J. Biochem. Mol. Biol. Vol. 28, No. 3 pp. 275~280 May 31, 1995

Nucleotide Binding Component of the Respiratory Burst Oxidase of Human Neutrophils

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(Received January 18, 1995)

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 and p67, are in the cytosol, while the rest are in the plasma membrane. Recent evidence has suggested that at least some of the cytosolic oxidase components exist as a complex. The cytosolic complex with a molecular weight of ~240 kDa was found to bind to blue-agarose and 2',5'-ADP-agarose, which recognize nucleotide requiring enzymes. In order to identify the nucleotide binding component of the cytosolic complex we purified recombinant p47 and p67 fusion proteins using the pGEX system. Pure recombinant p47 was retained completely on 2',5'-ADP-agarose, whereas pure recombinant p67 did not bind to these affinity beads. On the basis of these results, we infer that p47 may contain the nucleotide binding site.

Key words: 2',5'-ADP-agarose, cytosolic components, NADPH oxidase, pGEX system.

The respiratory burst oxidase, a multicomponent enzyme so far found exclusively in phagocytes and B-lymphocytes, catalyzes the reduction of oxygen to superoxide anion using NADPH as the preferred electron donor (Babior and Woodman, 1990). The active oxidase is found in the plasma membrane of stimulated neutrophils (Dewald *et al.*, 1979), but it is known that in resting cells the oxidase components are distributed between the plasma membrane and cytosol (Bromberg and Pick, 1984; Curnutte, 1985; McPhail *et al.*, 1985), and that at least some of the cytosolic components are transferred to the membrane when the enzyme is activated (Clark *et al.*, 1990; Park *et al.*, 1992; Park and Babior, 1992). The cytosolic components transferred to the plasma membrane have been proposed as a complex with a molecular weight of about 240 kDa that contains the cytosolic components p47 and p67, which were found to be involved in the activation of the enzyme (Heyworth *et al.*, 1990; Park *et al.*, 1992).

It has been assumed that the cytosolic complex also contains an NADPH binding component. This assumption was tested by the ability of the cytolic complex to bind 2',5'-ADP-agarose, taking into account the similarity between 2',5'-ADP and NADPH (Abramovitz, 1985). The elution of the bound complex was affected by NADPH but not by NADH (Sha'ag and Pick, 1990). However, it was also shown that ATP is a very efficient elutor as well. Therefore, the possibilities that 1) there is nonspecific binding, 2) the cytosolic complex contains binding sites for both NADPH and ADP or 3) one site in the cytosolic complex can recognize the phosphate group at the ribose 2' position can all be suggested.

It has been shown that Cibacron blue 3GA (CB-3 GA) interacts with nucleotide-requiring enzymes and binds strongly to some of these enzymes that have a dinucleotide fold, presumably owing to the structural similarity between the dye and the nucleotides (Thompson and Stellwagen, 1976; Wilson, 1976). It has been also shown that CB-3GA acts as a very effective competitive inhibitor of the respiratory burst oxidase (Yamaguchi and Kakinuma, 1982). Therefore, we employed dye agarose affinity chromatography with CB-3GA as a ligand (blue-agarose) to either confirm or refute the proposal that the cytosolic complex contains nucleotide binding site(s).

Recently, the cytosolic complex of oxidase, consisting of at least two different proteins, p47 and p67, was isolated using immunoaffinity chromatography with an antibody that recognizes the C-terminus of p47 (Park *et al.*, 1994). It seems likely that the interaction between components of the complex is strong enough to survive any known chromatographic conditions (Sha' ag and Pick, 1990; Park *et al.*, 1992, 1994). Therefore, it would be helpful to purify each component in order

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to address the question of whether p47 or p67 interact with beads which have affinity with nucleotides. In the present studies, we have used recombinant DNA methodology to express large amounts of p47 and p67 as fusion proteins, and have utilized these highly purified components to identify the nucleotide binding component in the cytosolic complex of respiratory burst oxidase.

Materials and Methods

Materials

Cytochrome c (type VI), bovine superoxide dismutase, NADPH, GTPγS, glutathione (GSH), 2',5'-ADP-agarose, blue-agarose, and GSH-agarose were obtained from Sigma. SDS and immunoblotting reagents were purchased from Bio-Rad. pGEX-family vectors, dextran, and Ficoll-Hypaque were purchased from Pharmacia. Other reagents were the best grade commercially available and were used without further purification.

Preparation of neutrophil fractions

Neutrophil cytosol and 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-coagulated blood. The neutrophils were suspended at a concentration of 10^8 cells/ml in a modified relaxation buffer (100 mM KCl/3 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℃ until use.

Affinity 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; 0.75 ml portions of the concentrated cytosol was mixed with 0.75 ml portions of ice-cold 50 mM HEPES (pH 8.0)/25% (v/v) ethylene glycol and then applied to a 1.5-ml column of blue-agarose that had been equilibrated with the same buffer. The column was first eluted with the HEPES/glycol buffer until the A_{260} of the effluent had fallen to 0, and then with the HEPES/glycol containing 5 mM NADPH and 0.25 M KCl, collecting 1-ml fractions throughout the procedure, which was carried out at 4℃. A batch protocol whereby 0.2 ml of packed affinity beads was put into conical polypropylene 1.5 ml Effendorf tubes was also employed. The concentrated cytosol (0.3 ml) or purified fusion proteins (~10 μg in 0.3 ml relaxation buffer) was mixed with an equal volume of ice-cold HEPES/glycol buffer and added to prewashed beads in the Eppendorf tube. The tubes were rotated end-over-end for 1 h at 4℃ and spun for a few seconds at maximum speed in a microcentrifuge. The supernatant ("pass-through") was reserved, and the affinity beads were washed with four 1-ml portions of ice-cold HEPES/glycol buffer. Finally, the bound proteins were eluted by incubating the affinity beads for 30 min at 4℃ with 0.5 ml of HEPES/glycol buffer containing NADPH/KCl. The fractions were subjected to electrophoresis and immunoblotting as described below. Protein concentrations were measured using a Bio-Rad kit.

FPLC analysis of eluate

Eluates from affinity purification were gel-filtered by FPLC through a Superose 6 size-exclusion column 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 and p67 fusion proteins

The fusion proteins were prepared as described by Park *et al.* (1994) with minor modifications. Full-length cDNA for p47 and p67 were directionally cloned into the *Eco*RI and *Hin*dIII sites of the cloning vector pBluescript SK^+. Both p47 and p67 were expressed as fusion proteins linked to the C-terminal end of *Leishmania donovani* glutathione S-transferase (Smith and Johnson, 1988). For this purpose, the two cDNAs were excised from the pBluescript, 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) and pGEX-3X (p67) (Fig. 1). The constructs were introduced into JM 101, which were then grown on YT/ampicillin agar. Colonies were picked and plasmids isolated and examined by restriction mapping. 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 the inserts were in-frame with the glutathione S-transferase cDNA. Transformed JM 101 cells containing inserts were grown at 37℃ 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℃. The culture was then made up to 0.1 mM isopropyl β-D-thiogalactoside and grown for an additional 3 h at room temperature. All cultures were agitated vigorously during growth. At the conclusion of room temperature incubation, the bacteria were recovered by centrifugation at 5,000×*g* for 10 min

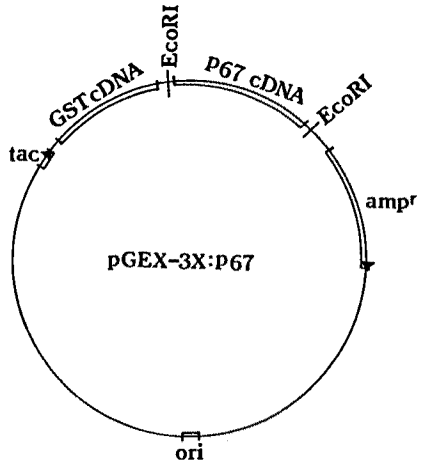

Fig. 1. Plasmid containing the cDNA for p67 from human neutrophils. The cDNA for p67 was inserted into pGEX-3X at the unique site for *Eco*RI.

at 4℃. The pellet was suspended in 10 ml of ice-cold MTPBS (Smith and Johnson, 1988) containing 0.5 mM DFP and 5 mM EDTA, and disrupted by sonication (three 10-sec pulses) at 0℃. The sonicate was clarified by centrifugation at 10,000×*g* for 5 min at 4℃. The fusion proteins were isolated from the clarified sonicate by purification over GSH-agarose as described by Smith and Johnson (1988).

Electrophoresis and immunoblotting

Protein samples were subjected to SDS-PAGE on either 8% or 10% 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 and p67 (1 : 20,000 and 1 : 1,000 dilutions, respectively) (Park *et al.*, 1992), detecting them with alkaline phosphatase-labeled goat anti-rabbit immunoglobulin antibodies (1 : 2,000 dilution) using the BCIP/nitroblue tetrazolium detection system (Bio-Rad).

Enzyme assay

O_2^--forming activity was measured as superoxide dismutase-inhibitable reduction of ferricytochrome c (Markert *et al.*, 1984). The assay mixture contained eluate from the affinity chromatography, 2×10^6 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 25℃ for 60 sec. Reactions were then started by the addition of NADPH in 25 μl of relaxation buffer, and cytochrome c reduction

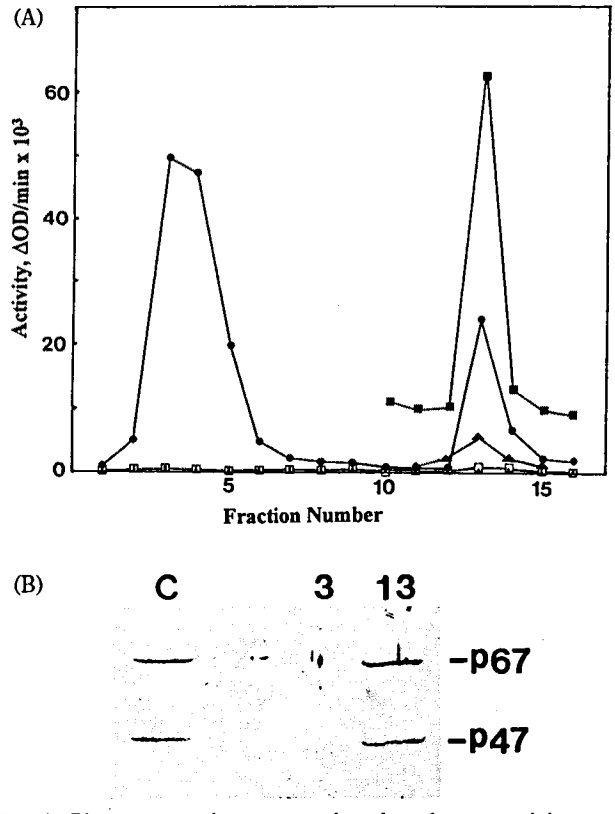

Fig. 2. Blue-agarose chromatography of crude neutrophil cytosol. Proteins were chromatographed on 1.5-ml blue-agarose column as described in Materials and Methods. *Panel A* □, Inherent activity of 100 μl eluate; ▲, activity of 100 μl eluate in the presence of 10 μM GTPγS; ■, activity of 100 μl eluate in the presence of 2×10^6 cell eq of cytosol; ●, protein, full scale=3 mg/ml. *Panel B* Immunoblot detection of fractions from blue-agarose chromatography. C, crude neutrophil cytosol; 3 and 13, fraction numbers 3 and 13, respectively.

was followed at 550 nm, 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.

Results and Discussion

When the cytosol from resting neutrophils was chromatographed on blue-agarose, absolutely no O_2^--forming activity was observed in pass-through. Subsequent elution with NADPH yielded a small but reproducible amount of O_2^--forming activity in the elution region in the presence of 10 μM GTPγS. (Fig. 2). We have previously shown that GTPγS augmented activity of superoxide generation in a cell-free system consisting of cytosol plus plasma membrane (Park and Babior, 1992). The small amount of activity in the eluate was increased as expected by supplementing the reaction mixtures with suboptimal amounts of neutrophil cytosol

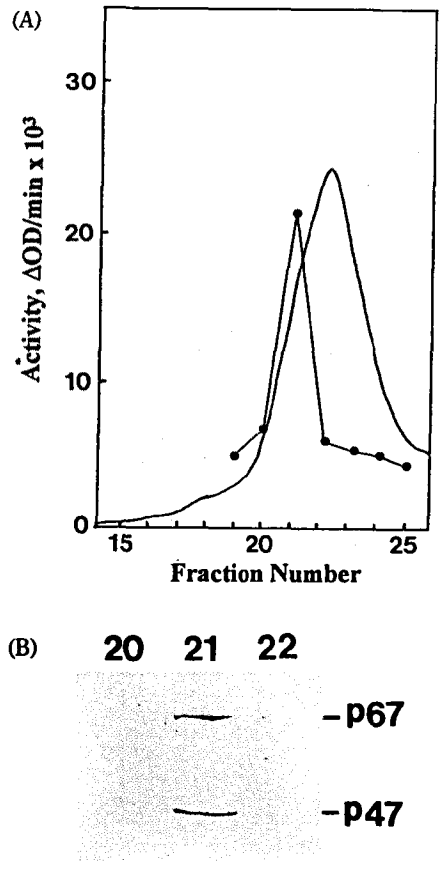

Fig. 3. Size of the complex in pooled eluates from blue-agarose column as estimated by FPLC over a Superose 6 size-exclusion column. FPLC was carried out as described in Materials and Methods. *Panel A* ●–●, Activity of 100 μl eluate in the presence of 1×10^6 cell eq of cytosol; ——, protein, full scale=0.3 A at 254 nm. *Panel B* Immunoblotting of FPLC fractions (20~22).

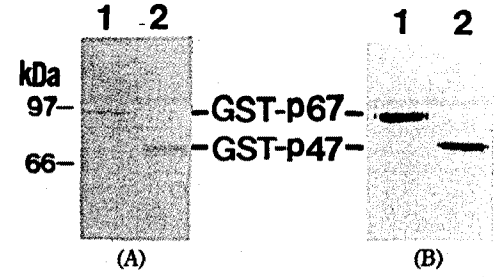

Fig. 4. Gel electrophoresis and immunoblot detection of recombinant cytosolic factors as fusion proteins. *Panel A* Lane 1 : p67 fusion protein; lane 2 : p47 fusion protein (10% polyacrylamide gel stained with Coomassie Blue B-250). *Panel B* Lane 1 : p67 fusion protein; lane 2 : p47 fusion protein (Immunoblot was probed with antibodies against synthetic peptides from p67 and p47).

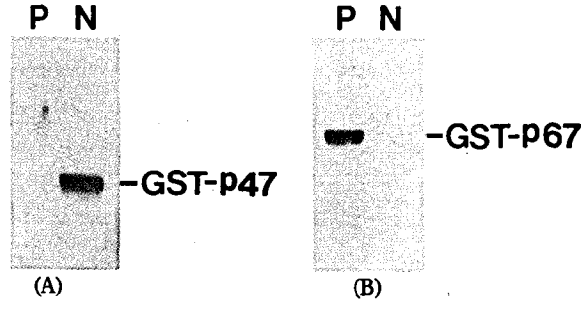

Fig. 5. 2′,5′-ADP-agarose chromatography of pure recombinant p47 and p67. Batch protocol was applied as described in Materials and Methods. Samples applied to each tube of beads; A : recombinant p47 fusion protein (10 μg); B : recombinant p67 fusion protein (10 μg). Pass-through fractions (P) represent proteins eluted in the washing buffer. NADPH eluates (N) were obtained from a step containing 5 mM NADPH and 0.25 M KCl in the same buffer. Eluted fractions were analyzed by SDS-PAGE on 8% polyacrylamide gels and immunoblotted as described in Fig. 4.

as shown in Fig. 2. Immunoblotting showed that both p47 and p67 were removed from resting neutrophil cytosol by the blue-agarose beads and remained attached to the beads through wash steps, but were eluted by a NADPH/KCl buffer. These results support the proposal that the complex contains a nucleotide binding site.

The rather low activity of the unsupplemented eluate could be explained by either the loss or inactivation of an essential constituent during the purification. We therefore conducted gel filtration experiments to estimate the approximate size of the complex obtained by blue-agarose affinity purification. The affinity-purified complex was subjected to FPLC over a Superose 6 column, detecting p47 and p67 in the eluted fractions by immunoblotting (Fig. 3). By this method, the apparent molecular weight of the complex was ~240 kDa, a value similar to that previously obtained with whole neutrophil cytosol (Heyworth *et al.*, 1990; Park *et al.*, 1992), or with the immunoaffinity-purified complex (Park *et al.*, 1994). Therefore, the possibility of the loss of the essential component of the complex during purification can be ruled out. Inactivation of p67 seems to be a reasonable possibility, since p67 is known to be highly unstable (Erickson *et al.*, 1992) and inactivation of p67 was also observed during the purification of the cytosolic complex by immunoaffinity chromatography (Park *et al.*, 1994).

The recombinant p47 fusion protein was the predominant polypeptide band observed in the bacterial lysate harvested at either 37℃ or room temperature. Although the p67 fusion protein forms inclusion bodies almost exclusively when bacteria are grown at 37℃, the crude lysate contained a soluble form of p67 when cells were grown at room temperature (~20℃) (data not shown). The purified p47 and p67 fusion proteins were characterized by immunoblotting with antipeptide antibodies against p47 and p67 respectively, after electrophoresis through a 10% SDS-PAGE gel. The principal band on the immunoblot of the p47 fusion protein migrated at the molecular weight of 69 kDa, while the

major band on the blot of the p67 fusion protein appeared at the molecular weight of 87 kDa (Fig. 4).

Pure recombinant p47 fusion protein was retained completely on 2′,5′-ADP-agarose and was subsequently eluted with the same buffer mentioned above containing 5 mM NADPH plus 0.25 M KCl, as shown in Fig. 5. In contrast, pure recombinant p67 fusion protein or glutathione S-transferase itself did not bind to this column and was entirely in the pass-through fraction. This finding suggests that p47 could be the nucleotide binding component of the respiratory burst oxidase. There is a possibility that an unidentified cytosol component other than p47 and p67 may contain the nucleotide binding site. However, the fact that the recombinant p47 and p67 plus plasma membrane can reconstitute the oxidase activity in the cell-free system (Uhlinger *et al.*, 1992) may exclude this possibility. In this regard, it can be inferred that p67 is retained by an association with p47 when resting cytosol is chromatographed on 2′,5′-ADP-agarose and blue-agarose beads.

There are many controversies concerning the identity of the nucleotide binding protein of respiratory burst oxidase. A number of investigartors have used affinity labeling techniques to identify nucleotide binding proteins. Although there is a proposal that the NADPH-binding protein may reside in the plasma membrane (Segal *et al.*, 1992), there is ample evidence to suggest that the nucleotide binding protein exists in the cytosol as a part of a complex. The activity of cytosolic oxidase components eluted from the 2′,5′-ADP-agarose affinity beads was retained by a 100 kDa cutoff membrane (Sha'ag and Pick, 1990); possibly suggesting that it consisted of a complex of oxidase components, one of which is the nucleotide binding subunit, rather than a single component of oxidase. There has been other evidence suggesting that a nucleotide (probably NADPH)-binding protein accompanies a cytosolic protein in resting neutrophils. Labeling of cytosol from resting neutrophils with NADPH 2′,3′-dialdehyde, an affinity label for NADPH-binding proteins, eliminates the cytosol's ability to support the cell-free oxidase activating system, while O_2^- production by this system was unaffected by preincubation of the plasma membrane with the affinity label (Smith *et al.*, 1989). Affinity labeling with radioactive material failed to identify the NADPH-binding oxidase component, however, when several cytosolic proteins were labeled by this method (Smith *et al.*, 1989). In a recent study, it was found that treatment of activated neutrophil membranes with 0.3 M KCl extracted an NADPH-binding protein which associated with the membrane as a peripheral protein, recognized by labeling with [^{32}P]NADPH-dialdehyde (Umei *et al.*, 1991). The authors suggested that an NADPH-binding component was located in the cytosol of resting neutrophils, and that the translocation of this protein to the plasma membrane occurred during activation. The major drawback of this approach is that due to the nonspecific reaction of labeling agents with amino groups, a large number of cytosolic proteins were labeled and no conclusion could be drawn concerning the molecular mass of the putative NADPH-binding components.

Our result cannot clarify whether p47 contains 1) two separate binding sites for NADPH and ATP, 2) one binding site for either NADPH or ATP or 3) one site which can recognize both nucleotides. The finding that recombinant p47 interact with the 2′,5′-ADP-agarose supports the notion that p47 may act as a nucleotide binding protein. In this regard, the application of the affinity labeling technique using nucleotide analogs to purified p47 will aid in elucidating the nature of the nucleotide binding site in the cytosolic component of respiratory burst oxidase.

Acknowledgement

This research was supported in part by a Genetic Engineering Research Grant (1994) and a Basic Sciences Research Institute Grant (BSRI-94-4403) from the Ministry of Education of Korea. We wish to thank Dr. Bernard M. Babior, the Scripps Research Institute, for providing the anti-p47 and -p67 antibodies.

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