

SH3-dependent Assembly of the Phagocyte NADPH Oxidase

By Linda C. McPhail

From the Department of Biochemistry and Department of Medicine, Division of Infectious Diseases, Wake Forest University Medical Center, Winston-Salem, North Carolina 27157

Phagocytic cells contain a complicated enzyme system, termed NADPH oxidase, that is responsible for the production of toxic oxygen species (1). The enzyme transfers electrons from NADPH to O_2 , forming O_2^- , which then can dismutate into H_2O_2 . Subsequently, other oxygen derivatives, such as hydroxyl radical and hypochlorous acid, may be formed. Collectively, these oxygen products are toxic to components in their environment, either phagocytosed or extracellular microorganisms or surrounding tissue (1, 2). Thus, the NADPH oxidase of phagocytic cells is an important participant in both host defense and inflammatory mechanisms. This is well-illustrated by the genetic disorder, chronic granulomatous disease (CGD), in which the NADPH oxidase enzyme is defective. Patients with CGD suffer from frequent and severe infections, as well as noninfectious complications, such as lymphadenopathy and hepatomegaly (3).

Components of NADPH Oxidase. The NADPH oxidase enzyme consists of at least four polypeptide components (1, 3, 4). Two of the components, termed gp91-phox and p22-phox, form an unusual heterodimeric cytochrome *b*, cytochrome b_{558} . The other two components, termed p47-phox and p67-phox, are cytosolic proteins that assemble with cytochrome b_{558} during activation of the enzyme. The nomenclature used designates the size of each protein by SDS-PAGE and indicates that each protein is a component of the phagocyte oxidase ("gp" denotes glycoprotein, "p" denotes protein). At least one additional protein, the small GTP-binding protein Rac (either Rac1 or Rac2), is needed for NADPH oxidase activation (5–7). The mechanism by which GTP-bound Rac influences the enzyme is unknown, but is under intense investigation (8, 9). A sixth oxidase-related protein, termed p40-phox, recently has been identified (10, 11), which has sequence similarity to p47-phox and p67-phox and appears to physically associate with p67-phox. Defects in any of the four genes that code for components of the NADPH oxidase enzyme system can cause CGD (3). In most cases, the gene defect results in the absence of the protein product and, thus, the absence of NADPH oxidase activity. Both gp91-phox and p22-phox are usually absent if either gene is defective, suggesting each is unstable without the other. A few cases have been reported where cytochrome b_{558} is present, but non-functional, and these are usually caused by point mutations in the gene for either gp91-phox or p22-phox. These have been particularly informative for gaining insights into structure/function relationships, as discussed below. Additional information on the molecular genetics of CGD can be found in recent excellent reviews (3, 12).

Activation of NADPH Oxidase. NADPH oxidase is inactive until the cell is stimulated by phagocytosis or various inflammatory mediators (e.g., chemoattractants, cytokines). Binding of an agonist to its cell-surface receptor triggers various signal transduction pathways (13). While a number of signaling intermediates (e.g., phospholipases, protein kinases) have been implicated as regulators of NADPH oxidase activation, a complete pathway has not been defined. Complexity is increased by the likelihood that multiple pathways are involved (14, 15). However, it is clear that activation of NADPH oxidase culminates in assembly of p47-phox and p67-phox with cytochrome b_{558} in the membrane (16–21). With the development of a cell-free system that models the assembly/activation process (3, 22–26) and the cloning of the NADPH oxidase components, it has been possible to begin addressing the structural features of the components involved.

Structure of NADPH Oxidase Components. Cloning of the four NADPH oxidase components (27–32) has revealed that the predicted sequence of each protein is unique, with only limited regions of similarity to other known proteins. The gp91-phox subunit of cytochrome b_{558} has weak homology to NADPH and FAD binding sites found in the ferredoxin reductase family of flavoproteins (33–36). This suggests that cytochrome b_{558} may be a flavocytochrome, capable of carrying out the entire electron transfer from NADPH to O_2 . Recent models (36, 37) postulate that a conformational change in gp91-phox is needed to either enhance NADPH binding and/or facilitate electron transfer between NADPH and FAD. Such a conformational change could be induced by assembly of the cytosolic components with the cytochrome. Experimental support for cytochrome b_{558} as a flavocytochrome has appeared from several laboratories (20, 33–35, 38, 39), although the model is not universally accepted (40, 41).

Several predicted structural features of p47-phox and p67-phox also are of interest. The COOH-terminal region of p47-phox contains six to eight clustered putative phosphorylation sites. It has been shown that p47-phox is phosphorylated during stimulation of intact neutrophils, yielding up to eight phosphospecies ranging from \sim pI 6.8 to 10 (42–44). Phosphorylation of the clustered sites could dramatically alter the conformation of the protein (43). However, direct evidence that phosphorylation of p47-phox is required for NADPH oxidase activation is lacking (45–48). Both p47-phox and p67-phox contain another important structural feature, Src homology 3 (SH3) domains. Each protein contains two of these regions (Fig. 1). The newly described p40-phox also contains one SH3 region (10). SH3 domains were originally described

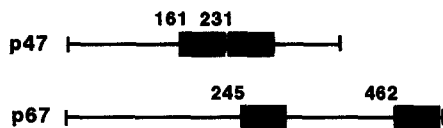


Figure 1. Schematic diagram showing positions of the SH3 domains of the cytosolic NADPH oxidase components p47-*phox* and p67-*phox* (32). Each SH3 region is comprised of about 60 amino acid residues.

in the Src family of tyrosine kinases and are found in a variety of proteins involved in signal transduction (49). They appear to mediate protein-protein interactions via binding to proline-rich sequences in target proteins (50). Several of the oxidase components contain proline-rich sequences that could be binding sites for SH3 domains (51–53). It is likely that SH3-mediated protein-protein interactions are involved in NADPH oxidase assembly, as discussed more fully below.

Requirement for SH3 Domains in NADPH Oxidase Activation. The SH3 regions of both p67-*phox* and p47-*phox* are necessary for NADPH oxidase activation. A role for the SH3 domain of p40-*phox* has not been explored. Studies by de Mendez et al. (54) showed that deletion mutants of p67-*phox*, lacking one or both SH3 regions, were unable to restore the ability to activate NADPH oxidase when transfected into p67-*phox*-deficient B cell lines derived from patients with this form of CGD. In contrast, p67-*phox* mutant proteins lacking the SH3 regions were active in a cell-free reconstitution system. This suggests that the SH3 domains of p67-*phox* are required for a signaling function in the intact cell, but this function is bypassed or replaced by the activator in the cell-free system. The non-SH3 portion of p67-*phox* is still needed in the cell-free system and, thus, must have an additional function in the assembly/activation process. Evidence is emerging that the two SH3 regions of p47-*phox* also are critical for assembly of an active NADPH oxidase, in both transfected cell and cell-free systems. Preliminary results from Leto et al. (55) indicate that truncated forms of p47-*phox* lacking one or both SH3 regions are inactive in the B cell transfection model. Also, Sumimoto et al. (53) demonstrated inhibition of cell-free activation of NADPH oxidase by a glutathione-S-transferase (GST)-fusion protein containing the two SH3 domains of p47-*phox* (GST-p47-SH3), presumably acting as a competitive inhibitor for SH3-dependent interactions mediated by the native protein.

Interaction of SH3 Domains with Pro-rich Sequences in NADPH Oxidase Components. Several groups recently have identified interactions between proline-rich sequences in NADPH oxidase components and SH3 domains of p47-*phox* and p67-*phox*. Table 1 lists the Pro-rich sequences present in NADPH oxidase components, based on consensus sequences identified in other proteins (50). At least three possible SH3 region/Pro-rich sequence interactions have experimental support, and each will be described separately. The first is binding between the second SH3 domain of p67-*phox* and the COOH-terminal Pro-rich sequence in p47-*phox*. Finan et al. (52) showed that a GST-fusion protein, containing the second SH3 domain of p67-*phox*, bound to p47-*phox*. Leto et al. (51), in a complemen-

Table 1. Proline-rich Sequences in NADPH Oxidase Components

Component*	Sequence†
p22- <i>phox</i>	
133–142	<u>P</u> <u>E</u> PKPR <u>R</u> EP
149–162	K <u>Q</u> PPSN <u>PP</u> PP <u>PP</u> AE
176–190	AG <u>GG</u> PP <u>GG</u> P <u>Q</u> V <u>N</u> P PV
p47- <i>phox</i>	
70–83	R I <u>PH</u> L <u>P</u> AP <u>K</u> W <u>F</u> EDG
338–351	<u>Q</u> AR <u>PG</u> P <u>Q</u> SP <u>GS</u> PLE
358–371	<u>R</u> SK <u>P</u> Q <u>PA</u> V <u>PP</u> RP <u>SA</u>
p67- <i>phox</i>	
219–235	<u>PL</u> Q <u>P</u> QAA <u>E</u> PP <u>PP</u> R <u>P</u> KT <u>PE</u>

* Residue numbers corresponding to sequences are provided.

† Given in single letter code. Positions of prolines conserved in functional Pro-rich sequences of other proteins are underlined (50–52).

tary series of experiments, came to a similar conclusion. Both groups pinpointed the SH3 binding site to a COOH-terminal Pro-rich sequence in p47-*phox* (aa 362–369), using either synthetic peptide inhibitors or GST-fusion proteins containing truncated forms of p47-*phox*. Sumimoto et al. (53) also have proposed an SH3-dependent interaction between p47-*phox* and p67-*phox*. A GST-fusion protein containing the SH3 domains of p47-*phox* bound p67-*phox*, only in the presence of the cell-free reconstitution system activator arachidonic acid. Binding site(s) in p67-*phox* were not identified. Thus, this interaction differs from that described by Finan et al. (52) and Leto et al. (51), in that the SH3 domains of p47-*phox*, rather than an SH3 region of p67-*phox*, mediates binding of the two proteins. It remains to be determined whether either or both interactions occur in the intact cell.

The second interaction involves binding of the SH3 domains of p47-*phox* to a cytoplasmic region of p22-*phox* containing three Pro-rich sequences. Evidence to support this interaction comes from three different groups. Sumimoto et al. (53) showed that a GST-fusion protein containing both SH3 domains of p47-*phox* bound to GST-fusion proteins containing the two most COOH-terminal, or only the middle, Pro-rich sequence of p22-*phox*. A natural mutation in the middle Pro-rich region in p22-*phox* (P156Q) has been reported in a patient with CGD (56). This mutation was introduced into GST-fusion proteins and resulted in markedly reduced binding of GST-p47-SH3. Leto et al. (51) obtained the same results using a similar series of GST-fusion proteins. This group also showed that synthetic peptides containing the middle Pro-rich sequence of p22-*phox* (aa 149–162) abolished the binding between GST-p47-SH3 and GST-p22 (aa 127–195), while peptides based on the COOH-terminal Pro-rich sequence (aa 170–195) or containing the P156Q mutation, were ineffective. These results suggest that the interaction between the SH3 regions of p47-*phox* and p22-*phox* depends on Pro

156 and raise the possibility that this Pro-dependent interaction is critical for assembly of an active enzyme.

Leto et al. (51) explored this possibility in transfected cell lines. Transfection of wtp22-*phox*, but not p22-*phox* containing the P156Q mutation, into p22-*phox*-deficient B cell lines corrected the defect in NADPH oxidase activation. Also, the ability of p47-*phox* to translocate to the membrane depended on the presence of wtp22-*phox* in K562 cells transfected to express both proteins. In contrast, p47-*phox* did not translocate in K562 cells co-transfected with the P156Q mutant form of p22-*phox*. An article by Leusen et al. (57), appearing in this issue of *The Journal of Experimental Medicine*, extends these results in a study using neutrophils from a patient with the P156Q mutation in p22-*phox*. Cells from the patient expressed normal levels of a cytochrome *b*₅₅₈ that was nonfunctional for activation of NADPH oxidase, in agreement with a previous report (56). Translocation of p47-*phox* and p67-*phox* to the membrane did not occur in either stimulated intact cells from the patient or the cell-free reconstitution system. The “nonfunctional” cytochrome was capable of electron transfer in an artificial cell-free system not requiring cytosolic components (37). Thus, the P156Q mutation in p22-*phox* found in this patient prevents assembly of the NADPH oxidase complex, and no enzyme activation occurs. Collectively, these data support a model in which assembly of the cytosolic components with cytochrome *b*₅₅₈, mediated by SH3-region/Pro-rich sequence interactions between p47-*phox* and p67-*phox* and between p47-*phox* and p22-*phox*, results in a conformational change in gp91-*phox* necessary for transport of electrons from NADPH to O₂ (Fig. 2).

The P156Q mutation in p22-*phox* is the second example of a genetic disease caused by disruption of protein-protein interactions mediated by SH3 domains. Zhu et al. (58) recently reported a deletion in the SH3 region of Bruton's tyrosine kinase, which resulted in X-linked agammaglobulinemia.

The third SH3-dependent interaction of NADPH oxidase components involves intramolecular binding of the SH3 domains of p47-*phox* to Pro-rich sequences within the molecule. GST-fusion proteins containing the SH3 regions of p47-*phox* were shown by Leto et al. (51) and Sumimoto et al. (53) to bind to regions of p47-*phox* containing Pro-rich sequences. Data from the two groups are consistent in implicating the most COOH-terminal Pro-rich sequence in p47-*phox* (aa 358–390) as one binding site. Leto et al. also provided evidence suggesting the most NH₂-terminal Pro-rich sequence (aa 70–84) is a second target. Additional studies with synthetic peptides and mutant or truncated proteins are needed to pinpoint the interaction sites more clearly. Leto et al. (51) speculate that such an intramolecular interaction in p47-*phox* folds the molecule in an “inactive” state, and the interaction must be disrupted in order for assembly of NADPH oxidase

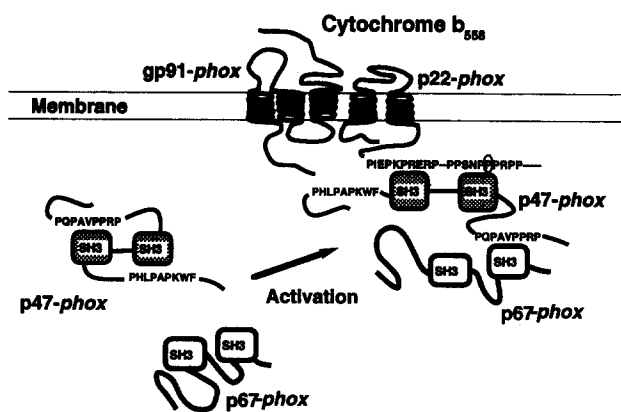


Figure 2. Model for SH3-dependent assembly of NADPH oxidase. In the resting state, p47-*phox* is folded such that the SH3 regions undergo an intramolecular interaction with Pro-rich sequences near the NH₂- and COOH-terminal portions of the protein. Activation induces a conformational change in p47-*phox*, allowing the SH3 domains to bind Pro-rich sequences in the cytoplasmic tail of p22-*phox*. The P156Q mutation in p22-*phox* (circled) disrupts this interaction. After activation, the COOH-terminal Pro-rich sequence of p47-*phox* is also free to interact with the COOH-terminal SH3 region of p67-*phox*. A complex between p47-*phox* and p67-*phox* may exist before activation and could involve other interactions (not illustrated). The consequence of the SH3-mediated assembly process may be the induction of conformational changes in gp91-*phox*, which are permissive for electron transfer between NADPH and O₂ (not illustrated). (Reproduced from *Proc. Natl. Acad. Sci. USA*, 1994, 91:10650, by copyright permission of the National Academy of Sciences [Washington, DC].)

components to occur (Fig. 2). Experimental evidence to support this model was provided by Sumimoto et al. (53). A monoclonal antibody specific for the SH3 domains of p47-*phox* immunoprecipitated native p47-*phox* (present in cytosolic fractions from HL-60 cells differentiated along the neutrophilic pathway) only in the presence of arachidonic acid or SDS. These amphiphilic molecules are used in cell-free reconstitution systems to induce assembly and activation of NADPH oxidase. Possibly, the amphiphiles induce a conformational change in p47-*phox*, “unmasking” the SH3 regions to allow interaction with other NADPH oxidase components. One can speculate that a physiological mechanism to achieve this conformational change is phosphorylation of multiple sites in the COOH-terminal region of p47-*phox*.

Conclusion. Considerable progress has been made in understanding the SH3-dependent interactions between components of NADPH oxidase. Such interactions appear to be crucial for assembly of an active enzyme. An intriguing model for SH3-mediated assembly, consistent with the data summarized here, has been put forth by Leto et al. (51) (see Fig. 2 and discussions above). Future research with this complex enzyme system will test and modify this model. What a fascinating story is unfolding!

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