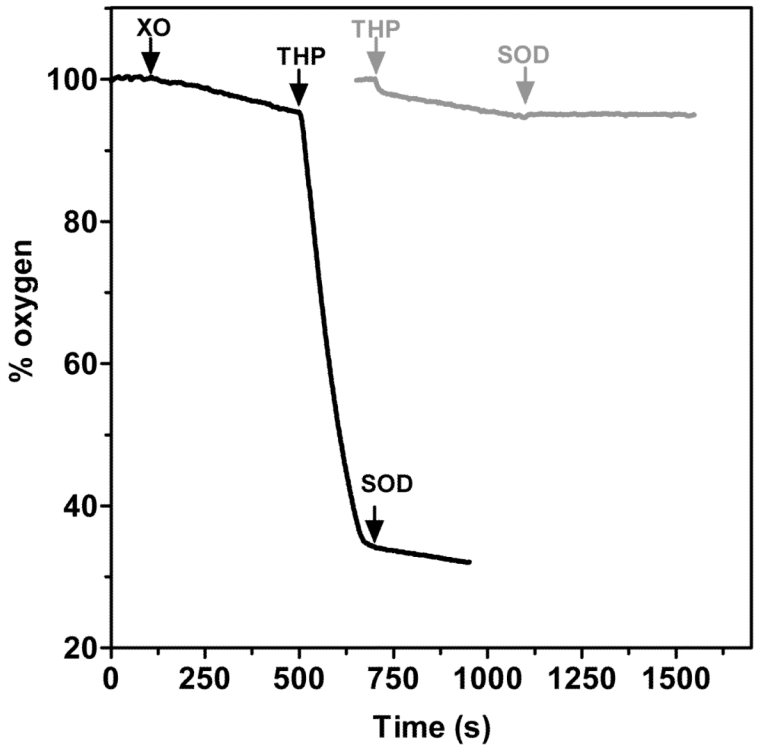

Figure 5.

Superoxide accelerates the aerobic oxidation of THP. Oxygen consumption was recorded at 25 °C in 2 mL of 50 mM Tris/HCl buffer, pH 7.5, containing 0.3 mM EDTA after the serial additions of 0.5 mM xanthine, 39 nM XO, 5 mM THP, and 80 units/mL of SOD as indicated. The grey line corresponds to a control following the addition of xanthine, THP and SOD under otherwise identical conditions.

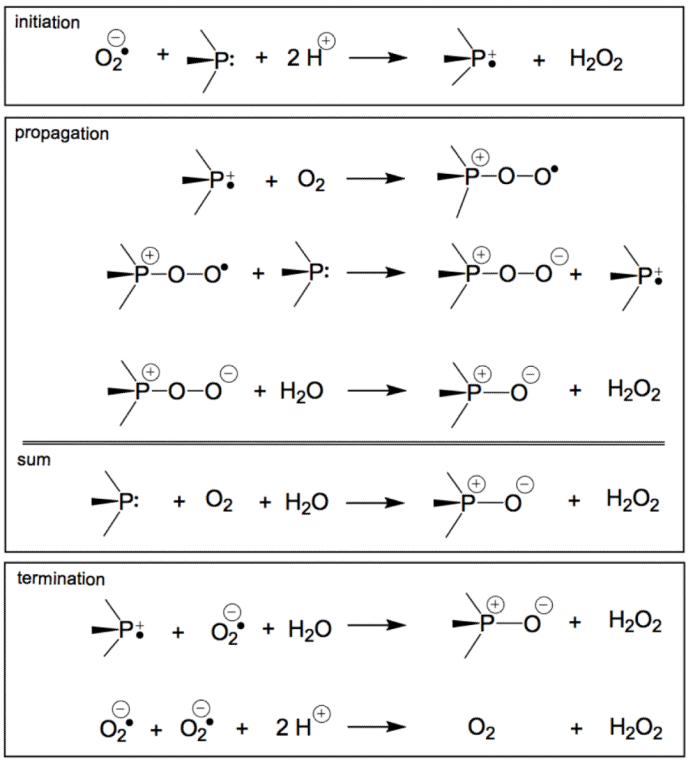

Figure 6.
Proposed steps in the superoxide-initiated oxidation of water-soluble phosphines. The chain reaction leads to the generation of phosphine oxide and hydrogen peroxide (see the Text).

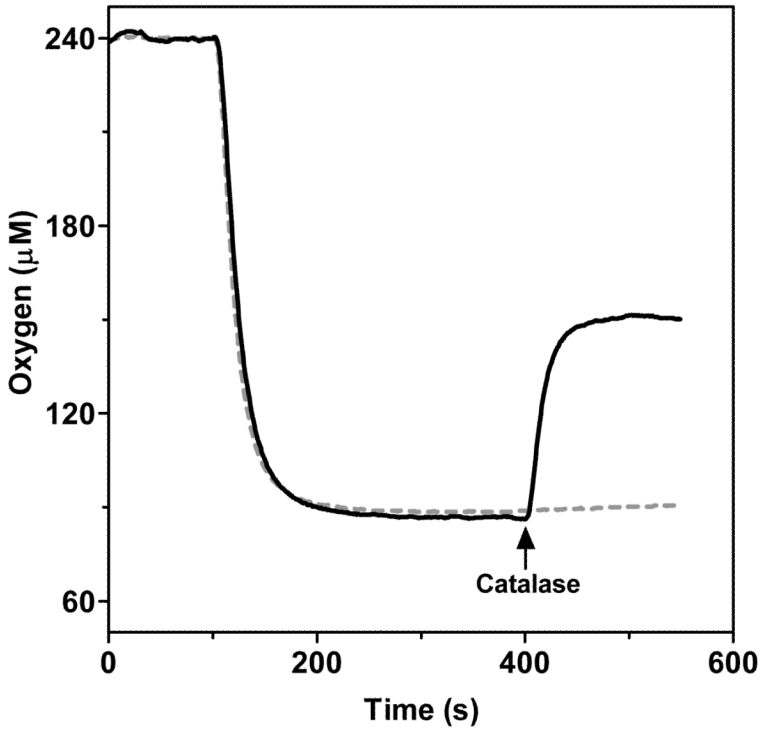

Figure 7.

Hydrogen peroxide is a major product of the phosphine-mediated radical chain reaction. A limiting amount of THP was added (200 μM) to 2.0 μM Erv2p in 2 mL of air-saturated 50 mM Tris/HCl buffer, pH 7.5 at 25 °C, containing 0.3 mM EDTA. The arrow corresponds to the subsequent addition of 250 nM catalase. A control experiment omitting catalase is shown by the dotted line.

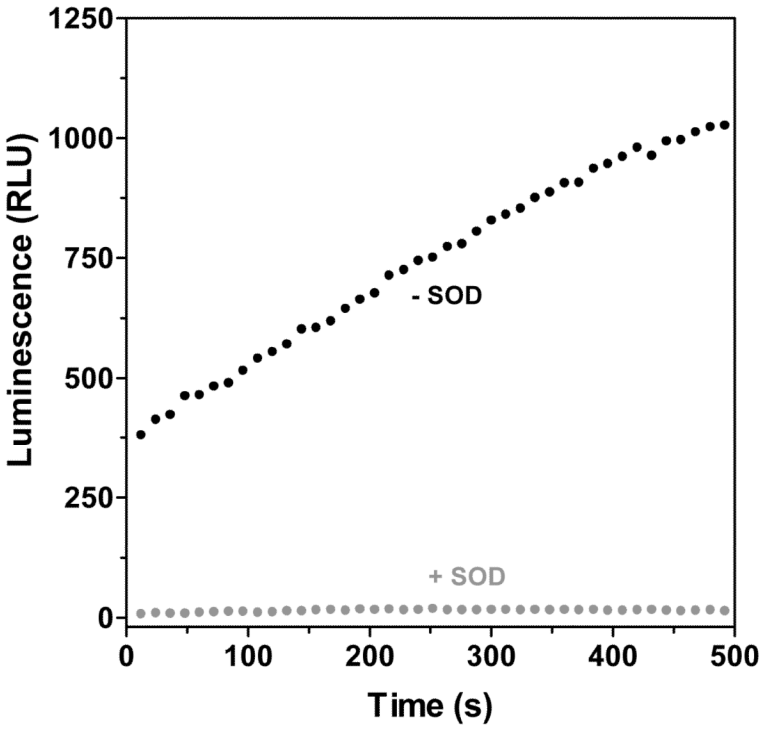

Figure 8.

Chemiluminescence detection of superoxide anion released during the oxidation of DTT by Erv2p. Erv2p (1 μM in 0.2 mL of 50 mM phosphate buffer, pH 7.5 containing 0.3 mM EDTA) was mixed with 2 mM DTT in the presence of 50 μM MCLA. Luminescence was recorded at 465 nm using a plate reader (see Methods) and was completely quenched in the presence of 75 U/mL of SOD (grey circles).

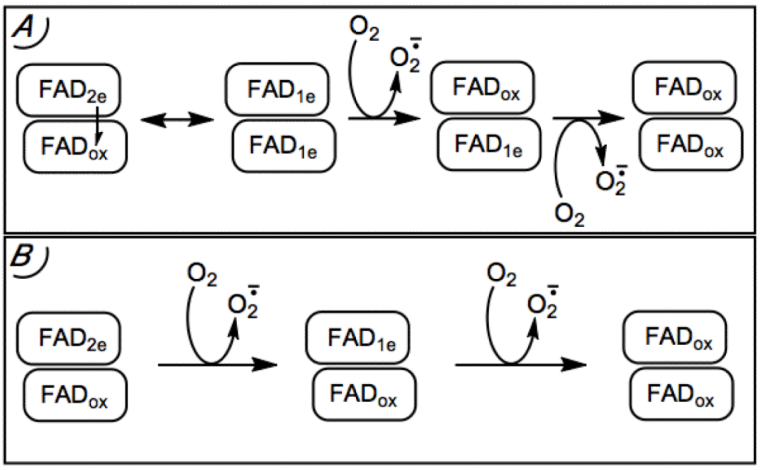

Figure 9.

Two model for superoxide generation in dimeric ALR and Erv2p. In panel A, an inter-subunit disproportionation reaction leads to generation of superoxide on both subunits. In panel B, two successive 1-electron transfers in one subunit (as in Figure 2C) generate superoxide without involvement of the other subunit.

Table 1

Superoxide and Hydrogen Peroxide Release from Selected Flavoprotein Oxidases[a,b]

Enzyme	Quaternary structure	Subunit MW (~ kDa)	% superoxide release [b]	% hydrogen peroxide release [b]
Erv2p	Dimer	20	23	77
lfALR	Dimer	25	33	67
sfALR	Dimer	15	21	79
*Hs*QSOX1	Monomer	67	5	95
*Bt*QSOX1	Monomer	62	1	99
*Tb*QSOX	Monomer	55	1	99
Glucose oxidase	Dimer	80	0.0	100

[a] Data are averages of duplicate experiments.

[b] Calculation of percentage of reduced oxygen species released as superoxide and hydrogen peroxide were calculated as in Methods. To calculate the corresponding percentage in terms of the release of reducing equivalents, the superoxide percentages should be halved and the balance added to the corresponding entry for hydrogen peroxide.