Activation of the Respiratory Burst Oxidase

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The respiratory burst oxidase of phagocytes and B lymphocytes catalyzes the reduction of oxygen by NADPH to form O2−, the precursor of a group of reactive oxidants that are employed by phagocytes as microbicidal agents. The enzyme is active in stimulated cells but dormant in resting cells. It is composed of several subunits: p22phox, gp91phox, p47phox, p67phox, and a low molecular weight guanine nucleotide-binding protein. The components p22phox and gp91phox form cytochrome b556, a flavohemoprotein that resides in the cortical cytoskeleton and in the membranes of the specific granules. The other components are found in the cytosol of resting cells, but migrate to the cortical cytoskeleton when the neutrophils are activated, where they assemble the active oxidase. Migration to the cortical cytoskeleton is caused in part by the appearance of a membrane binding site on one or more of the cytosolic subunits, possibly due to the phosphorylation of p47phox that takes place during cell activation. — Environ Health Perspect 102(Suppl 10):53–56 (1994)

Key words: respiratory burst oxidase, phagocytes, B lymphocytes

Introduction

The respiratory burst oxidase is a membrane-bound enzyme found in phagocytes and B lymphocytes (1). It catalyzes the one-electron reduction of oxygen to O2 at the expense of NADPH:

\[
2O_2 + NADPH \rightarrow 2O_2^- + NAPD^+ + H^+
\]

Dormant in resting cells, the respiratory burst oxidase is rapidly activated when the cells are exposed to appropriate stimuli.

The role of the respiratory burst oxidase in lymphocytes is obscure, though it might be speculated that the O2− generated by these cells is a signaling molecule, inasmuch as H2O2, which is rapidly produced by the spontaneous dismutation of O2−, activates the expression of genes that are under the control of the NF-κB promoter (2,3).

\[
2O_2^- \rightarrow H_2O_2 + O_2
\]

In phagocytes, however, the function of the oxidase is clear. The O2− generated by this enzyme is the precursor of a variety of powerful oxidants used by these cells as microbicidal agents. Included among these oxidants are a variety of oxidized halogens, including hypohalite ions (4,5) and an immense variety of chloramines (6). These are generated by the H2O2-mediated oxidation of halide ions under catalysis by myeloperoxidase or eosinophil peroxidase and the subsequent oxidation of amines by the product hypohalites:

\[
H_2O_2 + X^- \xrightarrow{\text{peroxidase}} \text{HOX} + OH^- [X^- = Cl^-, Br^-, I^-]
\]

\[
\text{HOX} + R-\text{NH}-R' \xrightarrow{\text{Fe or Cu}} R-\text{NX}-R' + H_2O
\]

Another group of reactive oxidants that are produced from O2− are the oxidizing radicals (7,8), generated in a transition metal-catalyzed reaction between O2− and a hydroperoxide (this is the well-known Haber-Weiss reaction, if R = H),

\[
O_2 + ROOH \xrightarrow{\text{Fe or Cu}} RO + OH^- + H_2O [R = H, -C, -(C=O)]
\]

or in a reaction between a previously generated oxidizing radical and another compound:

\[
\text{OH}^- + RH \rightarrow R^* + H_2O
\]

Finally, singlet oxygen has been found to be produced by neutrophils and eosinophils (9-11), possibly through a reaction between hypohalite and H2O2:

\[
\text{HOX} + H_2O_2 \rightarrow \text{O}_2 + X^- + H_2O
\]

It is evident that the production of large quantities of oxidants with the reactivity of the foregoing will generate an environment that is lethal for any microorganism exposed to it, but is at the same time highly destructive to nearby tissues. It therefore makes sense that the activity of this enzyme is tightly regulated both spatially and temporally, so that it releases its dangerous product only under appropriate circumstances. As a concomitant feature of its highly regulated nature, the enzyme is structurally complex, composed of a number of subunits that are distributed between two locations in resting cells but come together to assemble the active oxidase when the cell is stimulated. The following section first describes the properties of the components of the oxidase, followed by a review of what is known about oxidase activation at a molecular level.

The Components

In the resting cell, the components of the respiratory burst oxidase are distributed between the membrane and the cytosol.

Membrane-associated Components

Membrane-associated components were first discovered by two groups of Japanese workers some 30 years ago (12,13) and were later connected to the respiratory burst oxidase by experiments with neutrophils from patients with chronic granulomatous disease (CGD), an inherited disorder in which the respiratory burst oxidase is missing or fails to function (14,15). Purification and cloning disclosed two oxidase-specific components, gp91phox and p22phox, that together formed a flavohemoprotein known as cytochrome b558.

\[
\text{gp91phox}
\]

This is a membrane-associated glycoprotein consisting of a 56-kDa polypeptide that is glycosylated with several high-mannose oligosaccharides that raise its molecular weight to a value of approximately 90 kDa (16,17). It has five to six transmembrane regions and a cytoplasmic C-terminus, -PRGVHIFNKENF, that represents a docking site for a complex of cytosolic subunits (18).
**p22^phox**. The other membrane-associated subunit is a simple protein with a single transmembrane region (15,20). It contains a sequence, -PPSNPPRP-, that is highly homologous to the recently discovered sequence of an SH3 domain-binding region (21).

**Cytochrome b556**. Cytochrome b556 is an electron-transporting component (some think it is the electron-transporting component) of the respiratory burst oxidase. It is probably a trimer containing one gp91^phox^ and two p22^phox^ subunits. It contains two hemes shown by resonance Raman spectroscopy to be in an unusual environment and currently thought to be shared between the three subunits (22). It also appears to contain FAD (23–25).

This cytochrome has some characteristic properties. Its redox potential is −245 mV, a very low value for heme proteins. On optical spectroscopy, the oxidized cytochrome shows a Soret peak at 428 nm that increases in size and shifts to 413 nm when the cytochrome is reduced. The reduced cytochrome also shows α and β peaks at 558 and 528 nm, respectively. Heme ligands such as CN^−^, N_3^−^, CO, and butyryl isonitrile bind weakly or not at all. As to the role of the cytochrome in electron transport, it is generally held that the heme is the terminal electron carrier, passing electrons directly to oxygen, but this idea is not supported by kinetic experiments, which suggest that the rate of reduction of the heme is not compatible with an obligatory role in electron transport.

**Rap 1A**. Rap1A is a low molecular weight GTP-binding protein in neutrophil membranes. A possible role for rap1A in the function of the respiratory burst oxidase was suggested by the finding that rap1A copurified with cytochrome b558 (26). This protein, however, is found in membranes from CGD neutrophils that lack cytochrome b558 so it is not an intrinsic component of the cytochrome.

**Components in the Cytosol**

**p47^phox^**. The cytosolic protein p47^phox^ is an oxidase component with a molecular weight of about 45 kDa and a pI of 5.10. It contains a highly cationic C-terminal region with several protein kinase A and protein kinase C target sequences, and two SH3 domains, a feature often seen in proteins that associate with the actin cytoskeleton. During neutrophil activation, p47^phox^ becomes extensively phosphorylated on serine residues. It is likely that the phosphorylation of this protein has something to do with the activation of the respiratory burst oxidase, but unequivocal evidence establishing a cause-and-effect relationship between these two events is not yet available.

**p67^phox^**. A second cytosolic component is p67^phox^, a protein of 65 kDa that is also necessary for oxidase activity. Apart from its two SH3 regions, p67^phox^ seems to have no distinguishing features, and its function in the respiratory burst oxidase is not understood.

**A Cytosolic Complex**. When neutrophil cytosol is subjected to gel filtration over Superose 6, p47^phox^ and p67^phox^ appear together, both migrating with an M, of ~240 K. Some p47^phox^ is also run with an Mr of ~50 K, but all the p67^phox^ in the cytosol is found in the ~240 K peak. These findings suggest that p47^phox^ and p67^phox^ exist in the cytosol as a ~240 K complex. They further suggest that the quantity of p47^phox^ in the cytosol exceeds that of p67^phox^.

**rac1/rac2**. It has been known for some time that the respiratory burst oxidase requires an active guanine nucleotide-binding protein (GN-binding protein) for its operation. Thus GTP (or, better, a non-hydrolyzable GTP analog such as GTPγS) must be added to the cell-free oxidase-activating system to enable it to manufacture O_2_. It has recently been shown that rac1 and rac2 can fulfill the G-binding protein requirement of the oxidase-activating systems from mouse macrophages and human neutrophils, respectively (27,28). Furthermore, rac1 was isolated from mouse macrophage cytosol as a complex with rhGDI, a protein that retards the activation of rac1 by preventing the dissociation of GDP from its active site, suggesting that rhGDI may participate in the mechanism of activation of the respiratory burst oxidase (28). On the other hand, it is not clear which of several GN-binding proteins is the "right" one. At least three of these proteins — rac1, rac2, and the membrane-associated protein rap1A (29) — seem to work in the cell-free oxidase activating system, suggesting that any or all of them might be the right GN-binding protein. Moreover, there may be others yet to be discovered that also work in the cell-free oxidase activating system.

**Mechanism of Activation of the Respiratory Burst Oxidase**

The respiratory burst oxidase appears to be activated by the assembly of its various components into a catalytically functioning enzyme in response to a stimulus. Activation is associated with the transfer of the p47^phox^/p67^phox^ complex to the plasma membrane. At a molecular level, there is evidence that p47^phox^ binds directly to the membrane and that at least one of the sites of interaction between the membrane and the cytosolic complex consists of the 13 C-terminal amino acid residues of gp91^phox^ (18), the larger of the two subunits that make up cytochrome b558. The association between the p47^phox^/p67^phox^ complex and cytochrome b556 may be necessary to establish a complete electron transport chain between NADPH and oxygen, because the NADPH binding site seems to be associated with a cytosolic component, while a flavin required for oxidase activity is in the membrane, probably bound to cytochrome b558.

In both resting and activated neutrophils, cytochrome b556 is associated with the cytoskeleton (30, 31). In the activated neutrophil, the p47^phox^/p67^phox^ complex also is associated with the cytoskeleton (32), and the O_2_-forming activity of the activated plasma membranes is found in the cytoskeletal fraction. The attachment of the respiratory burst oxidase to the cytoskeleton presumably serves to allow oxidase activation by a particulate stimulus (e.g., a microorganism) to be restricted to the area of membrane in contact with the particle.

Cross-linking experiments suggest that the transfer of the p47^phox^/p67^phox^ complex to the plasma membrane results from a change in the structure of the complex that creates a membrane-binding site upon oxidase activation (33). When neutrophil cytosol was briefly exposed to a 3,3'-dithio-bis(sulfosuccinimidylpropionate) (DTSSP), a cleavable cross-linking agent, it lost a substantial fraction of its ability to support O_2_ production by the cell-free oxidase-activating system in response to SDS. Much of this activity was restored, however, when the DTSSP was cleaved with mercaptoethanol. Control cytosols treated with the noncleavable cross-linker bis(sulfosuccinimidyl)suberate (BS^S^) also lost much of their O_2_ -generating activity in the cell-free system, but mercaptoethanol had no effect on the BS^S^-treated cytosol. DTSSP treatment greatly inhibited the SDS-induced transfer of p47^phox^ from neutrophil cytosol to the plasma membrane in the cell-free system, but transfer was partly restored when the cross-linked cytosol was treated with mercaptoethanol. It was concluded from these results that some sort of structural alteration in the cytosolic oxidase components was necessary for oxidase activation to take place. Gel filtration experiments indicated that this structural change did not involve a major alteration in the molecular weight of the p47^phox^/p67^phox^ complex.
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