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J Clin Invest. 1996;98(4):977-983. https://doi.org/10.1172/JCI118882.

Research Article

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The Cytosolic Subunit p67^{phox} Contains an NADPH-binding Site that Participates in Catalysis by the Leukocyte NADPH Oxidase

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Abstract

The NADPH-dependent respiratory burst oxidase of human neutrophils catalyzes the reduction of oxygen to superoxide using NADPH as the electron donor and is essential for normal host defenses. To gain insight into the function of the various oxidase subunits that are required for the full expression of catalytic activity, we studied the interactions between the 2′,3′-dialdehyde derivative of NADPH (NADPH dialdehyde) and neutrophil cytosol. NADPH dialdehyde treatment of cytosol resulted in the loss of the ability of the cytosol to participate in cell-free oxidase activation; this inactivation was blocked by NADPH but not by NAD, NADP, or GTP. Partial purification of neutrophil cytosol yielded a single peak which could restore the activity lost in cytosol treated with NADPH dialdehyde. This peak contained p67^{phox}, but not p47^{phox} or Rac2. Purified recombinant p67^{phox} was similarly able to restore the activity lost in NADPH dialdehyde–treated cytosol and bound [^{32}P]NADPH dialdehyde in a specific fashion. The activity of recombinant p67^{phox} in cell-free oxidase assays was lost on treatment with NADPH dialdehyde. Together, these data suggest p67^{phox} contains the catalytic NADPH-binding site of the leukocyte NADPH oxidase. (*J. Clin. Invest.* 1996. 98:977–983.) Key words: NADH • NADPH oxidoreductases • neutrophils • cytosol • respiratory burst • affinity labels

Introduction

An essential feature of normal host defense is the ability of phagocytic cells to deploy a group of highly reactive oxygen intermediates as microbicidal agents (1, 2). The initial step in the production of these reactive oxidants is the one-electron reduction of oxygen to superoxide utilizing NADPH as the electron donor, a reaction catalyzed by a unique multicomponent enzyme system known as the leukocyte NADPH oxidase or respiratory burst oxidase (2–5). Inherited defects in any of the components of the oxidase result in chronic granulomatous disease (CGD),[1] a disorder characterized by frequent life-threatening infections and early mortality (6–8). Examination of individuals afflicted by CGD has allowed identification of the essential components of the oxidase. These components include the two membrane-bound polypeptides p22^{phox} and gp91^{phox} (9–11), which together make up cytochrome b_{558}, and the two cytosolic polypeptides p47^{phox} and p67^{phox} (12–15), which exist largely in the form of a complex (16, 17). The components are distributed in separate compartments in the unstimulated cell, but exposure of the cell to any of a wide variety of particulate or soluble agonists induces the association of the cytosolic components with the membrane-associated components to assemble the active oxidase (16, 18, 19). This process has been reproduced in vitro using cell-free systems in which oxidase assembly can be induced by the addition of an amphopathic agent to reaction mixtures containing all of the oxidase components (20–23). Experiments using these cell-free oxidase-activating systems have shown that in addition to the components named above, the guanine nucleotide-binding protein Rac2 is also required for oxidase activation (24–28).

Despite agreement that these oxidase components are required for enzyme activity, aspects of the function of these components are unclear. During oxidase activation in intact cells, p47^{phox} is extensively phosphorylated (29–32), an event thought to participate in the initiation of enzyme assembly in vivo. The function of p67^{phox} is less well understood, although data suggest that it may interact with Rac2 (33). The membrane associated cytochrome b_{558}, which contains both FAD and heme, is generally thought to be the terminal electron carrier of the oxidase (34, 35). This cytochrome has also been postulated to contain the catalytic NADPH-binding site of the oxidase, based on sequence homologies and some affinity-labeling experiments (34–36). Earlier studies using the 2′,3′-dialdehyde analog of NADPH (NADPH dialdehyde), however, suggested that a catalytically significant NADPH-binding component was present in the cytosol. NADPH dialdehyde was found to be a substrate for the oxidase, but treatment with the dialdehyde resulted over time in the irreversible loss of the ability of the cytosol to participate in cell-free oxidase systems (37–39). Examination of this interaction showed that only one oxidase component was affected by the dialdehyde. Furthermore, the K_m for NADPH dialdehyde as an oxidase inactivator was the same as its K_m as substrate, while NADPH protected the oxidase against inactivation by the dialdehyde with a K_i that was the same as its (i.e., NADPH's) K_m as substrate (38). On the other hand, the ability of neutrophil membranes (containing cytochrome b_{558}) to support oxidase activity in the cell-free system was completely unaffected by treatment with NADPH dialdehyde (37, 39). Therefore, any NADPH binding site present on cytochrome b_{558} had to be inaccessible to the dialdehyde, at least before oxidase assembly.

To understand the significance of these observations with NADPH dialdehyde, we undertook to identify the cytosolic oxidase component that contained the dialdehyde-sensitive

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Received for publication 2 November 1995 and accepted in revised form 14 June 1996.

1. *Abbreviations used in this paper:* CGD, chronic granulomatous disease; *phox*, phagocyte oxidase, used to designate protein components of the leukocyte NADPH oxidase of phagocytes.

The Journal of Clinical Investigation
Volume 98, Number 4, August 1996, 977–983

NADPH-binding site. We now present evidence that the dialdehyde-sensitive NADPH-binding site is on p67^{phox}, and that this NADPH binding site is essential for oxidase activity.

Methods

Materials. All reagents were obtained from the Sigma Chemical Co. (St. Louis, MO) unless noted otherwise.

Preparation of neutrophil fractions. Neutrophil membrane and cytosol for cell-free assay of the leukocyte NADPH oxidase were prepared from cells harvested by leukapheresis from normal donors as described previously (38). Cell fractions were stored at −70°C until use.

Synthesis of 2′,3′ NADPH dialdehyde and treatment of neutrophil cytosol. NADPH dialdehyde was prepared by periodate oxidation of NADP followed by reduction to NADPH dialdehyde using isocitric dehydrogenase (37). [^{32}P]NADPH dialdehyde was synthesized as previously described (40). Neutrophil cytosol in which the dialdehyde-sensitive oxidase component has been inactivated with NADPH dialdehyde was prepared and used as previously described (38). Briefly, neutrophil cytosol was incubated with 0.1 mM NADPH dialdehyde in the presence of 0.5 mM $NaCNBH_3$ for 18 h at 4°C. After the incubation, the inactivated cytosol was dialyzed against cell-fractionation buffer to remove unbound NADPH dialdehyde and $NaCNBH_3$, and then stored in aliquots at −70°C for use in assays of complementing activity. As we have previously shown (37, 38), the resulting product has a markedly reduced ability to participate in a cell-free oxidase activation assay (> 95% loss of activity compared to untreated cytosol).

For examination of the sensitivity of cytosol or recombinant p67^{phox} to treatment with NADPH dialdehyde, the incubation time was reduced to 6 h and various nucleotides were added (1.0 mM final concentration) to test their ability to block the inactivation process (38).

Assay of NADPH oxidase activity. For cell-free assay of the oxidase, solubilized neutrophil membranes and cytosol, 6×10^5 and 2×10^6 cell equivalents, respectively, are usually combined in the well of a 96-well plate in the presence of 0.1 mM ferricytochrome c and 0.16 mM NADPH (38). Activation is achieved by the addition of SDS (0.04 mM final concentration) and the formation of ferrocytochrome is followed at 550 nm. Results are expressed as nanomoles ferricytochrome reduced per min per 10^7 cell equivalents of membrane in the well containing the reaction mixture minus the rate of reduction in a well containing the identical components plus 0.045 mg of superoxide dismutase. For assay of activity complementing that missing in NADPH dialdehyde–treated cytosol (complementing activity), 2×10^6 cell equivalents of dialdehyde-treated cytosol was added in place of normal cytosol. Fractions to be assayed for activity (0.05 ml) were then added and the reaction initiated with the addition of SDS. Because the dialdehyde-treated cytosol possesses minimal activity on its own, background activity was not subtracted from the assay results. For calculation of recovery, 1 U of complementing activity was defined by the ability to increase superoxide production in this assay system by 1 nmol/min per 10^7 cell equivalents of membrane. For assay of activity contributed by various cytosolic oxidase components, a cell-free reaction mixture was used as described above, but with only sufficient normal neutrophil cytosol ($0.04–0.08 \times 10^6$ cell equivalents) to supply a threshold amount of each cytosolic oxidase component. The addition of preparative fractions containing one or more of any of the cytosolic oxidase components will then lead to increased oxidase activity after activation (41). The exact quantity of added cytosol was established by titration before each set of assays, and the amount of cytosol that contributed ≈ 1–3 nmol/min per 10^7 cell equivalents of membrane of oxidase activity was used in the subsequent assays. This background signal was subtracted for calculation of activity present in chromatographic fractions; the mean background for all assays was 2.4 ± 0.4 nmol/min per 10^7 cell equivalent membrane.

Partial purification of the NADPH dialdehyde–sensitive cytosolic component of the NADPH oxidase. Chromatographic purification was conducted at 4°C. To 25 ml neutrophil cytosol, sufficient ammonium sulfate was added with gentle stirring to bring the solution to 40% saturation. Complementing activity (see above), assayed with NADPH dialdehyde–treated cytosol, was found in the precipitate, which was isolated by centrifugation (12,000 *g*, 30 min at 4°C), dissolved in 10 ml buffer (0.1 M Tris pH 8.0, 10% ethylene glycol, 0.5 mM PMSF, 0.002 mM leupeptin, 1 mM EGTA, and 3.5 mM $MgCl_2$) and dialyzed overnight against the same buffer (but with 0.02 M Tris) before assay. The dialyzed solution containing the complementing activity was applied to a 5 × 0.5 cm Mono-Q column that had been

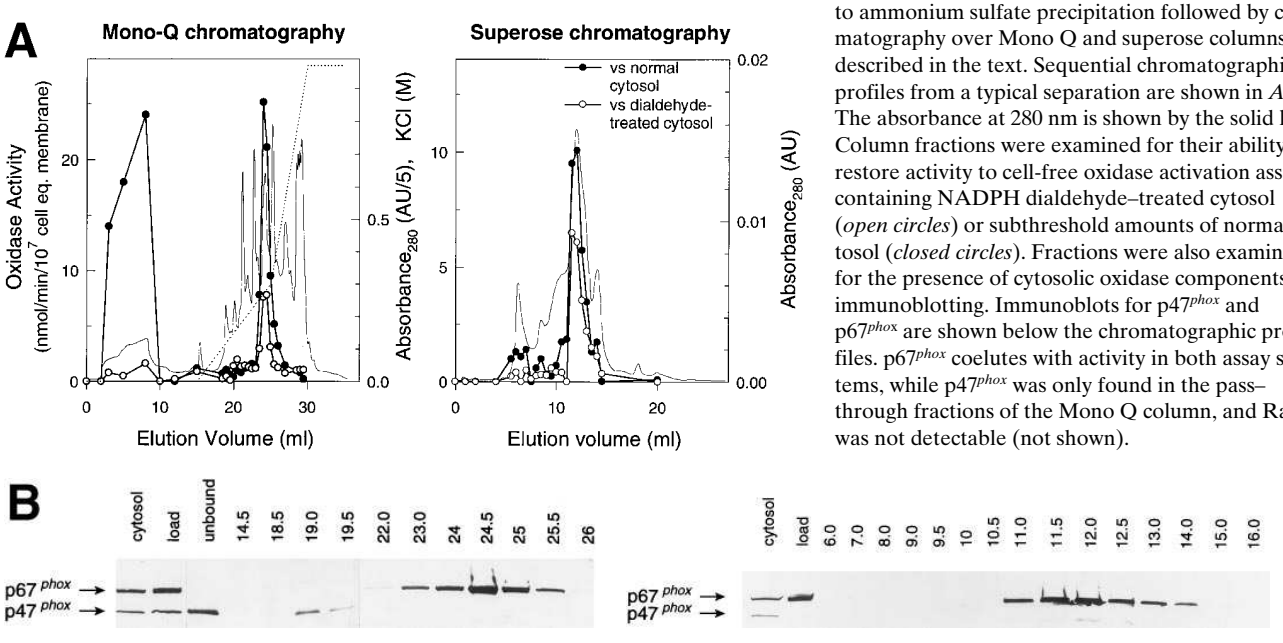

Figure 1. Partial purification of the NADPH dialdehyde–sensitive cytosolic component of the leukocyte NADPH oxidase. Neutrophil cytosol was subjected to ammonium sulfate precipitation followed by chromatography over Mono Q and superose columns as described in the text. Sequential chromatographic profiles from a typical separation are shown in *A*. The absorbance at 280 nm is shown by the solid line. Column fractions were examined for their ability to restore activity to cell-free oxidase activation assays containing NADPH dialdehyde–treated cytosol (*open circles*) or subthreshold amounts of normal cytosol (*closed circles*). Fractions were also examined for the presence of cytosolic oxidase components by immunoblotting. Immunoblots for p47^{phox} and p67^{phox} are shown below the chromatographic profiles. p67^{phox} coelutes with activity in both assay systems, while p47^{phox} was only found in the pass–through fractions of the Mono Q column, and Rac2 was not detectable (not shown).

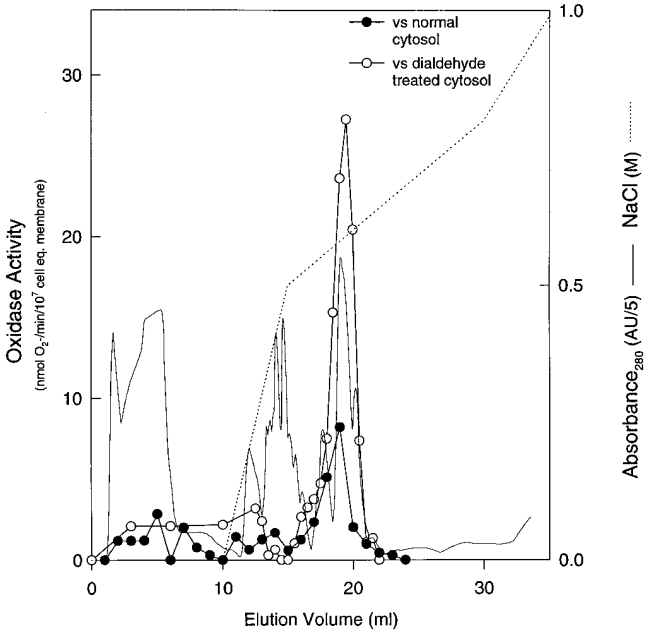

Figure 2. Ability of recombinant p67^{phox} to restore oxidase activity to a cell-free oxidase-activating system containing NADPH dialdehyde-treated cytosol. A spinner flask containing 2×10^8 SF9 cells was infected with a recombinant baculovirus expressing p67^{phox} (*left*), or with the AcMNPV vector without an insert (*right*), and then incubated at 28°C for 40 h. The cytosol fraction of the cells was prepared as described in Methods and then run over a Mono-Q column eluting with a gradient of NaCl (*dotted line*). Complementing activity in the Mono-Q fractions was assayed as described (*open and closed circles*) and the content and purity of p67^{phox} in the fractions was evaluated by immunoblotting and Coomassie blue staining. p67^{phox} was present in fractions eluting between 18 and 20.5 ml. The fractions containing antigenic p67^{phox} were coincident with those containing activity complementing NADPH dialdehyde–treated cytosol. A single polypeptide migrating at 67 kD was identified in this peak when aliquots of each fraction (15 μl) were subjected to SDS-PAGE and Coomassie staining. The fraction eluting at 19.0 ml was used in the labeling experiments shown in Fig. 3 where the Coomassie-stained gel is shown. Fractions obtained by the same purification procedure from cells infected with the wild-type virus contained no material crossreacting with anti-p67^{phox} and did not contain activity in the complementation assays using NADPH dialdehyde–inhibited cytosol or using a subthreshold amount of normal cytosol.

equilibrated with dialysis buffer, and then eluted at 0.5 ml/min by FPLC using a KCl gradient as shown in Fig. 1. Half milliter fractions were collected and 0.05-ml portions of each fraction were assayed for activity. The active fractions were pooled, concentrated to 0.25 ml in a Centricon 3 concentrator (Amicon Corp., Beverly MA) and applied to a 10×30 cm Superose 12 column equilibrated with modified relaxation buffer (0.05 M Pipes pH 7.4, 3.5 mM $MgCl_2$, 0.5 mM PMSF, 1 mM EGTA, 0.002 mM leupeptin, and 10% ethylene glycol). The column was eluted with the same buffer, running the column at 0.25 ml/min, collecting 0.5-ml fractions and assaying for complementing activity as above. For immunoblotting, 0.05 ml of each fraction was subjected to SDS-PAGE over a 12% polyacrylamide gel (42). The separated proteins were transferred to a polyvinylidene difluoride membrane (43), and p47^{phox} and p67^{phox} were detected with a 1:5,000 dilution of a mixture of rabbit polyclonal antibodies raised against 11 amino acid peptides corresponding to the carboxy termini of p47^{phox} and p67^{phox}, respectively (44), visualizing bound antibody with goat anti–rabbit IgG conjugated to alkaline phosphatase and developing with 5-bromo-chloro-3-indolyl phosphate/nitro blue tetrazolium (Western Blue; ProMega Corp., Madison, WI). Rac2 was detected in a similar fashion using a 1:500 dilution of polyclonal anti-Rac2 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA).

Preparation of recombinant p67^{phox}. A recombinant baculovirus expressing p67^{phox} was the generous gift of Dr. T.L. Leto. A spinner flask containing 2×10^8 SF9 insect cells was infected with the recombinant baculovirus (45); another flask of cells were infected with AcMNPV virus without an insert (Invitrogen Corp., San Diego, CA). The cells were incubated for 40 h before they were isolated by centrifugation (400 *g*, 10 min), washed, treated with diisopropylfluorophosphate, and subjected to nitrogen cavitation as previously described for preparation of neutrophil fractions (38). Nuclei and unbroken cells were removed by centrifugation (2,000 *g*, 10 min) and the low speed supernatant was subjected to further centrifugation (100,000 *g*, 60 min). The high-speed supernatant of the cells was subjected to Mono-Q chromatography as described for neutrophil cytosol above, but using a gradient of NaCl as shown in Fig. 2. Complementing activity in the Mono-Q fractions was assayed as described above, and the content and purity of p67^{phox} in the fractions were evaluated by immunoblotting and Coomassie blue staining, respectively.

Labeling of recombinant p67^{phox} by [^{32}P]NADPH dialdehyde. Purified recombinant p67^{phox} (10 μg, from the fraction eluting at 19 ml in the chromatogram shown in Fig. 2) was incubated for various times with 0.03 mM (~ 0.5–1.0 μCi) [^{32}P]NADPH dialdehyde in the absence and presence of 0.3 mM unlabeled NADPH dialdehyde or 5 mM NADPH in 0.1 ml buffer-containing triethanolamine HCl (0.05 M, pH 8.0) and lysine HCl (10 mM). Reaction mixtures were analyzed by SDS-PAGE followed by autoradiography. Quantitative labeling was accomplished as previously described (46). Briefly, aliquots of p67^{phox} were treated as above, and the reactions were allowed to proceed for 18 h to ensure maximum incorporation of NADPH dialdehyde. At the conclusion of the incubation, samples were exhaustively dialyzed ($n = 2$) or subjected to SDS-PAGE followed by hydrolysis in H_2O_2 of the band at 67 kD, and the incorporated radioactivity determined by scintillation counting.

Results

We isolated the NADPH-binding component from the cytosol using a modified cell-free oxidase activating system in which normal cytosol was replaced with cytosol that had been extensively inactivated (> 95%) by treatment with NADPH dialdehyde. The dialdehyde-mediated inactivation of neutrophil cytosol could be blocked by NADPH, as we had previously found (37), but not by NAD, NADP, or GTP (Table I). Fractions containing the dialdehyde-sensitive component were identified by their complementing activity, defined as their ability to restore oxidase activity to a system containing cytosol that had been treated with NADPH dialdehyde. Purification of normal neutrophil cytosol by ammonium sulfate fractionation followed by Mono-Q anion exchange chromatography and then gel filtration over a Superose column was carried out four times and consistently yielded a single major peak of complementing activity from each column (Fig. 1). Immunoblotting showed that the active fractions from both the Mono Q and Superose columns contained p67^{phox} (Fig. 1), but not p47^{phox} or Rac2 (not shown). Activity could also be detected in the pass-through fractions from the Mono-Q column when the assay was carried out with limiting quantities of normal cytosol rather than with dialdehyde-treated cytosol; these active fractions contained p47^{phox}. The fractions from the Superose column, however, showed only the single peak of activity that had been detected with dialdehyde-treated cytosol when the assays were repeated with normal cytosol. The protein content of this

Table I. Inactivation of a Cytosolic Component of the Leukocyte NADPH Oxidase by NADPH Dialdehyde

NADPH dialdehyde treatment	Added nucleotide	Complementing activity	
		cytosol	recombinant p67^{phox}
		nmol/min per 10^7 cell equivalents of membrane	
No	None	33.3±8.2	21.0±5.5
Yes	None	11.8±0.7*	10.2±2.3*
Yes	NADPH	26.1±1.2‡	20.4±6.0‡
Yes	NADP	12.9±3.4	12.9±4.6
Yes	NAD	13.7±2.1	11.4±4.3
Yes	GTP	7.2±0.5	3.9±1.1

Experiments were carried out as described previously (37), but incubations with NADPH dialdehyde were for 6 h. Blocking nucleotides were added to a final concentration of 1.0 mM. Results represent the mean± 1 SE of four separate experiments. Results were compared using a paired Student's *t* test with Bonferroni correction for multiple comparisons where appropriate (*$P < 0.05$ compared with reaction with no NADPH dialdehyde; ‡$P < 0.05$ compared with reaction with no blocking nucleotide).

peak was ~23 μg or 0.04% of the protein present in the initial aliquot of cytosol. There was a 25-fold enrichment of complementing activity (Table II), although the recovery was only ~1%. The greatest loss of activity occurred with the initial ammonium sulfate precipitation step, but significant additional losses of activity occurred over time and with each isolation step. Instability of the oxidase and its component parts during purification attempts has been noted previously and is enhanced when any individual component is separated from the original complex

Table II. Recovery of Activity Complementing NADPH Dialdehyde Treated Cytosol Through Successive Purification Steps of Normal Cytosol or SF9 Cell Extracts

	Protein	Activity	
	mg	*U/mg*	*Total units*
Neutrophil cytosol			
Starting material	56	725	40600
40% ammonium sulfate pellet	6.5	843	5480
peak from Mono-Q column	0.465	4133	1920
peak from Superose column	0.023	17950	415
SF-9 cell lysate			
peak from Mono-Q column	0.142	12394	1760

Activity complementing that lost in NADPH dialdehyde–treated cytosol was isolated as described in the text. The yields of activity from the ammonium sulfate precipitation and the successive chromatography stages displayed in Fig. 1 are shown. 1 U of complementing activity was defined as that able to increase superoxide production by 1 nmol/min per 10^7 cell equivalents of membrane in a cell-free oxidase assay performed in the presence of NADPH dialdehyde treated cytosol as described in Methods. There was a 25-fold enrichment in activity contained in neutrophil cytosol over the three steps, although there was substantial loss of total activity at each step. Chromatography of extracts of SF-9 cell infected with recombinant baculovirus expressing p67^{phox} over the Mono-Q column yielded a peak of activity that was comparable to that of the purified neutrophil cytosol fraction.

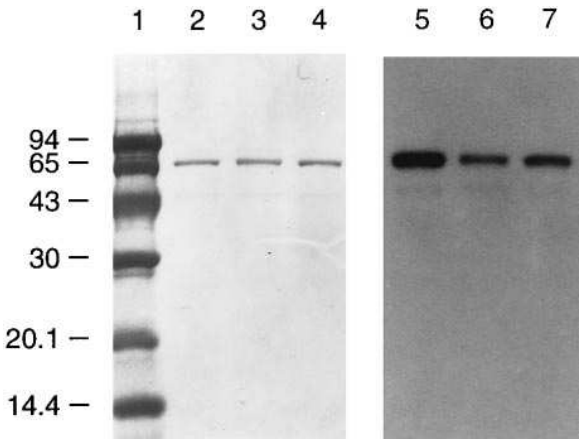

Figure 3. Labeling of recombinant p67^{phox} by [^{32}P]NADPH dialdehyde. Purified recombinant p67^{phox} (10 μg, from fraction eluting at 19.0 ml in chromatogram shown in Fig. 2) was incubated for 120 min at room temperature with 0.03 mM (≈ 0.5–1.0 μCi) [^{32}P]NADPH dialdehyde in the absence (*left*, lanes *2* and *5*) and presence of 0.3 mM unlabeled NADPH dialdehyde (lanes *3* and *6*), or 5 mM NADPH (lanes *4* and *7*) in 0.1 ml total volume in buffer containing triethanolamine HCl (0.05 M, pH 8.0) and lysine HCl (10 mM). Reaction mixtures were analyzed by SDS-PAGE (as described in Fig. 2) followed by autoradiography. The experiment was performed three times and a representative Coomassie blue–stained gel (lanes *1–4*) and autoradiograph (lanes *5–7*) are shown. Molecular weight markers were run in lane *1* and the estimated molecular weights shown to the left of the lane. The time course of labeling of p67^{phox} by [^{32}P]NADPH dialdehyde was examined in a similar fashion (*right*). Aliquots of a labeling reaction were removed at various time points and treated with SDS sample buffer to stop the reaction. The samples were analyzed by SDS-PAGE and autoradiography, and the extent of incorporation of label was examined by scanning densitometry (Ultrascan; Pharmacia LKB Biotechnology, Uppsala, Sweden). Incorporation of label in the presence of an excess of unmodified NADPH dialdehyde was interpreted as nonspecific. When this background signal was subtracted, the difference could be fit to the equation $y = 0.221(1\text{-}e^{(-0.197*t)})$ (*dashed line*), suggesting an approximate half time for labeling of 3.5 h.

of cytosolic components (41, 47, 48). SDS-PAGE of the final Superose peak showed at least six polypeptides, one of which migrated an apparent molecular weight of 67 kD and was identified as p67^{phox} by immunoblotting. Thus of the three cytosolic components required for the cell-free oxidase system (i.e., p47^{phox}, p67^{phox}, and Rac2) only p67^{phox} was found to be present in the fractions containing complementing activity. These findings suggested p67^{phox} may be the NADPH dialdehyde–sensitive cytosolic component of the leukocyte NADPH oxidase.

To confirm that p67^{phox} contained an essential NADPH-binding site and was responsible for reconstituting the activity in our assay system, we carried out experiments to determine whether recombinant p67^{phox} expressed in a baculovirus system and purified to homogeneity demonstrated complementing activity. The results showed that purified recombinant p67^{phox} restored activity to cytosol treated with NADPH dialdehyde (Fig. 2 and Table II), while a similar preparation from SF9 cells infected with wild-type baculovirus had no effect. The complementing activity of the recombinant p67^{phox} was abolished by treatment with NADPH, though not by NAD, NADP, or GTP (Table I). That the loss of p67^{phox}-complementing activity by treatment with NADPH dialdehyde was

Table III. Similarities Between the Sequence of p67^{phox} and the Sequences of Putative Pyridine Nucleotide-binding Sites of NADPH-dependent Enzymes

Protein	Putative NADPH-binding sequence
p67^{phox} (4–23)	VEAISLWNEGVLAADKKDWK
p67^{phox} (193–212)	LAKKDYLGKATVVASVVDQD
p67^{phox} (235–254)	EIFRALEGEAHRVLFGFVPE
gp91^{phox} (400–418)	YEVVMLVGAG-IGVTPFASR
Nitric oxide synthase (rat)	ISSCILVGPG-TGIAPFRSF
Pyridine nucleotide transhydrogenase (*Escherichia coli* 307–326)	HSVIITPGYGMAVAQAQYPV
Glutathione reductase (human)	PGRSVIVGAGYIAVEMAGIL
Mercuric reductase (*Shigella*)	PKRLAVIGSSVVALELAQAF
Pyridine nucleotide transhydrogenase (*E. coli* 421–440)	IVFKRSMNTGYAGVQNPLFF

The p67^{phox} sequence contains regions homologous to NADPH-binding proteins and to the proposed NADPH-binding region of gp91^{phox} (11, 12).

not associated with apparent fragmentation of the molecule was shown by examination of reaction mixtures by SDS-PAGE and subsequent Coomassie staining (Fig. 3) or immunoblotting (data not shown).

Affinity labeling studies showed that p67^{phox} was labeled by [^{32}P]NADPH dialdehyde (Fig. 3). Labeling by the ^{32}P-tagged dialdehyde was decreased significantly by unlabeled NADPH dialdehyde and by NADPH. Excesses of NAD, NADP, or GTP had no effect on the labeling of the protein (not shown). When [^{32}P]NADPH dialdehyde labeling was carried out overnight, quantitation of bound radioactivity showed that 0.83±0.11 mol of NADPH dialdehyde were bound per mol of p67^{phox} ($n = 4$ using two different p67^{phox} preparations). Binding of [^{32}P]NADPH dialdehyde in the presence of an excess of cold NADPH dialdehyde was 0.16±0.01 mol/mol p67^{phox}. If this signal is interpreted as nonspecific binding, then the stoichiometry of [^{32}P]NADPH dialdehyde binding to p67^{phox} is 0.67 mol/mol protein. Examination of the time course of labeling (Fig. 3, *right*) suggested that specific incorporation of radioactivity parallelled the time course of inactivation of cytosol by NADPH dialdehyde we have previously reported (37). Incorporation of label into p67^{phox} at 6 h (subtracting background labeling in the presence of an excess of cold NADPH dialdehyde) was ~ 70% of the maximum value, a value similar to the extent of inactivation of recombinant p67^{phox} by NADPH dialdehyde shown in Table I.

Discussion

Some time ago, we extended a study of Minakami's (49) by examining the effect of the 2′,3′-dialdehyde analog of NADPH on the activity of a cell-free O_2^--forming system from resting neutrophils. We found that NADPH dialdehyde was a substrate for the oxidase, but that over time the dialdehyde destroyed the ability of resting cytosol to support O_2^- production in the cell-free system (37). Kinetic studies showed that the K_m for NADPH dialdehyde as an oxidase inactivator was the same as its K_m as substrate, while native NADPH protected the cytosol against inactivation by the dialdehyde with a K_i that was the same as its (i.e., NADPH's) K_m as substrate. These results indicated that NADPH dialdehyde inactivated the oxidase by behaving as an affinity label, forming a Michaelis complex at the NADPH binding site of a cytosolic component of the oxidase, and over time attaching itself there irreversibly to destroy catalytic activity of the enzyme:

$$\text{E + dNADPH} \underset{\text{fast}}{\rightleftharpoons} \text{E·dNADPH} \xrightarrow[\text{slow}]{} \text{E-dNADPH (inactive)}$$

(where dNADPH is NADPH dialdehyde)

We have now identified the cytosolic NADPH binding component of the leukocyte NADPH oxidase as p67^{phox}, and have demonstrated further that the NADPH-binding function of p67^{phox} is essential for normal oxidase activity.

Several proteins have been nominated as candidate NADPH binding components of the oxidase, including a 66-kD membrane protein of undefined location (p67^{phox}?), an 84-kD membrane protein distinct from gp91^{phox}, a 52–57-kD membrane protein, and, as discussed above, gp91^{phox} itself (34–36, 49–51). Most of these identifications were based on sequence homologies and affinity-labeling experiments, although there is good enzymological evidence for a catalytically functional NADPH binding site on gp91^{phox} (see below). In the present study, the identification of p67^{phox} as an NADPH binding oxidase subunit was suggested by sequence homologies between p67^{phox} and the postulated pyridine nucleotide-binding sites of a number of NADP(H)-dependent enzymes (Table III). However, the inability of other investigators to define consensus canonical cofactor binding sequences for NADP(H) dependent enzymes (52) suggest that sequency homologies are likely to provide only weak evidence about the role of p67^{phox} as an NADPH-binding protein, and that conclusions regarding NADPH binding by p67^{phox} have to be based on other approaches. Final identification of p67^{phox} as an NADPH-binding protein was accomplished by affinity labeling of p67^{phox} with radioactive NADPH dialdehyde and by examining the activity of NADPH dialdehyde-treated and untreated p67^{phox} in the cell-free oxidase activating system. The latter measurements established the significance of the NADPH binding capacity of p67^{phox} for the catalytic activity of the oxidase. In an earlier study, we found a ~ 30-kD polypeptide that was translocated from cytosol to membranes of activated neutrophils and could be labeled with [^{32}P]NADPH dialdehyde. The properties of this polypeptide suggested it might be the catalytic NADPH-binding component of the oxidase (40); we now believe it was a fragment of p67^{phox}, whose susceptibility to proteolysis is well known.

Mizunari et al. studied the binding of several nucleotides to the cytosolic components of the leukocyte NADPH oxidase (53). From their finding that p67^{phox} from neutrophil cytosol

treated with [^{32}P]GTP dialdehyde contained 10 times as much stably bound nucleotide as p67^{phox} from cytosol treated with [^{32}P]NADPH dialdehyde, they concluded that the incorporation of NADPH dialdehyde into p67^{phox} represented labeling, not of a specific NADPH-binding site, but of an "ambiguous NTP-binding site" that was recognized by both nucleotides but preferentially took up GTP dialdehyde. Calculations of stoichiometry based on data from Table II of their report, however, indicate that their [^{32}P]GTP dialdehyde-labeled p67^{phox} (designated p63^{phox} in their paper) contained $\sim$ 10 mol of nucleotide/mol of protein, while their [^{32}P]NADPH dialdehyde-labeled material contained only $\sim$ 1 mol of nucleotide/mole of protein. It therefore seems likely that most if not all of their p67^{phox}-associated [^{32}P]GTP dialdehyde was attached nonspecifically, making interpretation uncertain.

With regard to binding of NADPH directly to cytochrome b_{558}, an NADPH-binding site on gp91^{phox} was first postulated on the basis of sequence homologies, and it was later shown that purified cytochrome b_{558}, if mixed with FAD and lipids of appropriate composition, can catalyze O_2^- production in the absence of any cytosolic factors, implying the presence of a catalytically significant NADPH-binding site (54, 55). The turnover number for O_2^- production in a system free of cytosol, however, was only 15–20% of the turnover number for cell-free systems containing cytosolic factors, and the K_m for NADPH was $\sim$ 0.125 mM, a value that is fourfold higher than the K_m of $\sim$ 0.035 mM that is routinely obtained with the complete cell-free system. These values indicate that with the oxidase working under V/K conditions, as most enzymes do, the rate of O_2^- production using the gp91^{phox} NADPH-binding site alone would be at most 6% of the rate seen with the complete oxidase, and this rate would prevail only if the cytochrome were in an ideal lipid environment. Some means of accelerating the O_2^- forming reaction would seem to be necessary.

Our results suggest a possible mechanism. We propose that the leukocyte NADPH oxidase contains two catalytically functional NADPH-binding sites, one of relatively low affinity on gp91^{phox} and the other of higher affinity on p67^{phox}, and that the kinetic properties of the oxidase reflect the balance between the two. When p67^{phox} is brought to the electron transport apparatus during oxidase activation, these two domains may cooperate to form the single catalytically efficient NADPH-binding site found on the active enzyme. Available evidence suggests that the active oxidase contains equimolar quantities of p47^{phox}, p67^{phox}, and cytochrome b_{558}, findings consistent with a role for p67^{phox} in enzyme catalysis (56, 57).

Support for this interpretation of electron transport through the oxidase is provided by a number of disparate observations. For instance, there are certain CGD patients with partial defects in O_2^- production, in whom the oxidase shows a K_m for NADPH of 0.125 mM or higher (58). A high K_m has also been demonstrated in cell-free oxidase activation systems at very low cytosol concentrations (59, 60), and can be corrected by the addition of cytosol-containing p67^{phox} (59) but not by cytosol treated with NADPH dialdehyde (38). Finally, the activation of mononuclear phagocytes is associated with a reduction in the K_m of the oxidase for NADPH from values of $\sim$ 0.15 mM or greater to levels similar to those measured in neutrophils, i.e., $\sim$ 0.035 mM (61–64). It is possible that this low affinity state of the oxidase may arise through inadequate quantities of p67^{phox} or through faulty interactions between p67^{phox} and cytochrome b_{558} such that the substrate associates directly with the NADPH binding site on gp91^{phox} rather than that on p67^{phox}.

Why might this complicated system involving NADPH-binding sites functioning sequentially or in parallel have evolved? The leukocyte NADPH oxidase is a very dangerous enzyme, responsible for the generation of highly reactive oxidizing agents that have the potential for inflicting great harm. The activity of the oxidase must therefore be stringently controlled as to time and location and the leakage of electrons to oxygen through the resting enzyme must be kept to an irreducible minimum. Distributing the active site of the oxidase between two components that are only brought together when the enzyme is activated represents a unique solution to this problem.

Acknowledgments

This work was supported in part by United States Public Health System grants HL23584, AI24227, AI30742, and RR00833.

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