# Activation of the Leukocyte NADPH Oxidase Subunit p47$^{phox}$ by Protein Kinase C. A Phosphorylation-Dependent Change in the Conformation of the C-Terminal End of p47$^{phox}$ †

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ABSTRACT: The leukocyte NADPH oxidase of neutrophils is a membrane-bound enzyme that catalyzes the production of $O_2^-$ from oxygen using NADPH as electron donor. Dormant in resting neutrophils, the enzyme acquires catalytic activity when the cells are exposed to appropriate stimuli. During activation, the cytosolic oxidase components p47$^{phox}$ and p67$^{phox}$ migrate to the plasma membrane, where they associate with cytochrome $b_{558}$, a membrane-bound flavohemoprotein, to assemble the active oxidase. An essential element of the activation process is the phosphorylation of p47$^{phox}$, an event that accompanies oxidase activation in whole cells and can activate the oxidase in a cell-free system. We show here that the phosphorylation of p47$^{phox}$ leads to a substantial decrease in the reactivity of cysteine C378 toward *N*-ethylmaleimide, indicating the occurrence of a conformational change involving the C-terminal region of p47$^{phox}$. A similar conformational change occurs when p47$^{phox}$ is exposed to arachidonate, one of a number of anionic detergents that activate the oxidase in the cell-free system. We propose that this change in conformation results in the appearance of a binding site through which p47$^{phox}$ interacts with cytochrome $b_{558}$ during the activation process.

The NADPH oxidase of phagocytes (the leukocyte NADPH oxidase), an important element of host defense against microbial infection, catalyzes the reduction of oxygen to $O_2^-$ using NADPH as the electron donor (Chanock et al., 1994). The oxidase is dormant in resting neutrophils, but acquires catalytic activity when cells are exposed to appropriate stimuli. Oxidase activity is located in the plasma membrane, but it is known that in resting cells the oxidase components are distributed between a membrane fraction and the cytosol, and that when activation takes place either in intact cells or in a cell-free system, the oxidase components p47$^{phox}$ and p67$^{phox}$, which exist in the cytosol as a ≈240 kDa complex (Clark et al., 1987; Park et al., 1992, 1994), and the small guanine nucleotide-binding protein Rac2, which also participates in oxidase activation (Knaus et al., 1991, 1992), migrate to the membrane, where the p47$^{phox}$·p67$^{phox}$ complex associates with the membrane cytochrome $b_{558}$ to assemble the functioning oxidase (Heyworth et al., 1991; Clark et al., 1990; Doussière et al., 1990; Quinn et al., 1993; Tyagi et al., 1992; Nakanishi et al., 1992; El Benna et al., 1994b).

The cytosolic oxidase component p47$^{phox}$ is a basic protein that becomes extensively phosphorylated when the oxidase is activated (Hayakawa et al., 1986; Rotrosen & Leto, 1990). Both in whole cells and under certain circumstances in the cell-free system, phosphorylation of p47$^{phox}$ appears to play an important role in the activation of the oxidase and the translocation of the p47$^{phox}$·p67$^{phox}$ complex from the cytosol to the membrane (Park & Ahn, 1995) [though in the cell-free system, oxidase activation and p47$^{phox}$·p67$^{phox}$ translocation is usually initiated with anionic detergents such as arachidonate or sodium dodecyl sulfate [SDS] (Bromberg & Pick, 1984; Shpungin et al., 1989)]. The phosphorylation target is known to be a group of serines (residues 303−379) in the highly basic carboxyl-terminal quarter of the polypeptide (El Benna et al., 1994a, 1996; Faust et al., 1995), but the biochemical basis for the transfer of p47$^{phox}$ to the membrane remains a matter of speculation, though it has been proposed that in intact neutrophils, the phosphorylation of p47$^{phox}$ is necessary to neutralize the strong positive charge in the arginine-rich carboxyl-terminal domain of the protein.

In the earlier study using the cell-free system, we found that the activation of the leukocyte NADPH oxidase by SDS was associated with the appearance of a membrane binding site on a cytosolic oxidase component (Park et al., 1992), presumably p47$^{phox}$ because p47$^{phox}$ can translocate by itself whereas p67$^{phox}$ cannot translocate without p47$^{phox}$ (Heyworth et al., 1991). This result suggested that oxidase activation involves a conformational change affecting the cytosolic components. Similarly, Sumimoto et al. showed that the SH3 domains of p47$^{phox}$, initially masked by a proline-rich stretch of peptide near the C-terminus of the molecule, could be exposed by arachidonate (Sumimoto et al., 1994). Therefore it is plausible to assume that both phosphorylation and anionic amphiphiles could lead to a change in the charge and conformation of p47$^{phox}$ that causes the translocation of p47$^{phox}$ to the membrane during the activation of the leukocyte NADPH oxidase.

To assess changes in the conformation of p47$^{phox}$ that occurred during activation, we have developed a method to covalently label p47$^{phox}$. The approach we used was based on the idea that such a conformational change may alter the accessibility of one or more of the five cysteine residues of

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p47$^{phox}$ (C48, C98, C111, C196, and/or C378) to −SH group-specific reagents. Our results indicate that C378, the cysteine residue in the C-terminal region of p47$^{phox}$, is the cysteine most readily alkylated by the -SH reagent *N*-ethylmaleimide (NEM) and suggest that modulation of p47$^{phox}$ by phosphorylation or arachidonate treatment appears to involve a conformational change that is reflected in a decrease in the susceptibility of C378 to alkylation by NEM.

## MATERIALS AND METHODS

*Materials*. Chemicals, enzymes, and reagents for molecular biology were obtained from the following sources: pGEX-1$\lambda$T vector, dextran, Ficoll-Hypaque (Pharmacia); bovine erythrocyte superoxide dismutase (SOD), luminol, phosphatidylserine, diacylglycerol, sucrose, isopropyl $\beta$-D-thiogalactoside (IPTG), NADPH, ATP, cytochrome *c* (type VI), GTP$\gamma$S, glutathione (GSH)-agarose, phenylmethylsulfonyl fluoride (PMSF), thrombin, CNBr, NEM, iodoacetamide, CM-Sepharose, ampicillin (Sigma), *N*-[2-$^3$H]ethylmaleimide ([$^3$H]NEM; specific activity, 50.08 Ci/mmol), 1.0 mCi/mL (Dupont−New England Nuclear); Amplify (Amersham); rat brain protein kinase C, horseradish peroxidase (Calbiochem); restriction enzymes (New England BioLabs); *Eco*RI linker (Stratagene); Bio-Rad protein assay kit, electrophoresis, and immunoblotting reagents (Bio-Rad); molecular weight markers for the Tris-Tricine SDS−PAGE (Gibco BRL).

*Preparation of Neutrophil Fractions*. Neutrophil cytosol and membranes were prepared as described previously (Borregaard et al., 1983). Briefly, neutrophils were obtained from normal subjects by dextran sedimentation and Ficoll-Hypaque fractionation of freshly drawn citrated 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. Both cytosol and membrane were divided into aliquots and stored at −70 °C until used.

*Preparation of Recombinant p47$^{phox}$*. *E. coli* transformed with pGEX-1$\lambda$T containing an insert of p47$^{phox}$ cDNA were grown and lysed and the glutathione-*S*-transferase−p47$^{phox}$ fusion protein purified on GSH-agarose as described elsewhere (Faust et al., 1995). The recombinant p47$^{phox}$ was separated from the glutathione-*S*-transferase by treatment with thrombin (10 units/mL) in elution buffer [50 mM Tris (pH 8.0)/5 mM GSH/150 mM NaCl/2.5 mM $CaCl_2$] for 2 h at room temperature and was then purified by chromatography over a 0.9 × 10 cm CM-Sepharose column equilibrated with 5 mM potassium phosphate buffer (pH 7.0)/0.1 mM PMSF, eluting with a 40-mL gradient of 0−0.4 M NaCl in the same buffer. Glutathione-*S*-transferase appeared in the pass-through; p47$^{phox}$ was eluted at 0.33 M NaCl. The fractions containing p47$^{phox}$ were concentrated to 1 mg/mL and stored at −70 °C. The concentration of proteins was determined with the Bio-Rad assay kit using bovine serum albumin as a standard.

*Assay of Activity of Recombinant p47$^{phox}$*. The activity of recombinant p47$^{phox}$ was measured by its ability to support $O_2^-$ formation by the leukocyte NADPH oxidase in a cell-free system initiated by protein kinase C (El Benna et al., 1995). For activation by protein kinase C, the reaction mixture contained 2.5 × $10^7$ cell equiv cytosol, 0.5 unit of protein kinase C, 5 $\mu$g of p47$^{phox}$, 25 $\mu$g of phosphatidylserine, 2.5 $\mu$g of diacylglycerol, 0.5 mM $CaCl_2$, 50 $\mu$M GTP$\gamma$S, 0.1 mM ATP, and relaxation buffer (Borregaard et al., 1983) in a total volume of 0.5 mL. Oxidase activity was followed by measuring chemiluminescence at room temperature in a Monolight 2010 luminometer (El Benna et al., 1995).

*In Vitro Phosphorylation*. The reaction mixture contained 2 $\mu$g of recombinant p47$^{phox}$, 1 mM ATP, 10 mM magnesium acetate, 0.5 mM $CaCl_2$, 160 $\mu$g of phosphatidylserine, 20 ng of protein kinase C, and relaxation buffer in a total volume of 0.1 mL. The samples were incubated 20 min at 37 °C and then gel filtered using a Bio-Spin column (Bio-Gel-6, Bio-Rad) equilibrated with relaxation buffer.

*Labeling of p47$^{phox}$ by [$^3$H]NEM*. Preparations of p47$^{phox}$ (2 $\mu$g) were treated at room temperature with 2 $\mu$L (2 $\mu$Ci) of [$^3$H]NEM in a total volume of 0.1 mL. After incubation for the indicated time, the sample was placed on ice for 5 min and the labeled protein was precipitated by adding 50% (w/v) trichloroacetic acid to a final concentration of 10%. Transfer RNA (250 $\mu$g) was also added as a carrier. After a further 10 min on ice, the proteins were pelleted by centrifugation at 10000*g* for 10 min at 4 °C. The precipitate was suspended in 100 $\mu$L of $H_2O$, mixed with water-compatible scintillation fluid (Ready Safe, Beckman), and assayed for radioactivity in a liquid scintillation counter. For SDS−PAGE and Tris-Tricine SDS−PAGE, the labeled samples were precipitated with acetone for 1 h at −20 °C and the precipitates isolated by centrifugation for 20 min at 13000*g* (4 °C). For analysis, the precipitates were dissolved either in Laemmli sample buffer or, when CNBr cleavage samples were analyzed, in sample buffer for Tris-Tricine gel electrophoresis (Schagger & von Jagow, 1987).

*Immunopurification of p47$^{phox}$ from Neutrophil Cytosol*. Cytosolic p47$^{phox}$ was prepared and the C-terminal CNBr peptide isolated by immunoaffinity chromatography as described previously, using agarose that had been coupled with an antibody against the C-terminal decapeptide of p47$^{phox}$ (El Benna et al., 1994a; Park et al., 1994). The agarose was placed in a column and equilibrated with relaxation buffer for 1 h at 4 °C. Resting cytosol ($10^9$ cell equiv) was then mixed with the agarose and incubated overnight at 4 °C with end-over-end rotation. The column was then washed extensively with relaxation buffer, and the adsorbed p47$^{phox}$ was eluted with 0.1 M glycine $Cl^-$ buffer, pH 2.8, neutralized by the addition of 1 M Tris-HCl, pH 9.2 (28 $\mu$L of Tris/mL of eluate), concentrated using an Amicon-Centriprep 10, and used for labeling with [$^3$H]NEM.

*CNBr Cleavage and Tris-Tricine SDS−PAGE of [$^3$H]-NEM-Labeled p47$^{phox}$*. Purified samples of $^3$H-labeled p47$^{phox}$ isolated by acetone precipitation as described above were cleaved at methionine residues by incubation with 12.5 mg/mL of CNBr in 0.15 mL of 70% (v/v) formic acid for 16 h at room temperature in the dark. The reaction mixtures were then quenched with an equal volume of $H_2O$ and lyophilized in a Speed-Vac (Savant). The cleavage products were separated by Tris-Tricine SDS−PAGE (Schagger & von Jagow, 1987).

*Electrophoresis and Immunoblotting*. SDS−PAGE of protein samples was carried out according to Laemmli (1970) using a 7.5% running gel. Small peptides from CNBr cleavage reactions were separated by Tris-Tricine SDS−

PAGE using a 16.5% running gel (Schagger & von Jagow, 1987). After SDS−PAGE, the separated proteins and the small peptides from CNBr cleavage were electrophoretically transferred onto a nitrocellulose sheet (Towbin et al., 1979), probed with a 1:5000 dilution of partially purified C-terminal rabbit polyclonal antibody raised against the C-terminal decapeptide of p47$^{phox}$, and finally detected with a 1:2000 dilution of alkaline phosphate-labeled goat anti-rabbit Ig antibody (CALTAG) using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium as substrate (Bio-Rad).

*Autoradiography of Polyacrylamide Gels*. The detection of radiolabeled p47$^{phox}$ and its peptide fragments on Laemmli or Tris-Tricine gels were accomplished by soaking the gels in Amplify (Amersham) with agitation for 30 min to increase the signal, then drying the Laemmli gels, and finally placing the dried (Laemmli) or undried (Tris-Tricine) gels in direct contact with X-ray film and an intensifying screen at −80 °C, first wrapping the undried gels in Saran Wrap. For more accurate quantitation, the regions of the gels containing the radioactive polypeptides were excised using an autoradiogram as a template, and the gel slices were placed in counting vials and dissolved by incubating overnight at room temperature with gentle agitation in a freshly prepared mixture of 150 $\mu$L of ammonium hydroxide (20−22%), 1 mL of NCS solubilizer (Amersham) and 10 mL of scintillator fluid as described above. The resulting solutions were assayed by liquid scintillation counting. Background counts were determined by the same procedure using a piece of gel of similar size excised from a tritium-free region of the gel.

*Replicates*. Unless otherwise indicated, each result described in this paper is representative of at least three separate experiments.

## RESULTS

*Labeling of Cysteine C378 by N-[2-$^3$H]Ethylmaleimide ([$^3$H]NEM)*. During the activation of the leukocyte NADPH oxidase in whole cells, p47$^{phox}$ becomes extensively phosphorylated on serine residues lying between S303 and the C-terminus of the 390-residue molecule. This occurrence suggests that during oxidase activation, p47$^{phox}$ undergoes a significant conformational change involving the C-terminal quarter of the molecule. We investigated this possibility by examining the accessibility of cysteine residues to a sulfhydryl-specific reagent before and after exposure of p47$^{phox}$ to agents known to activate the oxidase—specifically, protein kinase C and arachidonic acid.

The oxidase subunit p47$^{phox}$ contains five cysteine residues: C48, C98, C111, and C196 in the N-terminal half of the molecule, and C378 in a location very close to the C-terminus. When p47$^{phox}$ was incubated with [$^3$H]NEM, one or more of these cysteines was labeled by the reagent (Figure 1). Specificity of the labeling was confirmed by the finding that the reaction of the protein with [$^3$H]NEM was blocked by preincubation with either non-radioactive *N*-ethylmaleimide or iodoacetamide, another sulfhydryl reagent.

When p47$^{phox}$ is cleaved by CNBr, each of its cysteines is found in a different peptide (Table 1). It was therefore possible to identify the alkylated cysteines by cleaving [$^3$H]-NEM-treated p47$^{phox}$ with CNBr, separating the resulting peptides by gel electrophoresis and assaying them for radioactivity. The most heavily labeled peptide migrated

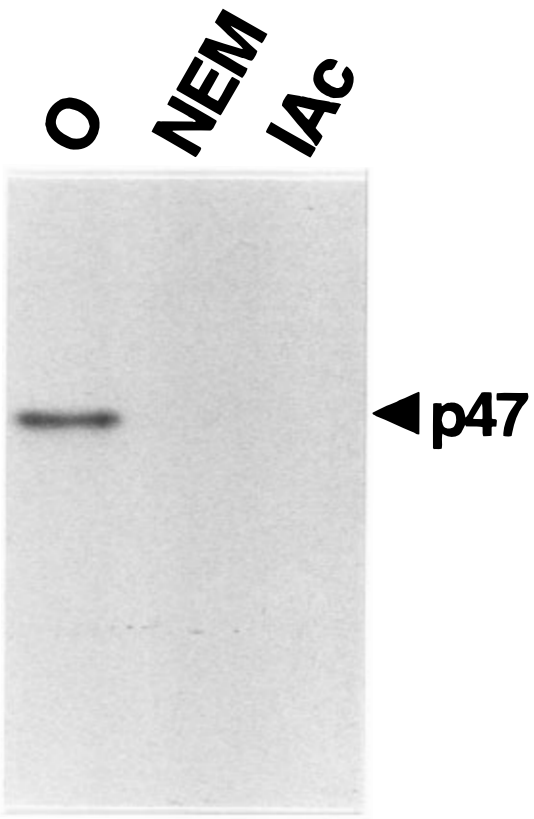



FIGURE 1: Labeling of p47$^{phox}$ by [$^3$H]NEM. Recombinant p47$^{phox}$ was treated with a sulfhydryl reagent as indicated, then incubated for 3 min with [$^3$H]NEM and analyzed by SDS−PAGE followed by autoradiography. (O) No treatment; (NEM) treated with *N*-ethylmaleimide; (IAc) treated with iodoacetamide.

Table 1: Cysteine-Containing CNBr Peptides of p47$^{phox}$

| residues | sequence |
|---|---|
| 28-57 | FLVKWQDLSE KVVYRRFTEI CEFHKTLKEM |
| 58-102 | FPIEAGAINP ENRIIPHLPA PKWFDGQRAA ENRQGTLTE YCSTLM |
| 103-141 | SLPTKISRCP HLLDFFKVRP DDLKLPTDNQ TKKPETYLM |
| 176-198 | ALSTGDVVEV VEKSESGWWF CQM |
| 329-390 | YLQKSGQDVS QAQRQIKRGA PPRRSSIRNA HSIHQRSRKR LSQDAYRRNS VRFLQQRRRQ ARPGPQSPGS PLEEERQTQR SKPQPAVPPR PSADLILNRC SESTKRKLAS AV* |

with an $M_r$ of ≈14000, identifying it as the C-terminal peptide Y279−V390, which contained cysteine C378 (Figure 2). Confirming this assignment were the observations that the peptide was recognized by an antibody that had been raised against the C-terminal decapeptide of p47$^{phox}$ (anti-p47$^{phox[381-390]}$), and that it was stripped from the CNBr cleavage mixture by an anti-p47$^{phox[381-390]}$ immunoaffinity column but could then be eluted from the column by a low-pH glycine buffer (Figure 3).

The second most heavily labeled peptide in the CNBr cleavage mixture was A176−M198, which contained C196 and was the smallest of the cysteine-containing CNBr peptides ($M_r$ < 3000). This peptide, however, appeared to contain much less than half the amount of radioactivity found in the C378 peptide (Figure 2). Consistent with the foregoing

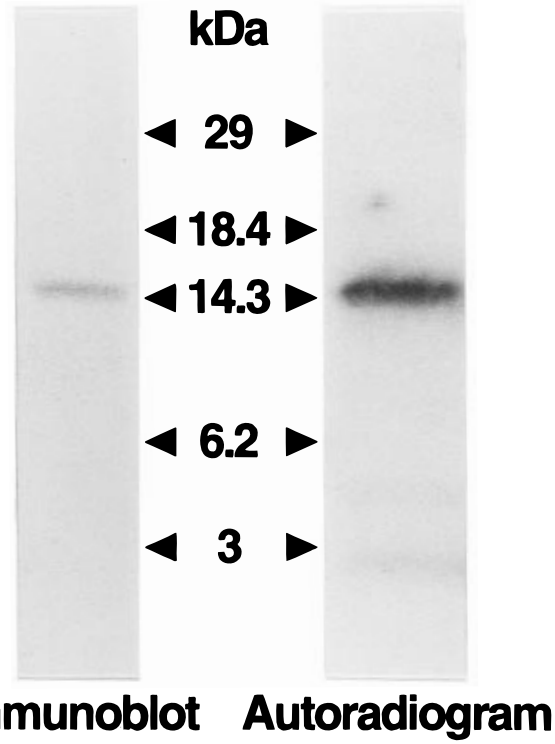



FIGURE 2: Radioactivity of peptides obtained from [$^3$H]NEM-labeled p47$^{phox}$ by CNBr cleavage. Recombinant p47$^{phox}$ was treated for 3 min with [$^3$H]NEM and was then precipitated with acetone and cleaved with CNBr as described in the text. The resulting peptides were separated by Tris-Tricine SDS−PAGE and either analyzed by autoradiography or transferred to nitrocellulose and probed for C-terminal peptide as described in Materials and Methods.

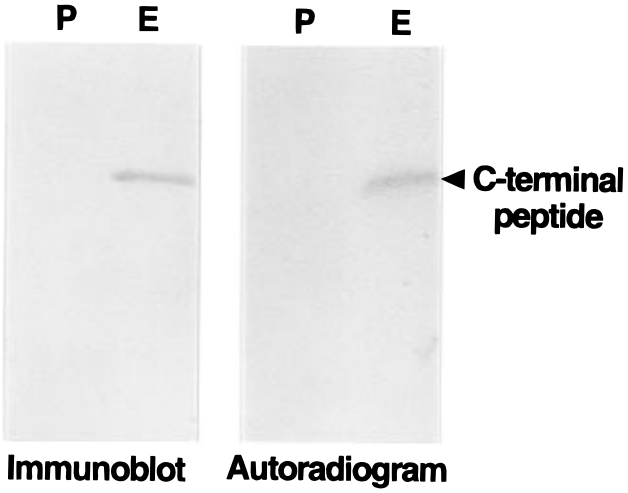



FIGURE 3: Affinity purification of the most heavily labeled CNBr peptide from [$^3$H]NEM-labeled p47$^{phox}$. Recombinant p47$^{phox}$ was treated for 3 min with [$^3$H]NEM and was then precipitated with acetone and cleaved with CNBr as described in the text. The resulting peptides were subjected to immunoaffinity chromatography as described in Materials and Methods. The pass-through and glycine eluate fractions were characterized by Tris-Tricine SDS−PAGE followed either by autoradiography or by immunoblotting using the antipeptide antibody as the detection reagent. (P) Pass-through; (E) Glycine eluate.

conclusions is the time course of labeling of these two peptides. Figure 4 shows that, at least in the first 2 min, C378 reacted with [$^3$H]NEM more than 6 times as rapidly as did C196, which as previously indicated was labeled more rapidly than either of the three other cysteines in p47$^{phox}$.

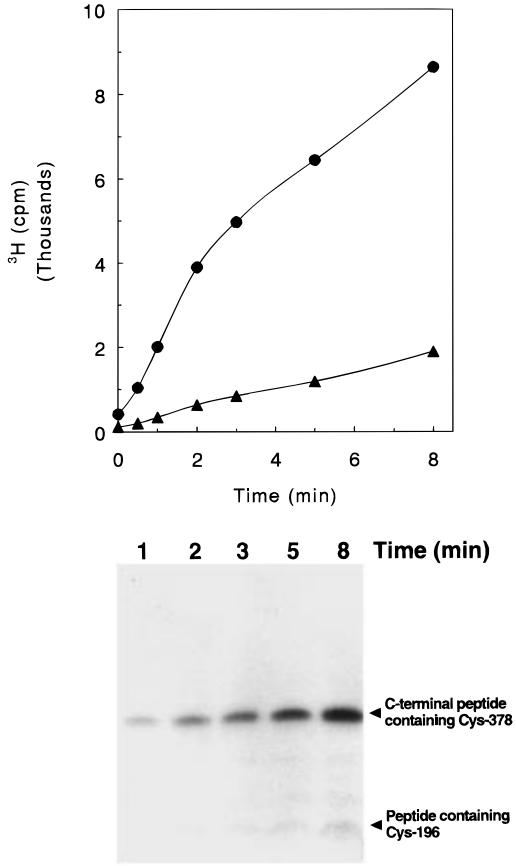



FIGURE 4: Labeling of p47$^{phox}$ C378 and C196 by [$^3$H]NEM as a function of time. Recombinant p47$^{phox}$ was treated with [$^3$H]NEM for the times indicated and was then precipitated with acetone and cleaved with CNBr as described in the text. The resulting peptides were separated by Tris-Tricine SDS−PAGE and analyzed by autoradiography as described. Top panel, radioactivity in labeled bands excised from the gel and assayed by liquid scintillation counting. (●) Peptide containing C378 (C-terminal peptide); (▲) peptide containing C196. Lower panel, autoradiogram.

*Burial of C378 after Activation of p47$^{phox}$.* As discussed above, earlier work on the phosphorylation of p47$^{phox}$ suggested that a change in the conformation of the C-terminal quarter of the molecule is important for the activation of the leukocyte NADPH oxidase. To obtain evidence for such a change, we examined the effect of agents that activate the oxidase in the cell-free system on the availability of cysteine C378 of p47$^{phox}$ to alkylating agents. For this purpose, protein kinase C was employed as the activating agent, with arachidonic acid serving as a positive control, because it had previously been shown that arachidonic acid induced a conformational change in p47$^{phox}$ that appeared to involve the C-terminal portion of the molecule (Sumimoto et al., 1994). Preliminary experiments with protein kinase C showed that the phosphorylation of p47$^{phox}$ in the cell-free system resulted in $O_2^-$ production without the addition of oxidase-activating detergents such as SDS, arachidonic acid, or phosphatidic acid (Figure 5), and that under the incubation conditions used the these experiments, phosphate was incorporated into p47$^{phox}$ at a stoichiometry of ≈6 to 1 (6.1 and 5.9 phosphates per molecule of p47$^{phox}$ in two separate experiments).

We found that the treatment of p47$^{phox}$ with either protein kinase C or arachidonic acid resulted in a considerable reduction in the rate of alkylation of the protein by [$^3$H]-NEM (Figure 6). This was seen not only with recombinant

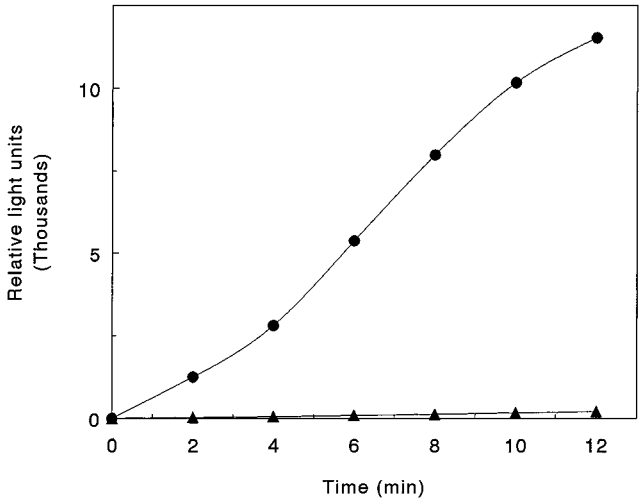
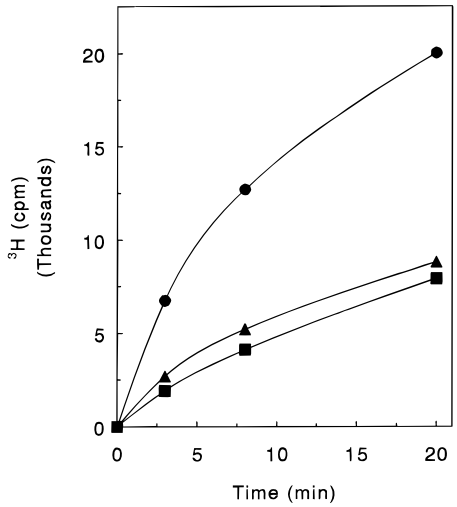



FIGURE 5: Activation of the leukocyte NADPH oxidase in the cell-free system by protein kinase C. The experiments were carried out as described in Materials and Methods. RLU, relative luminescence units. (●) Complete assay mixture; (▲) omit protein kinase C.



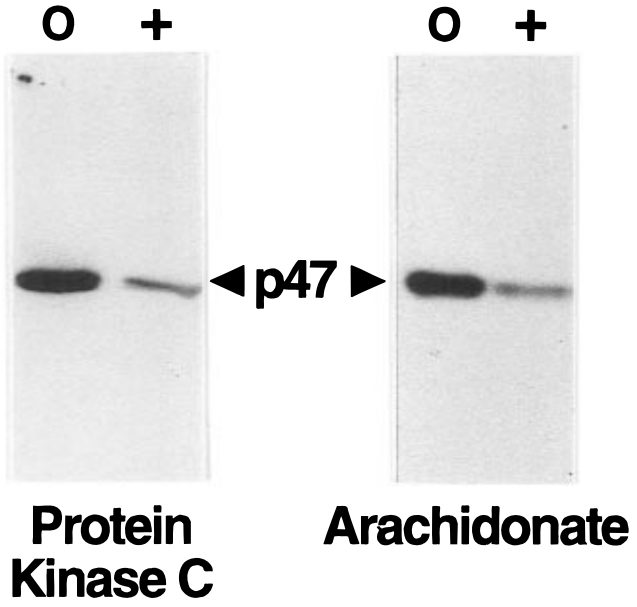




FIGURE 6: Effect of phosphorylation by protein kinase C or treatment with arachidonate on the labeling of recombinant p47$^{phox}$ by [$^3$H]NEM. Top panel, rates of phosphorylation of recombinant p47$^{phox}$ as measured by liquid scintillation counting [(●) no treatment; (▲) Pr kinase C; (■) arachidonate]; lower panel, labeling of untreated, phosphorylated, or arachidonate-treated recombinant p47$^{phox}$ during a 5 min incubation with [$^3$H]NEM, as determined by SDS−PAGE and autoradiography. (O) No treatment; (+) treated as indicated.

p47$^{phox}$ but also with p47$^{phox}$ that had been purified from resting neutrophil cytoplasm (Figure 7). In particular, the activation of p47$^{phox}$ reduced the rate of labeling of C378, the one cysteine located in the region of the molecule whose conformation was postulated to be altered by activation (Figure 8). These findings strongly imply that as a result of activation, a change occurs in the conformation of the C-terminal region of p47$^{phox}$ that reduces the access of alkylating agents to C378. To the extent that both phosphorylation and exposure to arachidonic acid induce similar changes in the kinetics of labeling of C378, the conformational alteration that occurs in response to these two chemically unrelated activating agents seems to be the same.

The conformational alteration, however, does not appear to involve the entire protein. This is suggested by the finding that the alkylation of C196 is only slightly altered by the phosphorylation of p47$^{phox}$. The results in Table 2 show that after phosphorylation, the accessibility of C378 for alkylation was decreased 4-fold, as expected from earlier results. In contrast, the accessibility of C196 for alkylation was almost unchanged. To the extent that inferences regarding conformation can be drawn from cysteine labeling studies, these results imply that the conformation of the region of p47$^{phox}$ in the vicinity of C196 is relatively unaffected by the phosphorylation of the protein.

## DISCUSSION

Earlier studies have suggested that a conformational change involving the leukocyte NADPH oxidase subunit p47$^{phox}$ participates in the activation of the enzyme. These studies showed (1) that exposure of neutrophil cytosol to SDS at a concentration that activates the enzyme in the cell-free system results in the development of a membrane binding site on the p47$^{phox}$·p67$^{phox}$ complex (Park et al., 1992) and (2) that SH3 domains that are occluded in resting p47$^{phox}$ by a proline-rich region near the C-terminus of the molecule are exposed by arachidonic acid, another oxidase-activating lipid (Sumimoto et al., 1994). In the foregoing studies, which were carried out with the cell-free system, an anionic detergent was used as the oxidase activating agent. In intact phagocytes and B lymphocytes, however, the phosphorylation of p47$^{phox}$ appears to be the key event in the activation of the leukocyte NADPH oxidase. Phosphopeptide maps of p47$^{phox}$ from neutrophils and EBV-transformed B lymphocytes activated by phorbol myristate acetate suggest that this phosphorylation is accomplished by protein kinase C (El Benna et al., 1994a, 1996), a finding consistent with earlier studies using inhibitors of protein kinase C and phosphatases (Heyworth et al., 1995; Arai et al., 1993; Twomey et al., 1990; Ding & Badwey, 1992; Steinbeck et al., 1991; Garcia et al., 1992; Djerdjouri et al., 1995; Suzuki et al., 1995). We show here that the phosphorylation of p47$^{phox}$ by protein kinase C caused a change in the conformation of the protein in the vicinity of C378, which lies only 13 residues in from the C-terminus of the molecule. A change in conformation affecting the same region of p47$^{phox}$ was also induced by arachidonic acid, and both arachidonic acid and protein kinase C are able to activate the oxidase in

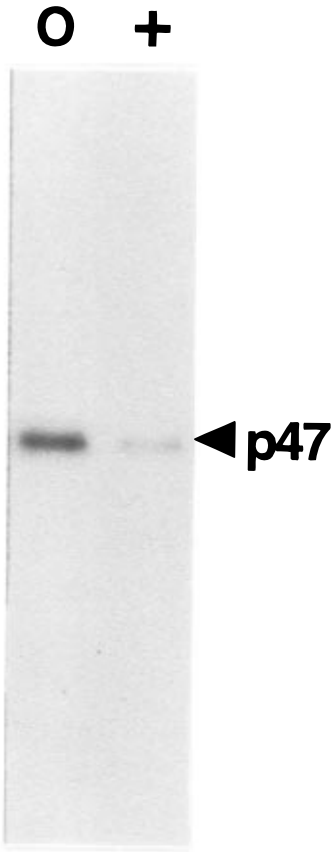



FIGURE 7: Effect of phosphorylation by protein kinase C on the labeling by [$^3$H]NEM of p47$^{phox}$ purified from resting neutrophil cytosol. Affinity-purified p47$^{phox}$ from resting neutrophil cytosol was treated with [$^3$H]NEM for 3 min before or after phosphorylation with protein kinase C as described in Materials and Methods. The labeled p47$^{phox}$ was analyzed by SDS−PAGE followed by autoradiography. (O) No treatment; (+) phosphorylated.

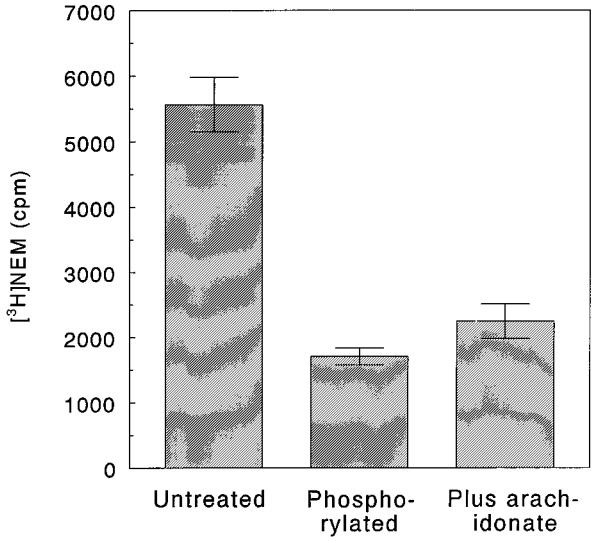



FIGURE 8: Effect of phosphorylation by protein kinase C on the labeling of cysteine C378 by [$^3$H]NEM in recombinant p47$^{phox}$. Recombinant p47$^{phox}$ was incubated with [$^3$H]NEM for 3 min before or after treatment with protein kinase C or arachidonate as described in Materials and Methods. The labeled protein was then cleaved with CNBr, and the resulting peptides were separated by Tris-Tricine SDS−PAGE. The labeled bands were located by autoradiography, and the bands corresponding to the peptide Y279−V390 were excised and counted.

Table 2: Labeling of C378 anc C196 by [$^3$H]NEM in Unphosphorylated and Phosphorylated p47$^{phox}$ [a]

| | [$^3$H]NEM (cpm) | |
|---|---|---|
| cysteine | expt 1 | expt 2 |
| C378 | | |
| untreated | 8660 | 8495 |
| phosphorylated | 2340 | 2015 |
| C196 | | |
| untreated | 1860 | 1900 |
| phosphorylated | 1665 | 1480 |

[a] Unphosphorylated and phosphorylated recombinant p47$^{phox}$ were treated with [$^3$H]NEM for 8 min and were then precipitated with acetone and cleaved with CNBr as described in the text. The resulting peptides were separated by Tris-Tricine SDS−PAGE, located by autoradiography, excised, and assayed by liquid scintillation counting as described.

the cell-free system (Curnutte, 1985; McPhail et al., 1985; Heynemen & Vercauteren, 1984; Cox et al., 1985; Park et al., 1997). We therefore suspect that the change in conformation observed in these experiments is related to oxidase activation.

The purpose of the conformational change is most likely to create binding sites for cytochrome $b_{558}$ on one or more of the cytosolic oxidase components. Supporting this notion is an earlier observation by Park and Ahn that phosphorylated p47$^{phox}$, like SDS-treated p47$^{phox}$, can translocate to neutrophil membranes (Park & Ahn, 1995), and a recent study showing that phosphorylated p47$^{phox}$, but not the unphosphorylated protein, can support oxidase activity in a kinase-activated cell-free system (Park et al., 1997). Several stretches of sequence in both gp91$^{phox}$ and p22$^{phox}$ have been shown to interact with p47$^{phox}$ (Sumimoto et al., 1994; Fuchs et al., 1995). Of particular interest in connection with the present results are the findings that SH3 domains near the middle of p47$^{phox}$ interact with proline-rich domains in both p22$^{phox}$ (P151−P160) and p47$^{phox}$ itself (Q357−P364) (Sumimoto et al., 1994) and that peptides from the C-terminal quarter of p47$^{phox}$ (A323−L332, Q334−G347, and Q357−P364) inhibit the activation of the oxidase and the transfer of p47$^{phox}$ to the membrane in the cell-free oxidase activating system (Nauseef et al., 1993; DeLeo et al., 1995; Finan et al., 1994). Those results suggest that these short stretches of sequence may be of special importance for the binding of p47$^{phox}$ to the membrane-associated components of the oxidase. These sequences are all located in the C-terminal quarter of p47$^{phox}$, and it is possible that the same conformational change that buries C378 could lead to their appearance on the surface of the rearranged protein, where they would be available to bind to the membrane-associated components of the oxidase.

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