NADPH Dehydrogenase Activity of p67^{PHOX}, a Cytosolic Subunit of the Leukocyte NADPH Oxidase†

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ABSTRACT: The leukocyte NADPH oxidase catalyzes the one-electron reduction of oxygen to O_2^- at the expense of NADPH. It is a multicomponent enzyme comprising a membrane-bound flavocytochrome (cytochrome b_{558}) and at least four cytosolic components: p47^{PHOX}, p67^{PHOX}, p40^{PHOX}, and Rac, a small GTPase. All the oxidase components except p40^{PHOX} are required for enzyme activity. Many aspects of their function, however, are unclear. Using the electron acceptor ferricyanide, we found that recombinant p67^{PHOX} from baculovirus-infected Sf9 cells could mediate the dehydrogenation of NADPH. NADPH dehydrogenation was not dependent on FAD and was insensitive to superoxide dismutase. Several control experiments showed that NADPH dehydrogenation was accomplished by p67^{PHOX}, not by a trace contaminant in the p67^{PHOX} preparation. The NADPH dehydrogenase activity of p67^{PHOX} was proportional to enzyme concentration, and showed saturation kinetics with NADPH (K_m 92 ± 5 μM), but was inhibited at high concentrations of ferricyanide. NADH was also used as a substrate by p67^{PHOX} (K_m 123 ± 38 μM). Taken together, these results show that p67^{PHOX} is able to mediate pyridine nucleotide dehydrogenation. These findings raise the possibility that p67^{PHOX} might participate directly in electron transfer between NADPH and the oxidase flavin.

Neutrophils reduce molecular oxygen at the expense of NADPH to produce superoxide anion (O_2^-) and secondarily derived oxidizing species (H_2O_2, HOCl, OH•) which can kill microorganisms efficiently (*1, 2*). Superoxide generation is catalyzed by a highly regulated multisubunit NADPH oxidase whose components include cytochrome b_{558} (a heterodimeric flavohemoprotein containing 91 and 22 kDa subunits) and at least four cytosolic proteins: p47^{PHOX}, p67^{PHOX}, p40^{PHOX}, and Rac, a small GTPase. In the absence of p47^{PHOX} or p67^{PHOX}, neutrophils produce little or no O_2^-, giving rise to the autosomal recessive form of chronic granulomatous disease, a disorder characterized by frequent life-threatening infections because of a failure of oxygen-dependent microbial killing (*3*).

In resting neutrophils, the NADPH oxidase is dormant, and its protein components are distributed between cytoplasmic and plasma membrane compartments. On activating the cells with appropriate stimuli, the cytosolic components migrate to the submembranous cytoskeleton where they associate with cytochrome b_{558} to form the catalytically active oxidase (*4*−*6*). The exact mechanism of activation of the oxidase, however, still remains unclear. Phosphorylation of p47^{PHOX} in intact cells accompanies the activation of the oxidase (*7*−*10*) and probably initiates the assembly of the enzyme. The function of p67^{PHOX} is less well understood, although it was shown to bind directly to Rac (*11*−*13*) and to be phosphorylated in human neutrophils upon stimulation by *N*-formylmethionylleucylphenylalanine (fMLP) and phorbol myristate acetate (PMA) (*14*). Two recent studies (*15, 16*) demonstrated that pronounced activation of NADPH oxidase could be achieved by exposing cytochrome b_{558} to high concentrations of p67^{PHOX} and Rac in the absence of p47^{PHOX}, suggesting that p67^{PHOX} might play a direct role in regulating electron transfer. This idea is supported by the fact that p67^{PHOX} was identified as the cytosolic NADPH-binding component of the leukocyte NADPH oxidase and that this NADPH-binding function of p67^{PHOX} was essential for normal oxidase activity (*17*).

In the present study, we show that recombinant p67^{PHOX} purified from baculovirus-infected Sf9 cells is able to mediate NADPH oxidation by ferricyanide in the absence of other NADPH oxidase components. These finding provide further evidence consistent with the idea that p67^{PHOX} might participate directly in the transfer of electrons between NADPH and the oxidase flavin.

MATERIALS AND METHODS

Materials

NADPH, NADH, FAD, dichlorophenolindophenol (DCIP),[1] and nitroblue tetrazolium (NBT) were obtained from the Sigma Chemical Co (St. Louis, MO). Potassium ferricyanide was from Mallinckrodt Inc. (Chesterfield, MO). BacPAK6, a derivative of the AcMNPV baculovirus strain, was from

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[1] Abbreviations: SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; PIPES, 1,4-piperazinediethanesulfonic acid; NEM, *N*-ethylmaleimide; DCIP, 2,6-dichlorophenolindophenol; NBT, nitroblue tetrazolium.

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Clontech (Palo Alto, CA). Cell cultures were grown in Ex Cell 401 medium (JRH Biosciences, Woodland Hills, CA).

Methods

Production and Purification of Recombinant Proteins. The recombinant baculovirus expressing p67^{PHOX} (*18*) was a generous gift of Dr. T. L. Leto. Large-scale production of recombinant p67^{PHOX} was obtained by infecting monolayer cultures of Sf9 cells in 150 cm^2 flasks at a density of 1−2 × 10^6 cells/mL (*18*). Control cells were infected with BacPAK6, a derivative of AcMNPV that expresses β-galactosidase. Cells were harvested 72 h post-infection, washed twice in phosphate-buffered saline by centrifugation at 400*g* for 10 min, and then resuspended to 5 × 10^7 cells/mL in lysis buffer (50 mM KCl, 3 mM NaCl, 2 mM $MgCl_2$, 0.1 mM DTT, 1 mM EDTA, 250 μg/mL leupeptin, 100 μg/mL pepstatin, 100 μg/mL aprotinin, 2 mM PMSF, 5.4 mM DFP, and 5 mM PIPES, pH 7.5). All subsequent work was conducted at 4 °C. The cells were disrupted by sonication (4 × 30 s) and centrifuged at 400*g* for 10 min. The pellet was discarded and the supernatant centrifuged at 100000*g* for 1 h. The 100000*g* supernatant fraction contained the p67^{PHOX}. To this supernatant was added ammonium sulfate with gentle stirring to bring the solution to 45% saturation. p67^{PHOX} was found in the precipitate, which was isolated by centrifugation (12000*g*, 30 min at 4 °C), dissolved in 10 mL of buffer A (20 mM Tris, pH 7.5, 0.1 mM DTT, 1 mM EDTA, 2 mM EGTA, 0.15 mM PMSF), and dialyzed overnight against the same buffer. Using Pharmacia FPLC equipment, the dialyzed solution was applied to a Mono Q Sepharose column (Pharmacia Biotech Inc.) equilibrated with buffer A, and washed with 5 volumes of the same buffer. Proteins were eluted with a 0.1−0.3 M NaCl gradient in the same buffer, eluting at a flow rate of 0.5 mL/min. Fractions were analyzed by SDS−PAGE and immunoblotting with an antibody raised against a synthetic peptide from p67^{PHOX} (*19*). The fractions containing p67^{PHOX} were pooled, concentrated to <0.4 mL in a Centricon concentrator (Amicon Corp.), and applied to a Superdex 75 (Pharmacia Biotech Inc.) column equilibrated with buffer A containing 0.3 M NaCl. The column was eluted with the same buffer using a flow rate of 0.25 mL/min and collecting 0.6 mL fractions. Fractions were analyzed by SDS−PAGE and immunoblotting, and those containing pure p67^{PHOX} were pooled and assayed for protein concentration with the Biorad assay kit using serum albumin as a standard. The activity of purified p67^{PHOX} was tested in a cell-free system which contained only sufficient normal neutrophil cytosol to supply a threshold amount of each cytosolic factor. The addition of the purified p67^{PHOX} leads to increased oxidase activity after activation (*17*). In some experiments, p67^{PHOX} was further applied to a DEAE-Sepharose column (Pharmacia Biotech Inc) and eluted with a 0−300 mM NaCl gradient or to a hydroxyapatite column (Biorad laboratories) and eluted with a gradient of 10−400 mM sodium phosphate buffer (pH 6.8).

E. coli transformed with pGEX-1λT vector containing an insert of p47^{PHOX} cDNA were grown and lysed, and the glutathione-*S*-transferase−p47^{PHOX} fusion protein was purified on GSH−agarose as described previously (*20*). Briefly, *E. coli* was grown at 37 °C overnight in 50 mL of ampicillin broth medium. The overnight culture was diluted into 950 mL of fresh medium and grown for an additional hour at 37 °C. The culture was then made up to 0.1 mM in isopropyl β-D-thiogalactoside and grown for an additional 3 h at 37 °C. The bacteria were isolated by centrifugation at 5000*g* for 10 min at 4 °C, resuspended in 5 mL of ice-cold bacteria lysis buffer (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 100 μg/mL leupeptin, 100 μg/mL pepstatin, and 0.5 mM DFP), and disrupted by sonication (4 × 30 s). The sonicate was clarified by centrifugation at 20000*g* for 10 min at 4 °C. The fusion protein was isolated from the clarified sonicate by purification over glutathione−Sepharose beads (Pharmacia) as described previously (*20*) and dialyzed against 20 mM Tris, pH 7.5, 1 mM EDTA, 5 mM $MgCl_2$, and 0.1 mM DTT. Crude p47^{PHOX} recombinant preparations from baculovirus-infected Sf9 cells were a kind gift from Dr. T. L. Leto. They were purified on a CM-Sepharose column as described elsewhere (*18*).

E. coli transformed with pGEX4T-3 vector containing an insert of Rac2 (a kind gift of Dr. U. Knaus) were grown and lysed, and the glutathione-*S*-transferase−Rac2 fusion protein was purified on GSH−agarose as described for p47^{PHOX}, except that after induction with isopropyl β-D-thiogalactoside, the culture was grown for 3 h at 30 °C instead of 37 °C.

Spectrophotometric Assays. All the assays were performed in quartz cuvettes using purified p67^{PHOX} in relaxation buffer (100 mM KCl, 3 mM NaCl, 3.5 mM $MgCl_2$, 10 mM PIPES, pH 7.3) in a final volume of 100 μL, conducting the incubations in a Uvikon 941 spectrophotometer thermostated at 30 °C. Reaction mixtures contained p67^{PHOX} at the indicated concentration, 0.1 mM NADPH, and 500 μM ferricyanide or 100 μM DCIP in relaxation buffer except were indicated, plus 10 μM FAD or 50 units/mL SOD as indicated. The reference cuvette contained the buffer in which the assays were performed. Reactions were initiated by the addition of the electron acceptor. Ferricyanide reduction was measured by recording the decrease in absorbance at 420 nm (ϵ = 1.0 mM^{-1} cm^{-1}); NADPH oxidation was measured by following the decrease in absorbance at 340 nm (ϵ = 6.22 mM^{-1} cm^{-1}), correcting for the difference in absorbance between $Fe(CN)_6^{3-}$ and $Fe(CN)_6^{4-}$ at 340 nm; and DCIP reduction was measured by the decrease in absorbance at 600 nm (ϵ = 21 mM^{-1} cm^{-1}). Column fractions to be assayed for activity were first concentrated to one-sixth of their initial volume. Ten microliters of the concentrated fraction was added to the reaction mixture described above (0.1 mM NADPH, 500 μM ferricyanide in relaxation buffer). Maximum rates were determined by the initial velocity of the reaction and are expressed as nanomoles of NADPH oxidized per minute per milligram of protein. Heat-inactivated p67^{PHOX} (70 °C, 5 min) was used for some assays. The effects of p47^{PHOX} and Rac2−GTPγS were studied by preincubation with p67^{PHOX} for 5 min; the reaction was then started by the addition of ferricyanide. Rac2 was preloaded with GTPγS by incubation with 25 mM EDTA and 100 mM GTPγS for 15 min at room temperature, and then adding $MgCl_2$ to an excess of 5 mM.

NBT-Reductase Activity Stain. p67^{PHOX} was subjected to PAGE on a nondenaturing gel using Laemmli's protocol except for the omission of SDS and mercaptoethanol. Following electrophoresis, the gel was incubated for 2 h at room temperature in NBT reaction mixture (50 mM phosphate buffer, pH 7.4, 0.4 mM NADPH, 0.05% NBT). When the stained band appeared, the gel was transferred to 7%

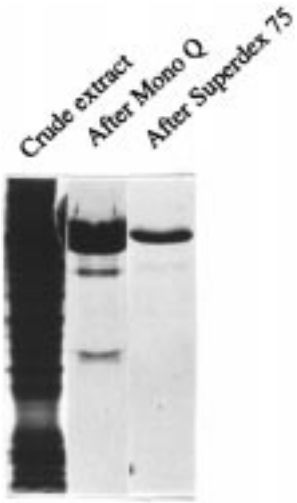

FIGURE 1: SDS−polyacrylamide gel electrophoresis of p67^{PHOX}. Lane 1 shows the crude extract; lanes 2 and 3 show p67^{PHOX} after purification on the Mono Q anion exchange column and the Superdex 75 gel filtration column, respectively. The amounts of protein in each lane were as follows: lane 1, 80 μg; lane 2, 30 μg; lane 3, 12 μg.

acetic acid to fix the band. In some experiments, the stained band was excised and analyzed by SDS−PAGE and immunoblotting.

SDS−PAGE and Immunoblotting. SDS−PAGE of proteins was carried out according to Laemmli, using a 12% running gel. p67^{PHOX} was blotted and visualized as described elsewhere (*20*).

RESULTS

p67^{PHOX} Mediates NADPH Oxidation. Previous studies have demonstrated that the cytosolic subunit p67^{PHOX} contains an NADPH binding site that is essential for oxidase activity (*17*). These NADPH binding properties suggested that p67^{PHOX} might be involved in the mediation of NADPH oxidation. To assess this possibility, studies were carried out to see whether pure recombinant p67^{PHOX} could mediate the oxidation of NADPH by artificial electron acceptors such as ferricyanide or DCIP. The recombinant p67^{PHOX} was purified from infected Sf9 cell supernatants using the procedure described under Materials and Methods. By this procedure, we obtained a protein of >98% purity (Figure 1).

The reductase activity of p67^{PHOX} was followed first by measuring the rate of NADPH oxidation at 340 nm using ferricyanide as electron acceptor. These experiments revealed that p67^{PHOX} induces NADPH oxidation in the presence of ferricyanide (Figure 2, top panel). No change in A_{340} was detected with reaction mixtures lacking either ferricyanide or NADPH; with reaction mixtures lacking p67^{PHOX}, however, NADPH was oxidized by ferricyanide, but at a rate considerably slower than observed in the presence of p67^{PHOX}. p67^{PHOX} was also able to pass electrons from NADPH to DCIP (Figure 2, botton panel) (ΔA 0.08/min vs 0.04/min without NADPH; the electron donor in the absence of NADPH was probably the DTT in the enzyme preparation). Negligible DCIP reduction was observed without p67^{PHOX}.

It is known that p67^{PHOX} can interact directly with p47^{PHOX} (*19*−*21*), p40^{PHOX} (*22*−*24*), and Rac2 (*11*−*13*). To determine whether the NADPH dehydrogenase activity of p67^{PHOX} could be increased by these components, we conducted experiments in which p67^{PHOX} was preincubated with 3 μM recombinant p47^{PHOX} from *E. coli* and/or 3 μM recombinant Rac2 preloaded with GTPγS, then adding ferricyanide to start the reaction. We failed to show any effect of p47^{PHOX} or Rac2−GTPγS on the dehydrogenase activity of p67^{PHOX} (data not shown). In addition, p67^{PHOX} was recently shown to be phosphorylated (14), and its phosphorylation state could also influence its activity. We therefore added to the reaction mixture 100 μM ATP together with the catalytic subunit of protein kinase C (i.e., protein kinase M), but saw no alteration of the dehydrogenase activity (not shown). Consequently, the interaction of p67^{PHOX} with these components as well as its phosphorylation is more likely to influence the assembly of p67^{PHOX} within the whole NADPH oxidase complex rather than the dehydrogenase activity that we identified. Finally, NADPH oxidation by p67^{PHOX} was found to be independent of FAD and insensitive to superoxide dismutase (Figure 3).

p67^{PHOX} Is Responsible for the NADPH Dehydrogenase Activity of the Enzyme Preparation. To confirm that the NADPH oxidation was mediated by p67^{PHOX} and not a trace contaminant, Sf9 cells were transfected with BacPAK6 (Clontech), a recombinant control baculovirus that contains the cDNA for β-galactosidase. Supernatants from cells infected by BacPAK6 were submitted to the same purification procedure as supernatants from cells infected by the baculovirus containing the p67^{PHOX} cDNA, and both preparations were tested for their NADPH oxidation activity. Figure 3 indicates that the activity of the BacPAK6 preparation was the same as seen in the control without p67^{PHOX}, indicating that the NADPH dehydrogenase activity is likely to be an intrinsic property of p67^{PHOX}. Further evidence that the dehydrogenase activity was specific to p67^{PHOX} were the findings that NADPH dehydrogenation was not catalyzed by recombinant p47^{PHOX} purified either from *E. coli* or from baculovirus-infected Sf9 cells (Figure 3). Heat treatment also abolished the dehydrogenase activity of p67^{PHOX} (Figure 3). Further evidence for the NADPH dehydrogenase activity of p67^{PHOX} was obtained by an activity stain of a nondenaturing gel on which p67^{PHOX} had been subjected to electrophoresis. When subjected to nondenaturing PAGE, p67^{PHOX} was able to reduce NBT in an NADPH-dependent manner, resulting in the appearance of a major stained band (Figure 4A, arrow) with some higher molecular weight bands that are weakly stained. When transferred to nitrocellulose, all these bands are recognized by an antibody against the whole p67^{PHOX} (data not shown). This suggests that high molecular weight bands are aggregated forms of p67^{PHOX}. In the absence of NADPH or NBT, no stained band was observed. To confirm that the protein responsible for the appearance of the stained band comigrated with p67^{PHOX}, the stained band was excised, subjected to SDS−PAGE, and immunoblotted with anti-p67^{PHOX}. As shown in Figure 4B, protein eluted from the excised band showed an M_r of 67 000 and was recognized by the p67^{PHOX} antibody. Finally, when the purified recombinant protein was subjected to chromatography over DEAE-Sepharose or hydroxyapatite, p67^{PHOX} and the NADPH dehydrogenase activity eluted in the same fractions (185 mM NaCl and 360 mM potassium phosphate, respectively; Figure 5, top and bottom panels). Taken together, these findings make it highly unlikely that the observed NADPH dehydrogenase activity is due to a minor contaminant, and furnish strong evidence that in the absence of the other components

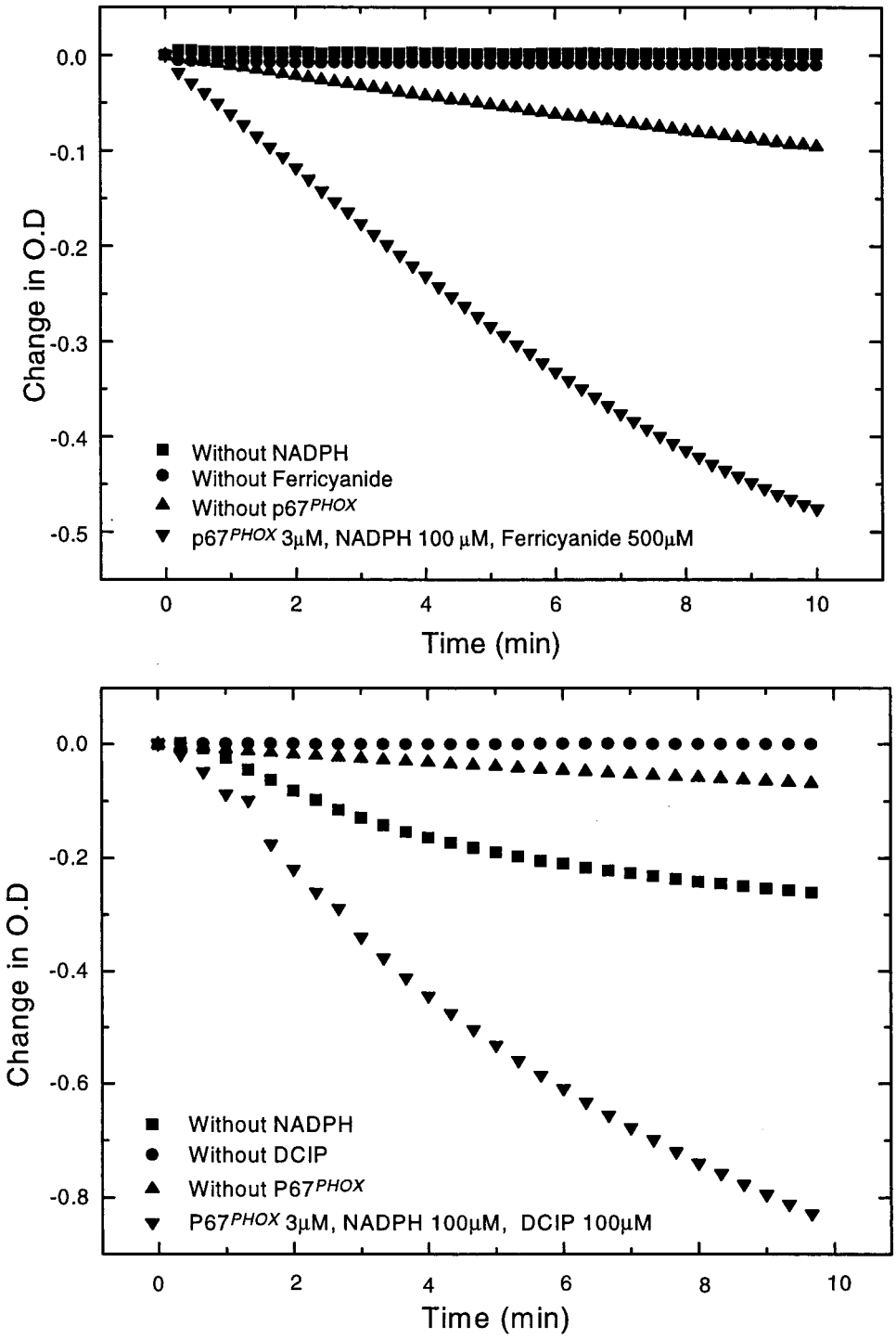

FIGURE 2: Catalysis of NADPH dehydrogenation and DCIP reduction by p67^{PHOX}. NADPH oxidation at 340 nm (top) and DCIP reduction at 600 nm (bottom) were measured as described under Materials and Methods. The reaction mixtures contained 3 μM p67^{PHOX}, 100 μM NADPH, and 500 μM ferricyanide or 100 μM DCIP in relaxation buffer. Reactions were initiated by the addition of the electron acceptor. To follow NADPH oxidation, ferricyanide was used in preference to DCIP because of the high absorbance of the latter at 340 nm.

of the NADPH oxidase complex, p67^{PHOX} is able to mediate NADPH dehydrogenation.

Characteristics of p67^{PHOX}-Mediated NADPH Dehydrogenation. To determine the stoichiometry of electron transfer, NADPH oxidation and ferricyanide reduction were followed in combination by repetitive scanning of the reaction mixture between 300 and 500 nm. Using 3 μM p67^{PHOX}, we found 2.07 $\pm$ 0.22 nmol of ferricyanide reduced per minute compared with 0.94 $\pm$ 0.10 nmol of NADPH oxidized per minute, the 2:1 stoichiometry expected for the reduction of $Fe(CN)_6^{3-}$ by NADPH. NADPH dehydrogenation by p67^{PHOX} exhibited a linear response with increasing amounts of protein (data not shown). Kinetic properties were determined by studying p67^{PHOX} activity as a function of increasing pyridine nucleotide concentration. The velocity versus substrate plot showed typical Michaelis−Menten kinetics for NADPH (Figure 6) and NADH (data not shown). The kinetic constants, calculated from a nonlinear least-squares fit to the untransformed data, were a K_m of 92 $\pm$ 5 μM and a V_{max} of 166 $\pm$ 4 nmol min^{-1} (mg of protein)$^{-1}$ for NADPH, and a

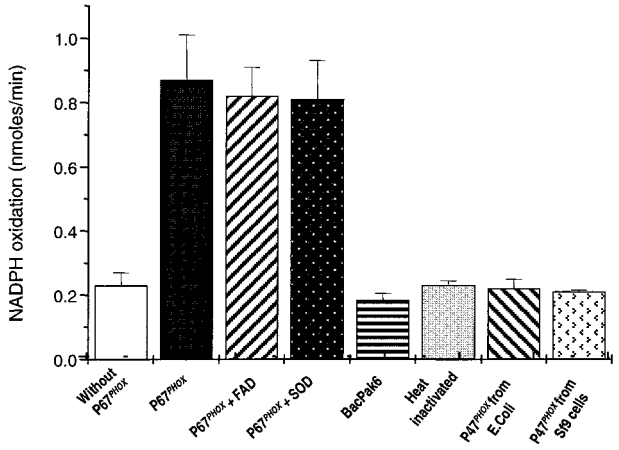

FIGURE 3: NADPH is oxidized by p67*PHOX*, not by a contaminant. Assays were carried out as described under Materials and Methods. The assay mixtures contained 3 μM p67*PHOX* or 3 μM p47*PHOX* or an equivalent volume of the BacPAK6 preparation that was submitted to the same purification procedure as the p67*PHOX* preparation. Heat inactivation was carried out by heating p67*PHOX* at 70 °C for 5 min. Values represent the mean ± range of at least three separate determinations.

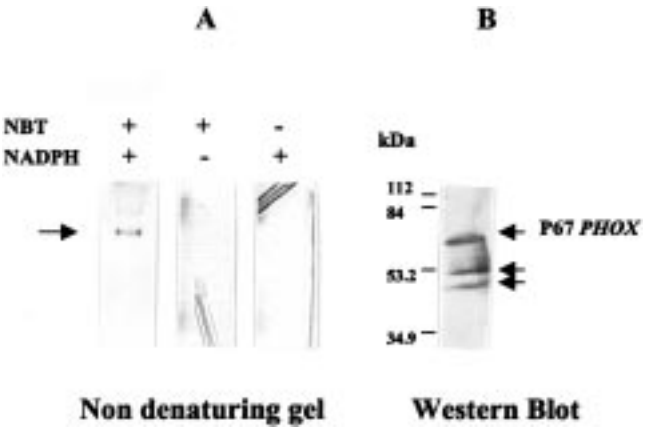

FIGURE 4: NADPH dehydrogenase activity activity of p67*PHOX* as measured by an activity stain. A 0.3 nmol sample of p67*PHOX* was subjected to PAGE under nondenaturing conditions as described under Materials and Methods. (A) Staining of gel slices incubated in a reaction mixture containing 0.05% NBT in 50 mM phosphate buffer, pH 7.4, with or without 0.4 mM NADPH and 100 μM mersalyl as indicated. (B) Immunoblot of proteins comigrating with the stained band as analyzed by SDS−PAGE and developed using anti-p67*PHOX*.

K_m of 123 ± 38 μM and a V_{max} of 123 ± 18 nmol min^{-1} (mg of protein)$^{-1}$ for NADH. Turnover numbers, expressed as electrons transferred per mole of protein, were 20.8 and 15.4 min^{-1} for NADPH and NADH, respectively. When p67*PHOX* activity was assayed with varying concentrations of ferricyanide, keeping NADPH concentration constant at 100 μM, the response increased in a linear manner with ferricyanide up to a concentration of about 15 μM (Figure 7). Above that concentration, however, the reaction was powerfully inhibited by ferricyanide, rendering it impossible to determine the true V_{max} of p67*PHOX* as a pyridine nucleotide dehydrogenase except to say that it is probably considerably higher than indicated by the preceding values, which were calculated from experiments carried out at 500 μM ferricyanide. We tried replacing ferricyanide with DCIP and $(NH_3)_6Ru^{3+}$, but were unable to overcome this problem with either of these alternative electron acceptors.

DISCUSSION

The electron transport pathway of the leukocyte NADPH oxidase still remains incompletely defined, although there is agreement that NADPH is the physiological electron donor, O_2^- the product, and FAD an obligatory electron carrier. Our results provide evidence that p67*PHOX* can mediate the dehydrogenation of NADPH in the presence of a suitable electron acceptor, thus suggesting that this subunit might participate directly in the transfer of electrons between NADPH and the flavin center of the oxidase.

It is generally believed that cytochrome b_{558} represents the only enzymatic component of the NADPH oxidase, since it contains all the prosthetic groups necessary for activity: FAD, two hemes, and a putative consensus sequence for pyridine nucleotide binding (*25*, *26*). Furthermore, several reports have suggested that gp91*PHOX* may actually be the NADPH-binding protein of the oxidase (*26*−*28*). Residues 403−417, 441−450, 504−518, and 533−538 are weakly homologous to putative consensus NADPH-binding sites of the ferridoxin−NADPH reductase family (*25*−*29*), and a missense mutation within one of these regions of gp91*PHOX* (P415H) has been shown to result in a form of X-linked CGD in which normal amounts of cytochrome b_{558} were present and normal translocation of cytosolic factor to the membrane occurred, but without the generation of O_2^-. In support of this proposal, Koshkin and Pick (*30*) have shown that purified cytochrome b_{558} in the presence of a defined lipid mixture was able to reduce oxygen to O_2^- using NADPH as electron donor. The turnover number for O_2^- production was low, however, and the K_m for NADPH considerably higher than that obtained with the complete oxidase (124−146 μM against 30−40 μM). This suggests that other components in addition to cytochrome b_{558} may be essential for the catalytic activity of the enzyme.

Several reports have presented evidence that p67*PHOX* might regulate or directly participate in electron flow within the NADPH oxidase complex. First, the possibility that p67*PHOX* is the NADPH-binding component has been suggested by several investigators. Early data suggesting that p67*PHOX* might be the NADPH-binding component were reported by Umei and co-workers, who showed that NADPH 2′,3′-dialdehyde specifically labeled a 66 kDa protein in the O_2^--generating complex of activated guinea pig neutrophils (*31*, *32*). In addition, Smith et al. (*33*) identified an oxidase component in the cytosol of resting neutrophils whose labeling with NADPH dialdehyde resulted in the inactivation of the enzyme. In more recent studies, the latter group identified the NADPH dialdehyde-labeled protein as p67*PHOX* and showed that dialdehyde treatment of recombinant p67*PHOX* blocked its activity in cell-free oxidase assay (*17*). Further supporting p67*PHOX* as an NADPH-binding protein, we have found that NADPH suppressed the fluorescence of tryptophan residues of p67*PHOX* (unpublished data). We are not concerned about the lack in p67*PHOX* of well-defined motifs related to NADPH binding, because there is abundant evidence that NADPH does bind to p67*PHOX*, and there is some doubt as to whether a consensus sequence for the binding of NADPH actually exists (*34*).

Adding to the evidence that p67*PHOX* may be an electron-transporting protein are the results of a study showing that the human neutrophil O_2^--generating NADPH oxidase

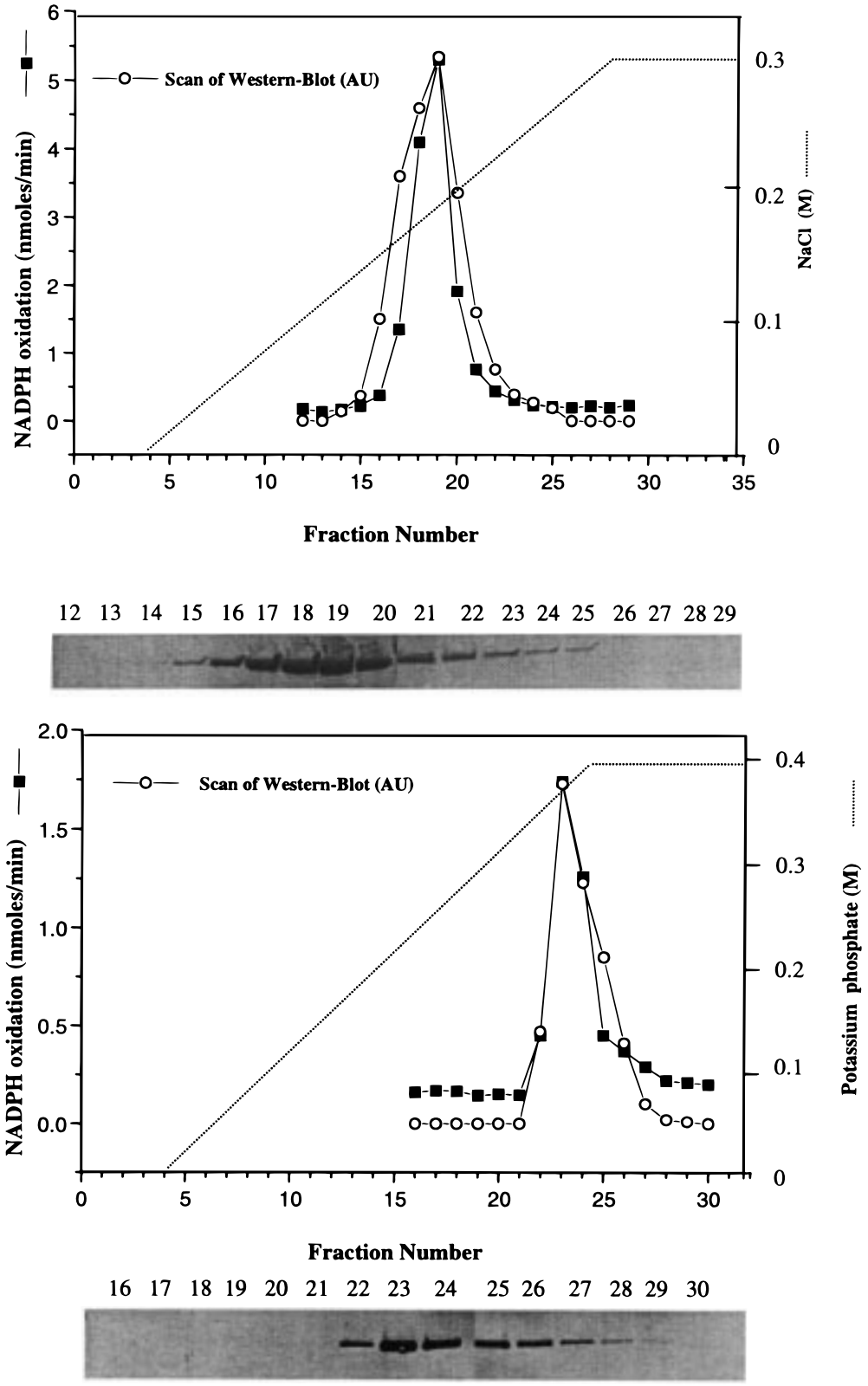

FIGURE 5: Coelution of p67^{PHOX} and NADPH dehydrogenase activity from DEAE Sepharose and hydroxyapatite columns. (Top panel) Elution from DEAE Sepharose. Four milligrams of purified recombinant p67^{PHOX} was applied to a 1 mL DEAE Sepharose column and eluted with a 0−300mM NaCl gradient. (Bottom panel) Elution from hydroxyapatite. Two milligrams of purified recombinant p67^{PHOX} was applied to a 5 mL hydroxyapatite column and eluted with a 10−400 mM gradient of potassium phosphate buffer (pH 6.8). The activities of the column fractions were measured as described under Materials and Methods after concentrating each fraction to one-sixth of its original volume. Ten microliters of each fraction from the DEAE Sepharose column and 10 μL of each fraction from the hydroxyapatite column were then assayed. Five microliters of each fraction from both columns was analyzed by SDS−PAGE followed by immunoblotting, developing with an antibody directed against p67^{PHOX}.

possesses a tetrazolium dye reductase activity which exhibits an absolute requirement for the cytosolic subunit p67^{PHOX} but not for p47^{PHOX} (*35*). Furthermore, using spectroscopy, the authors of that study demonstrated that incubation of

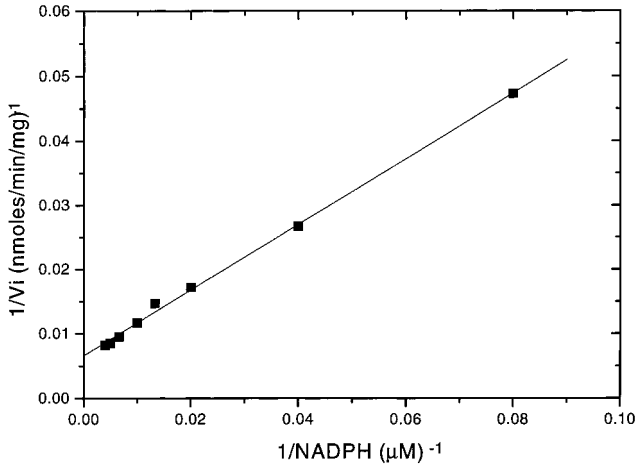

FIGURE 6: Activity of p67^{PHOX} as a function of NADPH concentration. Assays were run at constant ferricyanide concentration (500 μM) and protein concentration (3 μM) with varying concentrations of reduced pyridine nucleotide. Oxidation of NADPH was recorded at 340 nm. The Lineweaver−Burk plots are representative of results from three separate experiments. For each nucleotide, K_m and V_{max} values as given in the text were calculated from all the experimental data by nonlinear regression to a ligand binding curve.

membrane extract with p47^{PHOX}-deficient cytosol (which contained normal levels of p67^{PHOX}) resulted in the reduction of the membrane flavin but not cytochrome b_{558}, while neither was reduced when p67^{PHOX}-deficient cytosol was used (36). This suggests that p67^{PHOX} is at least capable of inducing electron flow from NADPH to the flavin cofactor of gp91^{PHOX}. Still other laboratories have shown that NADPH oxidase activity could be reconstituted in vitro in the absence of p47^{PHOX} if high concentrations of p67^{PHOX} and Rac were used (*15*, *16*), thus indicating that p67^{PHOX} and Rac were the cytosolic components directly involved in the passage of electrons to the flavin of membrane-associated cytochrome b_{558}. Finally, Han et al. (*37*) recently described an activation domain in p67^{PHOX} that could directly be involved in the regulation of electron transfer from NADPH to oxygen by interacting with cytochrome b_{558}.

In the present study, we demonstrated that p67^{PHOX}, in the absence of other components of the NADPH oxidase complex, is able to catalyze NADPH oxidation, a result consistent with the idea that it carries the NADPH-binding site and that it participates directly in electron transfer between NADPH and the FAD center. As with the complete oxidase, the K_m for NADPH (92 μM) was lower than the K_m for NADH (123 μM), though the K_m values were much closer to each other than the corresponding values for the complete oxidase. The differences in the K_m's for NADPH and NADH as measured by NADPH dehydrogenation using

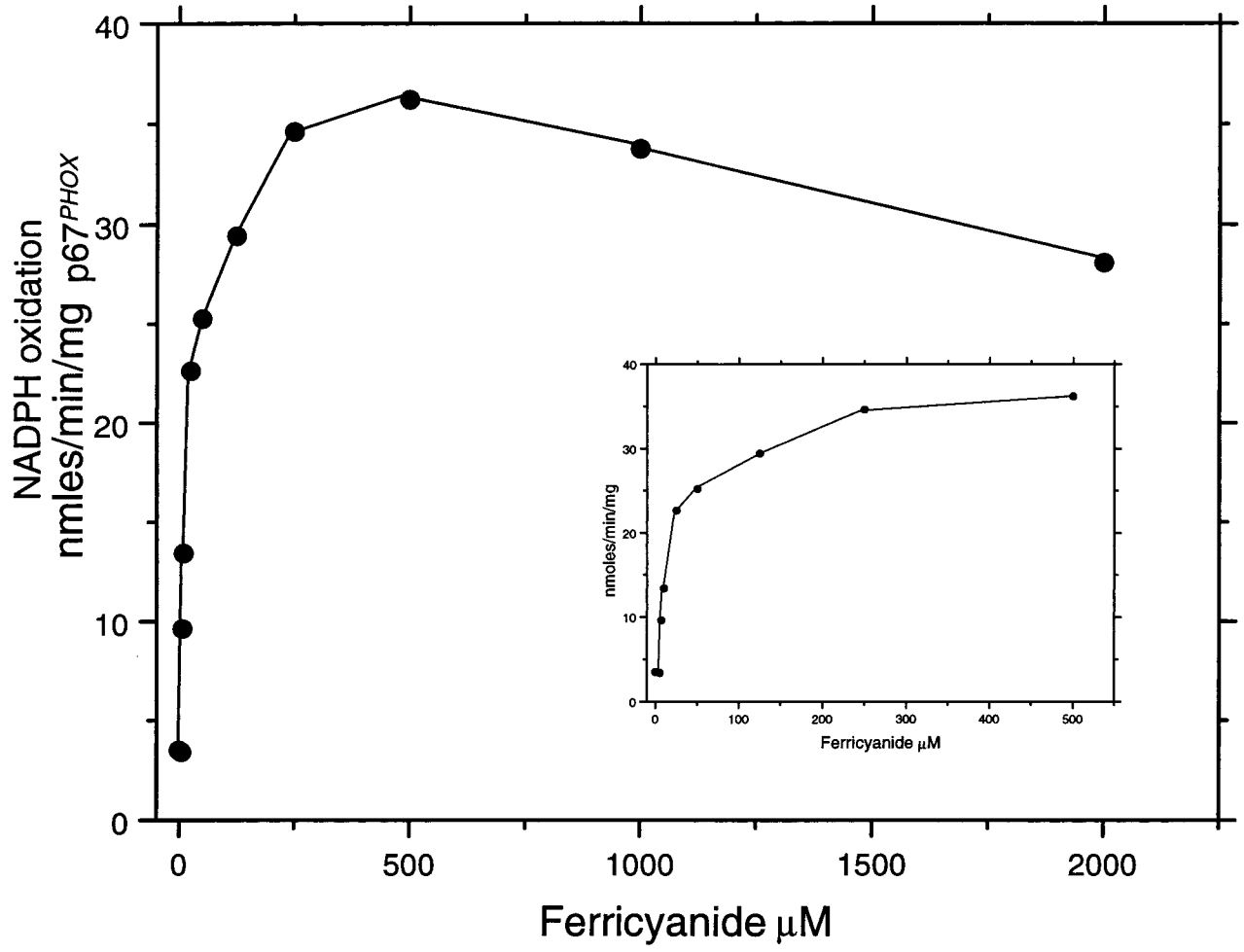

FIGURE 7: Ferricyanide dependence of p67^{PHOX} activity. Assays were conducted as described under Materials and Methods at a constant NADPH concentration (100 μM) and protein concentration (3 μM), with varying concentrations of ferricyanide. The activity in the absence of p67^{PHOX}, which increased as a function of ferricyanide concentration, was subtracted from the total activity to obtain the activity of the enzyme. The inset shows the results at the lowest concentrations of ferricyanide using an expanded *x*-axis scale.

p67^{PHOX} alone vs O_2^- production using the complete oxidase are not unexpected, because a K_m determined from the catalytic activity of an enzyme is a complex function of the rate constants of all the steps in the catalytic mechanism. It would be hard to imagine that the mechanisms, much less the rate constants, would be the same for NADPH dehydrogenation catalyzed by p67^{PHOX} and O_2^- production catalyzed by the complete oxidase.

With both substrates, the V_{max} values were far from the values observed with the complete oxidase. In part this reflects the effect of ferricyanide on the dehydrogenase activity of the oxidase; indeed, if it is assumed that the enzyme has no catalytic binding site for ferricyanide so the reaction between enzyme and ferricyanide is second order at all concentrations, it can be calculated that at 1 mM ferricyanide, the true V_{max} of the NADPH dehydrogenase activity of p67^{PHOX} in the absence of ferricyanide inhibition would be ca. 3800 nequiv of electrons min^{-1} (mg of protein)$^{-1}$ (turnover number 240 min^{-1}). This is still a low turnover number in comparison with the activity of the complete oxidase, but it represents very significant activity.

At present, the most widely held view of electron transport by the NADPH oxidase holds that cytochrome b_{558} is the sole electron-transporting component, binding NADPH directly and transferring its electrons first to the flavin, then to the heme, and finally to oxygen. On the basis of the present results together with those obtained during earlier investigations of the interaction between NADPH and p67^{PHOX} (*17*, *31*, *33*, *38*, *39*), we offer the following alternative scheme to explain the early steps in the reaction. We propose as a first step that p67^{PHOX} translocates to the plasma membrane along with the other cytosolic subunits. It may carry a molecule of NADPH to cytochrome b_{558}, or it may pick one up after it has become associated with the cytochrome. Whether the final NADPH-binding domain is restricted to p67^{PHOX} or is shared between p67^{PHOX} and gp91^{PHOX} remains to be determined. In any case, p67^{PHOX} then catalyzes the transfer of electrons from NADPH to the flavin of gp91^{PHOX}. According to this scheme, both cytochrome b_{558} and p67^{PHOX} are electron-transporting oxidase components.

In summary, our study has demonstrated that p67^{PHOX} possesses a pyridine nucleotide dehydrogenase activity, a finding consistent with the idea that it participates directly in the transfer of electrons from the NADPH to the flavin center of gp91^{PHOX}. Distributing the active site of the oxidase between two components that possess little or no activity until they are brought together provides a very efficient way to control the leukocyte NADPH oxidase so as to ensure against the accidental production of the very reactive microbicidal oxidants whose generation is the chief function of this dangerous enzyme.

REFERENCES

1. Badwey, J. A., and Karnovsky, M. L. (1980) *Annu. Rev. Biochem. 49*, 695−726.
2. Chanock, S. J., El Benna, J., Smith, R. M., and Babior, B. M. (1994) *J. Biol. Chem. 269*, 24519−24522.
3. Curnutte, J. T. (1993) *Clin. Immunol. Immunopathol. 67*, S2−S15.
4. Clark, R. A., Volpp, B. D., Leidal, K. G., and Nauseef, W. M. (1990) *J. Clin. Invest. 85*, 714−721.
5. El Benna, J., Ruedi, J. M., and Babior, B. M. (1994) *J. Biol. Chem. 269*, 6729−6734.
6. Quinn, M. T., Evans, T., Loetterle, L. R., Jesaitis, A. J., and Bokoch, G. M. (1993) *J. Biol. Chem. 268*, 20983−20987.
7. Hayakawa, T., Suzuki, K., Suzuki, S., Andrews, P. C., and Babior, B. M. (1986) *J. Biol. Chem. 261*, 9109−9115.
8. Okamura, N., Malawista, S. E., Roberts, R. L., Rosen, H., Ochs, H. D., Babior, B. M., and Curnutte, J. T. (1988) *Blood 72*, 811−816.
9. El Benna, J., Faust, L. P., and Babior, B. M. (1994) *J. Biol. Chem. 269*, 23431−23436.
10. Levy, R., Dana, R., Leto, T. L., and Malech, H. L. (1994) *Biochim. Biophys. Acta Mol. Cell Res. 1220*, 253−260.
11. Dorseuil, O., Reibel, L., Bokoch, G. M., Camonis, J., and Gacon, G. (1996) *J. Biol. Chem. 271*, 83−88.
12. Diekmann, D., Abo, A., Johnston, C., Segal, A. W., and Hall, A. (1994) *Science 265*, 531−533.
13. Prigmore, E., Ahmed, S., Best, A., Kozma, R., Manser, E., Segal, A. W., and Lim, L. (1995) *J. Biol. Chem. 270*, 10717−10722.
14. El Benna, J., Dang, P. M. C., Gaudry, M., Fay, M., Morel, F., Hakim, J., and Gougerot-Pocidalo, M.-A. (1997) *J. Biol. Chem. 272*, 17204−17208.
15. Koshkin, V., Lotan, O., and Pick, E. (1996) *J. Biol. Chem. 271*, 30326−30329.
16. Freeman, J. L., and Lambeth, J. D. (1996) *J. Biol. Chem. 271*, 22578−22582.
17. Smith, R. M., Connor, J. A., Chen, L. M., and Babior, B. M. (1996) *J. Clin. Invest. 98*, 977−983.
18. Leto, T. L., Garrett, M. C., Fujii, H., and Nunoi, H. (1991) *J. Biol. Chem. 266*, 19812−19818.
19. Park, J.-W., Ma, M., Ruedi, J. M., Smith, R. M., and Babior, B. M. (1992) *J. Biol. Chem. 267*, 17327−17332.
20. Park, J.-W., El Benna, J., Scott, K. E., Christensen, B. L., Chanock, S. J., and Babior, B. M. (1994) *Biochemistry 33*, 2907−2911.
21. Iyer, S. S., Pearson, D. W., Nauseef, W. M., and Clark, R. A. (1994) *J. Biol. Chem. 269*, 22405−22411.
22. Fuchs, A., Dagher, M.-C., and Vignais, P. V. (1995) *J. Biol. Chem. 270*, 5695−5697.
23. Fuchs, A., Dagher, M. C., Fauré, J., and Vignais, P. V. (1996) *Biochim. Biophys. Acta Mol. Cell Res. 1312*, 39−47.
24. Ito, T., Nakamura, R., Sumimoto, H., Takeshige, K., and Sakaki, Y. (1996) *FEBS Lett. 385*, 229−232.
25. Rotrosen, D., Yeung, C. L., Leto, T. L., Malech, H. L., and Kwong, C. H. (1992) *Science 256*, 1459−1462.
26. Segal, A. W., West, I., Wientjes, F., Nugent, J. H. A., Chavan, A. J., Haley, B., Garcia, R. C., Rosen, H., and Scrace, G. (1992) *Biochem. J. 284*, 781−788.
27. Taylor, W. R., Jones, D. T., and Segal, A. W. (1993) *Protein Sci. 2*, 1675−1685.
28. Doussiere, J., Brandolin, G., Derrien, V., and Vignais, P. V. (1993) *Biochemistry 32*, 8880−8887.
29. Sumimoto, H., Sakamoto, N., Nozaki, M., Sakaki, Y., Takeshige, K., and Minakami, S. (1992) *Biochem. Biophys. Res. Commun. 186*, 1368−1375.
30. Koshkin, V., and Pick, E. (1994) *FEBS Lett. 338*, 285−289.
31. Umei, T., Takeshige, K., and Minakami, S. (1986) *J. Biol. Chem. 261*, 5229−5232.
32. Umei, T., Takeshige, K., and Minakami, S. (1987) *Biochem. J. 243*, 467−472.
33. Smith, R. M., Curnutte, J. T., and Babior, B. M. (1989) *J. Biol. Chem. 264*, 1958−1962.
34. Bork, P., and Grunwald, C. (1990) *Eur. J. Biochem. 191*, 347−358.
35. Cross, A. R., Yarchover, J. L., and Curnutte, J. T. (1994) *J. Biol. Chem. 269*, 21448−21454.
36. Cross, A. R., and Curnutte, J. T. (1995) *J. Biol. Chem. 270*, 6543−6548.
37. Han, C.-H., Freeman, J. L. R., Lee, T., Motalebi, S. A., and Lambeth, D. J. (1998) *J. Biol. Chem. 273*, 16663−16668.
38. Takasugi, S., Ishida, K., Takeshige, K., and Minakami, S. (1989) *J. Biochem. 105*, 155−157.
39. Smith, R. M., Curnutte, J. T., Mayo, L. A., and Babior, B. M. (1989) *J. Biol. Chem. 264*, 12243−12248.

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