# Isolation of a Complex of Respiratory Burst Oxidase Components from Resting Neutrophil Cytosol†

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ABSTRACT: The respiratory burst oxidase of neutrophils is a multicomponent enzyme, dormant in resting cells, that catalyzes the reduction of oxygen to $O_2^-$ at the expense of NADPH. In the resting neutrophil, some of the components of the oxidase, including proteins $p47^{phox}$ and $p67^{phox}$, are in the cytosol, while the rest are in a fraction that usually copurifies with plasma membrane. Recent evidence has suggested that at least some of the cytosolic oxidase components exist as a complex. We have now purified such a complex from the cytoplasm of resting neutrophils using an affinity column prepared with an antibody that recognizes the C-terminal decapeptide of $p47^{phox}$. Immunoblotting showed that the complex contained both $p47^{phox}$ and $p67^{phox}$. When supplemented with recombinant $p67^{phox}$, the complex displayed considerable activity in a cell-free oxidase-activating system, and even without added $p67^{phox}$, the complex could more than double $O_2^-$ production in an oxidase-activating system supplemented with suboptimal amounts of cytosol. Isolation of the complex was blocked by preincubating the affinity column with CFSTKRKLASAV, the peptide against which the antibody was raised. On gel filtration, the complex migrated with a molecular weight of 240–300K, similar to that observed with whole neutrophil cytosol. The $p47^{phox}/p67_{phox}$ ratio in the gel-filtered complex was approximately 1 to 1. These results indicate that in resting neutrophil cytosol, $p47^{phox}$ and $p67^{phox}$ exist as a complex.

The respiratory burst oxidase, a multicomponent enzyme so far found exclusively in phagocytes and B-lymphocytes, catalyzes the reduction of oxygen to $O_2^-$ using NADPH as electron donor (Babior & Woodman, 1990; Smith & Curnutte, 1991). In stimulated neutrophils, the active oxidase is found in the plasma membrane (Dewald et al., 1979), but in resting cells the components of the oxidase are distributed between cytosol and plasma membrane (Bromberg & Pick, 1984; Curnutte, 1985; McPhail et al., 1985; Heynemen & Vercauteren, 1984) [or a particle that cofractionates with plasma membrane (Sengelov et al., 1992)], with cytosolic components moving to the plasma membrane when the enzyme is activated (Clark et al., 1990; Park et al., 1992). Recently, we and others found that fractionation of cytosol from resting neutrophils over a size-exclusion column gave a peak of oxidase activity at molecular weight approximately 240–300K that contained the cytosolic components $p47^{phox}$ and $p67^{phox}$ (Park et al., 1992; Heyworth et al., 1990), a result suggesting the existence of a complex of cytosolic oxidase subunits. We now report the isolation of such a complex from the cytosol of resting neutrophils by immunoaffinity chromatography with an antibody that recognizes the C-terminus of $p47^{phox}$.

## MATERIALS AND METHODS

*Preparation of Neutrophil Fractions.* Neutrophil cytosol and plasma membrane were prepared as described previously (Park et al., 1992). Briefly, neutrophils were obtained from normal subjects by dextran sedimentation and Ficoll–Hypaque fractionation of freshly drawn citrate-anticoagulated blood. The neutrophils were suspended at a concentration of $10^8$ cells/mL in a modified relaxation buffer (100 mM KCl/3.0 mM NaCl/3.5 mM $MgCl_2$/10 mM PIPES buffer, pH 7.3), and plasma membrane and cytosol were prepared by nitrogen cavitation and centrifugation through Percoll by the method of Borregaard (Borregaard et al., 1983). Both cytosol and membrane were divided into aliquots and stored at −70 °C until use.

*Preparation of the Antibody Column.* The antibody used for affinity chromatography was a rabbit polyclonal anti-$p47^{phox}$ antibody that had been raised aginst the peptide CFSTKRKLASAV, which contains the C-terminal decapeptide of $p47^{phox}$ (Park et al., 1992). The antibody was purified from 62 mL of antiserum by ammonium sulfate precipitation followed by chromatography over a 10-mL affinity column (Harlow & Lane, 1988) prepared by attaching the crude peptide (Multiple Peptide Systems, La Jolla, CA) to a derivatized agarose (Sulfolink, Pierce), eluting with 0.1 M glycine·HCl (pH 2.8). The fractions containing anti-$p47^{phox}$ antibody were pooled, dialyzed overnight against two 2-L changes of coupling buffer (0.5 M NaCl/0.1 M $NaHCO_3$, pH 8.4), coupled to CNBr-activated agarose according to the supplier's directions, and finally stored at 4 °C in Dulbecco's phosphate-buffered saline/0.1% $NaN_3$/20% glycerol (v/v). The final product contained 2.7 mg of antibody/mL of packed gel. The immunoaffinity beads could be regenerated by washing with 3.5 M sodium thiocyanate followed by *N*-(2-

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hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES)[1]/glycol buffer [50 mM Na-HEPES (pH 8.0)/25% ethylene glycol (v/v)].

*Immunoaffinity Chromatography.* Cytosol (≈9 × $10^7$ cell eq/mL) was concentrated to one-fifth of its original volume by pressure filtration in an Amicon Centriprep 10; 0.6-mL portions of the concentrated cytosol were placed in 1.5-mL Eppendorf tubes, followed by 0.6-mL portions of ice-cold HEPES/glycol buffer and 50-μL portions of packed immunoaffinity beads that had been washed with the same buffer. The tubes were rotated end-over-end for 1 h at 4 °C and then spun for a few seconds at maximum speed in an Eppendorf centrifuge. The supernatant ("pass-through") was reserved, and the immunoaffinity beads were washed with four 1-mL portions of ice-cold HEPES/glycol buffer, centrifuging and reserving the supernatant after each wash. Finally, the bound complex was eluted by incubating the immunoaffinity beads for 30 min at 4 °C with 0.5 mL of 0.1 M glycine·HCl buffer, pH 2.8, and then neutralizing the eluate with 14 μL of 1 M Tris·HCl (pH 9.2) per 0.5 mL. The fractions were assayed for $O_2^-$-forming activity and subjected to electrophoresis and immunoblotting as described below.

For some experiments, the immunoaffinity beads were blocked with the peptide CFSTKRKLASAV, against which the antibody had been raised. For this purpose, the synthetic peptide was purified by reversed-phase HPLC over a Beckman Ultraphere C-18 column, eluting with a linear water/acetonitrile gradient containing 0.1% trifluoroacetic acid and lyophilizing to remove the solvents, and then dissolved in water at a concentration of 1 mg/mL. The immunoaffinity beads were blocked by incubating for 30 min at 4 °C with a 10-fold molar excess of peptide and then washing twice with HEPES/glycol buffer.

*FPLC Analysis of the Eluate.* Glycine eluates from three immunoaffinity purifications were pooled, concentrated to 0.5 mL in an Amicon Centriprep, and gel-filtered by FPLC through a Superose 6 column (Pharmacia, Alameda, CA) that had been equilibrated with relaxation buffer. The column was eluted with the same buffer at a flow rate of 0.2 mL/min, collecting 0.8-mL fractions.

*Recombinant p47$^{phox}$ and p67$^{phox}$ Fusion Proteins.* Where not otherwise stipulated, these recombinant proteins were cloned and expressed using methods described in standard cloning manuals (Sambrook et al., 1989; Ausubel et al., 1991). Full-length cDNAs for p47$^{phox}$ and p67$^{phox}$ were prepared by amplification from a retinoic acid/dimethylformamide-differentiated HL-60 λgt10 cDNA library generously furnished by Dr. Stuart Orkin, using the polymerase chain reaction and unique primers. For both cDNAs, the 5′ primer corresponded to the start of the open reading frame and the 3′ end matched a unique sequence at the end of the open reading frame (p47$^{phox}$) or a unique sequence in the 3′-untranslated region (p67$^{phox}$). For amplification of the p47$^{phox}$, a 1.3-kb insert was obtained with the 5′ and 3′ primers ATGGGGGGACACCTTCATCCGTCAC and CACTCCAAGCAACATTTATTG, respectively (Chanock et al., 1992); for p67$^{phox}$, a 1.8-kb insert was obtained with the 5′ primer ATGTCCCTGGTGGAGGCCATCAGC and the 3′ primer TGTATATGCCTTATGAGTAAC. These products were directionally cloned into the *Eco*RI and *Hin*dIII sites of the cloning vector Bluescript SK+ (Stratagene). Orientation was confirmed by sequencing (Sanger et al., 1977). The recombinant plasmids were purified by alkaline lysis and centrifugation over a CsCl gradient.

Both p47$^{phox}$ and p67$^{phox}$ were expressed as fusion proteins linked to the C-terminal end of *Leishmania donovani* glutathione *S*-transferase (Smith & Johnson, 1988). For this purpose, the two cDNAs were excised from Bluescript, their ends were filled in with Klenow fragment, and *Eco*RI linkers were attached. The cDNAs were then ligated into the *Eco*RI sites of pGEX-1λT (p47$^{phox}$) and pGEX-3X (p67$^{phox}$) (the pGEX-family vectors were purchased from Pharmacia). The constructs were transformed into JM101, which were then grown on YT/ampicillin agar. Colonies were picked and plasmids isolated and examined by restriction mapping (for the p47$^{phox}$ construct, a double digest with *Xba*I and *Apa*I; for the p67$^{phox}$ construct, digests with *Bgl*II, *Sma*I, *Eco*RI, and *Bam*HI). Plasmids with correctly oriented inserts were selected on the basis of the restriction maps and sequenced across the 5′ junction between the pGEX vector and the cDNA, to make sure that (1) the inserts were in-frame with the glutathione *S*-transferase cDNA and (2) the protease cleavage sites linking glutathione *S*-transferase to the phagocyte proteins were intact.

The proteins were expressed according to a modification of the protocol provided by the supplier. In summary, the plasmid construct was transformed into JM101 and grown at 37 °C overnight in 50 mL of YT/ampicillin broth. The overnight culture was diluted into 450 mL of fresh YT/ampicillin broth 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 room temperature. All cultures were agitated vigorouly during growth. At the conclusion of the room temperature incubation, the bacteria were recovered by centrifugation at 5000*g* for 10 min at 4 °C. The pellet was suspended in 10 mL of ice-cold MTPBS (Smith & Johnson, 1988) containing 0.5 mM DFP and 5 mM EDTA, and disrupted by sonication (three 10-s pulses) at 0 °C. The sonicate was clarified by centrifugation at 10000*g* for 5 min at 4 °C. The fusion proteins were isolated from the clarified sonicate by purification over glutathione agarose as described by Smith and Johnson (1988). The purified fusion proteins were eluted at final concentrations of 22.1 μg/mL (p47$^{phox}$) and 440 μg/mL (p67$^{phox}$). The purified p47$^{phox}$ and p67$^{phox}$ fusion proteins were characterized by immunoblotting with antipeptide antibodies against p47$^{phox}$ and p67$^{phox}$ (Park et al., 1992), respectively, after electrophoresis through a 10% SDS–PAGE gel. The principal band on the immunoblot of the p47$^{phox}$ fusion protein migrated at $M_r$ 69K, while the major band on the blot of the p67$^{phox}$ fusion protein appeared at $M_r$ 89K.

*Electrophoresis and Immunoblotting.* Proteins in the fractions from the immunoaffinity purification or the Superose 6 column were precipitated by the addition of an equal volume of cold acetone followed by centrifugation at 12000*g* for 15 min at 4 °C. The precipitates were air-dried and then dissolved in 60 μL of sample buffer and subjected to SDS–PAGE on either 8% or 12.5% polyacrylamide gels using the Laemmli buffer system (Laemmli, 1970). The separated proteins were electrophoretically transferred onto a nitrocellulose sheet (Towbin et al., 1979) and probed with a mixture of ammonium sulfate-purified rabbit polyclonal antibodies raised against synthetic peptides from p47$^{phox}$ and p67$^{phox}$ (1:20 000 and 1:1000 dilutions, respectively) (Park et al., 1992), detecting with alkaline phosphatase-labeled goat anti-rabbit immunoglobulin antibodies (1:2000 dilution) using the BCIP/nitroblue

[1] Abbreviations: FPLC, fast protein liquid chromatography; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; HPLC, high-pressure liquid chromatography; cell eq and c-eq, cell equivalent(s); SE, standard error; $\Gamma_{1/2}$, ionic strength.

tetrazolium detection system (Bio-Rad). Bands were visualized with alkaline phosphatase-labeled goat anti-mouse Ig antibody (1:2000) or anti-rabbit Ig antibody (1:2000) and the BCIP/nitroblue tetrazolium detection system.

Quantitation of p47$^{phox}$ and p67$^{phox}$ on the immunoblots was accomplished by scanning with an UltroScan XL (Pharmacia—LKB). Results were calibrated using known amounts of the recombinant p47$^{phox}$ and p67$^{phox}$ fusion proteins in separate lanes on the same blot. Since the proteins were detected with anti-peptide antibodies, calculations were based on the assumption that the area under a given peak was proportional to the *molar* quantity of protein in the corresponding band. For all bands, both recombinant standards and unknown, the areas under the peaks were in the linear range of the graph of peak area vs quantity of recombinant protein.

*Assays.* $O_2^-$-forming activity was measured as superoxide dismutase-inhibitable reduction of ferricytochrome *c* (Markert et al., 1984). Assay mixtures contained 50 μL of glycine eluate from the immunoaffinity beads or 100 μL of FPLC fraction, $2 \times 10^6$ cell eq of membrane, 0.1 mM cytochrome *c*, 90 μM SDS, 0.16 mM NADPH, relaxation buffer, and other components as indicated, in a final volume of 0.75 mL. For most assays, all components except NADPH were mixed in the cuvette and equilibrated at 15 °C for 60 s. Reactions were then started by the addition of NADPH in 25 μL of relaxation buffer, and cytochrome *c* reduction was followed at 550 nm in a Uvikon 941 dual-beam recording spectrophotometer (Kontron Instruments, Milan), reading against a reference containing the same components plus 45 μg of superoxide dismutase. For each assay, the reaction rate shown is the maximum rate of cytochrome *c* reduction observed during the course of the incubation.

Protein concentrations were measured using a Bio-Rad assay kit.

## RESULTS

*Complex Containing p47$^{phox}$ and p67$^{phox}$.* Earlier work had suggested that p47$^{phox}$ and p67$^{phox}$ existed in neutrophil cytosol at least in part in the form of a complex (Park et al., 1992). Passage of cytosol over anti-p47$^{phox}$ agarose confirmed the existence of such a complex. Immunoblotting (Figure 1, *left*) showed that both p47$^{phox}$ and p67$^{phox}$ were removed from neutrophil cytosol by the immunoaffinity beads and remained attached to the beads through four wash steps, but were eluted by a low-pH glycine buffer. Elution of p47$^{phox}$ and p67$^{phox}$ by the glycine buffer appeared to be complete, because subsequent treatment of the beads with 3.5 M sodium thiocyanate did not release any additional p47$^{phox}$ or p67$^{phox}$. To confirm that p47$^{phox}$ and p67$^{phox}$ are components of a cytosolic complex that is specifically taken up by the anti-p47$^{phox}$ on the immunoaffinity beads, as opposed to proteins that are nonspecifically adsorbed to the immunoaffinity beads, we repeated the experiments with immunoaffinity beads whose antibody binding site had been blocked by preincubation with the peptide CFSTKRKLASAV. The results showed that the uptake of both p47$^{phox}$ and p67$^{phox}$ was prevented by pretreatment of the immunoaffinity beads with the peptide (Figure 1, *center*).

Analysis of the same samples by SDS–PAGE followed by Coomassie staining showed little protein except for a heavy band at $M_r$ 47K and weaker bands at 53K and 70K. These bands were not oxidase subunits, but appeared instead to be unrelated proteins that bound nonspecifically to the immunoaffinity beads (see below), since their appearance was not affected by treatment of the beads with the peptide (not shown).

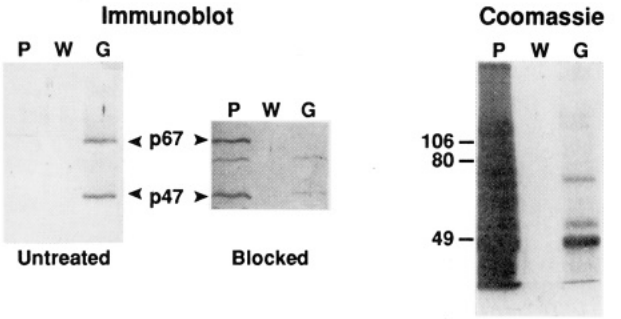


FIGURE 1: Isolation from neutrophil cytosol of a complex that contains the oxidase components p47$^{phox}$ and p67$^{phox}$. Isolation of the complex was carried out as described under Materials and Methods, using untreated immunoaffinity beads (*untreated*) and immunoaffinity beads that had been pretreated with the peptide CFSTKRKLASAV (*blocked*). For immunoblotting, 40 μL of each fraction was subjected to electrophoresis on an 8% SDS–PAGE gel, and antibodies were used at the following dilutions: anti-p47$^{phox}$, 1:10 000; anti-p67$^{phox}$, 1:1000; goat anti-rabbit immunoglobulin, 1:2000. In addition, 20 μL of each fraction was electrophoresed on a separate 8% SDS–PAGE gel that was stained with Coomassie Blue. Of the Coomassie Blue-stained gels, only the gel run with fractions from the untreated beads is shown; fractions from the peptide-treated beads gave a similar pattern. *P*, pass-through; *W*, final wash; *G*, glycine eluate.

Table 1: Support of $O_2^-$ Production in the Cell-Free Oxidase-Activating System by Fractions from Immunoaffinity Isolations[a]

| | activity [nmol of $O_2^-$ min$^{-1}$ (mg of membrane protein)$^{-2}$] | | | |
|---|---|---|---|---|
| | no peptide | | peptide | |
| fraction | no addition | plus cytosol | no addition | plus cytosol |
| pass-through | 0.18 ± 0.08 | 3.13 ± 0.65 | 1.10, 0.95 | 7.8, 6.98 |
| final wash | 0 | 2.48 ± 1.08 | 0 | 3.28, 3.00 |
| glycine eluate | 0.33 ± 0.06 | 8.73 ± 2.30 | 0 | 2.43, 2.80 |

[a] Immunoaffinity chromatography was carried out as described under Materials and Methods, using untreated immunoaffinity beads or beads that had been treated with the peptide CFSTKRKLASAV as indicated. Each assay mixture contained 50 μL of immunoaffinity fraction, $1.6 \times 10^6$ cell eq of membrane, 0.1 mM cytochrome *c*, 90 μM SDS, 0.16 mM NADPH, and relaxation buffer in a final volume of 0.75 mL. Some assay mixtures were supplemented with $2 \times 10^6$ cell eq of cytosol. The results shown represent the mean ± 1 SD for 3 separate sets of fractions obtained with untreated beads and the individual values for 2 sets of fractions obtained with peptide-treated beads.

*Activity of the Complex.* We investigated the ability of the glycine eluate to support $O_2^-$ production by the cell-free oxidase-activating system. By itself, the eluate showed a small but reproducible amount of activity when used in reaction mixtures that contained no other source of cytosolic oxidase components, suggesting that the complex contained all the elements necessary for $O_2^-$-forming activity, at least in this abbreviated system (Table 1). Supporting this interpretation is the finding that in experiments using peptide-blocked immunoaffinity beads, the $O_2^-$-forming activity was absent from the glycine eluate, being found instead in the pass-through. The small amount of activity in the glycine eluate was not enhanced by the pass-through fraction or by 10 μM GTPγS (not shown), but was increased as expected by supplementing the reaction mixtures with suboptimal amounts of neutrophil cytosol.

The rather unimpressive activity of the unsupplemented glycine eluate could in theory be explained by the loss or inactivation of an essential constituent during the isolation of the complex. Inactivation of p67$^{phox}$ seemed to be a reasonable

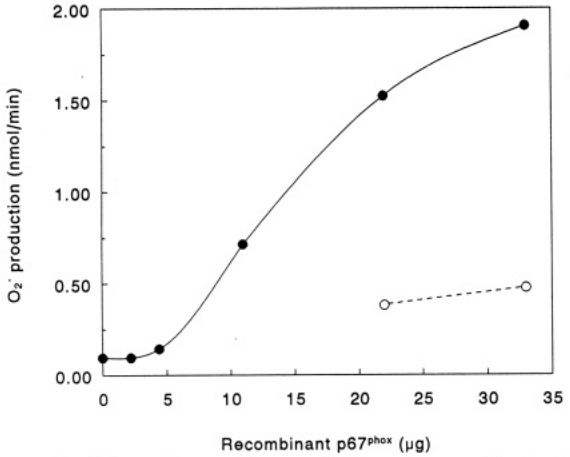


FIGURE 2: Effect of recombinant p67$^{phox}$ on $O_2^-$ production by a cell-free oxidase-activating system that uses the complex as the source of cytosolic oxidase components. Incubations were carried out as described in Table 1, except that recombinant p67$^{phox}$ was added to the assay mixtures in the quantities shown. $O_2^-$ production was followed at 550 nm in a Unikon 941 dual-beam recording spectrophotometer (Kontron Instruments, Milan), reading against a reference containing the same components plus 45 μg of superoxide dismutase. For each assay, the reaction rate shown is the maximum rate of cytochrome *c* reduction observed during the course of the incubation (●). Where indicated, 40 μg of superoxide dismutase (SOD) was present in the sample cuvettes (○).

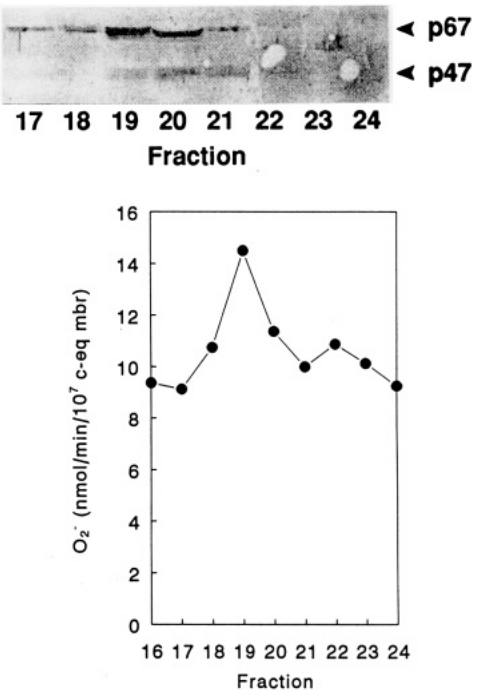


FIGURE 3: Size of the complex in pooled glycine eluates as estimated by FPLC over a Superose 6 size-exclusion column. FPLC was carried out as described under Materials and Methods. (*Top*) immunoblotting of the FPLC fractions. Immunoblotting was performed as described in Figure 1. (*Bottom*) $O_2^-$ production by the cell-free oxidase-activating system supplemented with FPLC fractions. Each assay mixture contained 100 μL of FPLC fraction, $2 \times 10^6$ cell eq of cytosol, $1.6 \times 10^6$ cell eq of membrane, 10 μM GTPγS, 0.1 mM cytochrome *c*, 90 μM SDS, 0.16 mM NADPH, and relaxation buffer in a final volume of 0.75 mL. $O_2^-$ production was followed at 550 nm in a Unikon 941 dual-beam recording spectrophotometer (Kontron Instruments, Milan), reading against a reference containing the same components plus 45 μg of superoxide dismutase. For each assay, the reaction rate shown is the maximum rate of cytochrome *c* reduction observed during the course of the incubation.

possibility, since p67$^{phox}$ is known to be highly unstable (Fujimoto et al., 1989; Erickson et al., 1992). We therefore conducted experiments to see if supplementation with recombinant p67$^{phox}$ could enhance the activity of the glycine eluate. The results (Figure 2) showed that the $O_2^-$-forming activity of the glycine eluate was increased very significantly by the recombinant p67$^{phox}$ fusion protein, the extent of the increase depending on the amount of p67$^{phox}$ added to the assay mixture. In contrast, the p47$^{phox}$ fusion protein had no effect on the $O_2^-$-forming activity of the complex.

*Size and Stoichiometry.* The approximate size of the complex obtained by immunoaffinity purification was estimated by gel filtration. The affinity-purified complex was subjected to FPLC over a Superose 6 column, detecting p47$^{phox}$ and p67$^{phox}$ in the eluted fractions by immunoblotting. By this method, the apparent $M_r$ of the complex was 240–300K, a value similar to that previously obtained with whole neutrophil cytosol (Park et al., 1992) (Figure 3, *top panel*). The fractions containing the complex were able to stimulate $O_2^-$ production by a cell-free oxidase-activating system that had been supplemented with suboptimal amounts of neutrophil cytosol (Figure 3, *bottom panel*).

The molar ratio of p47$^{phox}$ to p67$^{phox}$ in the complex could not be determined by analysis of the immunoprecipitate, because neutrophil cytosol contains uncomplexed p47$^{phox}$ (Park et al., 1992). The complex was therefore separated from the uncomplexed p47$^{phox}$ by FPLC over Superose 6, and the p47$^{phox}$/p67$^{phox}$ ratio was measured on the fraction that contained the largest amount of p67$^{phox}$, making the measurement by immunoblotting with recombinant standards as described under Materials and Methods. For the fractionation, cytosol from $10^8$ neutrophils was concentrated to 0.4 mL and chromatographed as described elsewhere (Park et al., 1992), collecting 1-mL fractions. Each lane of a blot contained protein from 20 μL of a fraction. The quantities of p47$^{phox}$ and p67$^{phox}$ in the selected fractions, as determined from measurements on samples from four separate fractionations, were 8.4 ± 1.7 and 9.4 ± 1.8 (SE) pmol/mL, respectively, values most consistent with the idea that the molar p47$^{phox}$/p67$^{phox}$ ratio in the complex is 1 to 1. In absolute terms, the results showed that resting neutrophil cytosol contains ≈15–20 pmol of p47$^{phox}$-p67$^{phox}$ complex/$10^8$ cells, assuming that the fractions selected for quantitation contained half the complex in the original sample (Park et al., 1992).

## DISCUSSION

Our findings show the presence in resting neutrophil cytosol of a complex of oxidase subunits of $M_r$ 240–300K consisting of at least two different proteins: p47$^{phox}$ and p67$^{phox}$. The complex appears to contain one p67$^{phox}$ per molecule of p47$^{phox}$, for a combined $M_r$ of 115K. Whether the 125–185K difference between the apparent $M_r$ of the complex and the combined $M_r$ of the subunits is due to the presence in the complex of one or more additional polypeptides, an additional p47$^{phox}$/p67$^{phox}$ pair, or both remains to be seen. In any case, it seems likely that the proteins in the complex migrate to the membrane as a unit when the oxidase is activated.

Indirect evidence for the existence of a complex between p47$^{phox}$ and p67$^{phox}$ has been available for some time. The earliest fractionation experiments showed that the active component of neutrophil cytosol migrated on gel filtration with $M_r$ 200–300K (Clark et al., 1987; Curnutte et al., 1987). When the molecular weights of p47$^{phox}$ and p67$^{phox}$ became known, it seemed reasonable to postulate that the $M_r$ 200–300K species represented a complex of some sort, although the composition of the complex could not be defined. Later

kinetic experiments specifically suggested that the complex might contain both $p47^{phox}$ and $p67^{phox}$. In those experiments, performed with a cell-free oxidase-activating system containing cytosol from neutrophils deficient in either $p47^{phox}$ or $p67^{phox}$, rates of $O_2^-$ production were found to be directly proportional to the amounts of normal cytosol added to the assay mixtures, indicating that in a cell-free activating system containing an excess of deficient cytosol and small amounts of normal cytosol the component (either $p47^{phox}$ or $p67^{phox}$) missing from the deficient cytosol but added with the normal cytosol was rate-limiting (Curnutte et al., 1989; Okamura et al., 1990). Consequently, it would be expected that with a cytosol deficient in both $p47^{phox}$ and $p67^{phox}$, each of the missing components would be limiting, and that therefore the rate of $O_2^-$ production would be proportional to the *square* of the concentration of normal cytosol added to the assay, since the normal cytosol was supplying both $p47^{phox}$ and $p67^{phox}$ to the assay mixture. When this experiment was carried out, however, the rate of $O_2^-$ production was found again to be directly proportional to the amount of normal cytosol added to the assay mixture (Fujimoto et al., 1989). The two proteins therefore behaved kinetically like a single component, suggesting that they existed in the cytosol in the form of a $p47^{phox}$-$p67^{phox}$ complex. Further evidence for such a complex was provided by the finding that on gel filtration of neutrophil cytosol, all the $p67^{phox}$ and part of the $p47^{phox}$ migrated at an $M_r$ of approximately 240K (Park et al., 1992), a result consistent with the presence in the cytosol of a $p47^{phox}$-$p67^{phox}$ complex. The existence of the $p47^{phox}$-$p67^{phox}$ complex, hitherto postulated on indirect grounds, has now been confirmed by its isolation.

Though the $p47^{phox}$-$p67^{phox}$ complex is stable enough to survive chromatography in the HEPES/glycol buffer employed in these studies, in a more physiological buffer the complex is probably in equilibrium with its components. This is suggested by the dependence of oxidase yield on the 2.5 power of the concentration of normal cytosol in the cell-free oxidase activation mixture (Babior et al., 1988), and by our repeated failures to isolate this complex from relaxation buffer ($\Gamma_{1/2}$ = 235 mM). The latter observation in turn suggests that the interaction between $p47^{phox}$ and $p67^{phox}$ is weakened by salts, and therefore that the two polypeptides are held together in the complex at least partly by electrostatic forces.

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