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Antagonists of Phosphatidylinositol 3-Kinase Block Phosphorylation-Dependent Activation of the Leukocyte NADPH Oxidase in a Cell-Free System

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Abstract : The NADPH oxidase of phagocytes catalyzes the reduction of oxygen to O_2^- at the expense of NADPH. The enzyme is dormant in resting neutrophils and becomes activated on stimulation. During activation, $p47^{phox}$ (phagocyte oxidase factor), a cytosolic oxidase subunit, becomes extensively phosphorylated at a number of serines located between S303-S379. Oxidase activation can also be achieved by the addition of phosphorylated recombinant $p47^{phox}$ by protein kinase C in the cell-free system in the presence of GTPγS. The cell-free activation is inhibited by wortmannin and LY294002, specific inhibitors of phosphatidylinositol 3-kinase (PI 3-kinase). These results indicate that PI 3-kinase may play a pivotal role in the activation of NADPH oxidase.

Key words : NADPH oxidase, $p47^{phox}$, PKC, PI 3-kinase, phosphorylation

NADPH oxidase (also called respiratory burst oxidase), a complex enzyme found in phagocytes and B lymphocytes, catalyzes the reduction of oxygen to O_2^- using NADPH as the electron donor and plays an important role in host defense against microbial infection. The O_2^- produced by this enzyme is itself weakly microbicidal, but serves as the precursor of a complex battery of highly reactive oxidants that act as powerful microbicidal agents. These oxidants are major components of the system used to defend the host against invading pathogens (Babior, 1978; 1992).

The active NADPH oxidase consists of four polypeptides; $gp91^{phox}$ (phagocyte oxidase factor) and $p22^{phox}$ which are tightly-associated subunits constituting a phagocyte-specific membrane-integrated cytochrome b_{558}, and two cytosolic proteins, $p47^{phox}$ and $p67^{phox}$, which have been identified through their absence in patients whose phagocytes are unable to generate O_2^- (Volpp *et al.*, 1989; Lomax *et al.*, 1989; Dinauer, 1993). Recently, two more cytosolic factors, Ras-related small GTP-binding protein Rac (either Rac1 or Rac2) (Abo *et al.*, 1991; Knaus *et al.*, 1991) and $p40^{phox}$ (Someya *et al.*, 1993; Wientjes *et al.*, 1993) have been shown to be involved in the activation of the enzyme. The oxidase is dormant in resting neutrophils but acquires catalytic activity when cells are exposed to appropriate stimuli. The catalytic activity of the oxidase is located in the plasma membrane, but it is known that in resting cells the oxidase components are distributed between the plasma membrane and cytosol (Bromberg and Pick, 1984; 1985; Heyneman and Vercauteren, 1984; Curnutte, 1985; McPhail *et al.*, 1985). When the cells are activated, the cytosolic components migrate to the plasma membrane, where they associate with cytochrome b_{558}, a flavohemoprotein bound to membranes, to assemble the catalytically active oxidase (Heyworth *et al.*, 1991; Park and Babior, 1992).

Among the cytosolic oxidase components of NADPH oxidase, $p47^{phox}$ is a basic protein that becomes extensively phosphorylated when the oxidase is activated. The phosphorylation targets are a group of serines in the highly basic carboxyl-terminal quarter of the polypeptide (Hayakawa *et al.*, 1986; El Benna *et al.*, 1994; Faust *et al.*, 1995). The relationship between the phosphorylation of $p47^{phox}$ and the initiation of O_2^- production by neutrophils exposed to activating agents such as phorbol-12-myristate 13-acetate suggested that phosphorylation of this protein is an essential step in the mechanism of the oxidase activation in whole cells. However, the mechanism of the oxidase activation in a cell-free system is unclear.

The oxidase can be activated in a cell-free system, but

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the activating agent usually employed is an anionic amphiphile such as arachidonic acid or SDS (Bromberg and Pick, 1984; Curnutte, 1985; McPhail *et al.*, 1985). Recently, the activation of the leukocyte NADPH oxidase in a cell-free system by protein kinase C (PKC), or by $p47^{phox}$ that had been pre-phosphorylated by PKC has been described (El Benna *et al.*, 1995; Park *et al.*, 1997). However, the involvement of other kinases in the activation of NADPH oxidase was also suggested. We describe here that both wortmannin and LY294002, potent and selective inhibitors of phosphatidylinositol 3-kinase (PI 3-kinase), inhibit the phosphorylation-dependent activation of NADPH oxidase in a cell-free system. The data indicate that PI 3-kinase is involved in the oxidase activation.

Materials and Methods

Materials

Chemicals, enzymes, and molecular biology reagents were obtained from the following sources: pGEX-1λT vector, dextran, Ficoll-Hypaque from Pharmacia (Uppsala, Sweden); luminol, phosphatidylserine, diacylglycerol, NADPH, guanosine 5'-O-(3-thiotriphosphate) (GTPγS), guanosine 5'-O-(2-thiodiphosphate) (GDPβS), ATP, glutathione (GSH), GSH-agarose, Nonidet P-40, N-formylmethionylleucylphenylalanine (fMLP) and phenylmethylsulfonyl fluoride (PMSF) from Sigma (St. Louis, USA); rat brain PKC, horseradish peroxidase, calyculin A, wortmannin, and LY294002 from Calbiochem (La Jolla, USA); anti-pan PKC antibodies were from Upstate Biotechnology Inc. (Lake Placid, USA); and Bio-Rad protein assay kit, electrophoresis and immunoblotting reagents from Bio-Rad (Hercules, USA).

Preparation of neutrophil fractions

Neutrophil cytosol and membrane were prepared as described previously (Park and Babior, 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°C until use.

Preparation of recombinant $p47^{phox}$ protein

Recombinant fusion proteins composed of an upstream glutathione S-transferase (GST) linked to a downstream $p47^{phox}$ were isolated from *E. coli* that had been transformed with pGEX-1λT plasmids containing cDNA inserts encoding the downstream proteins, as previously reported (Park, 1996a). The fusion proteins were purified by affinity chromatography on GSH-agarose as described elsewhere. The concentration of proteins was determined with the Bio-Rad assay kit using bovine serum albumin as a standard.

In vitro phosphorylation

Labeling of $p47^{phox}$ with PKC was performed by incubating a reaction mixture containing 1 μg of recombinant $p47^{phox}$, 0.1 μg of PKC, 10 mM magnesium acetate, 1 mM ATP, 5 μCi [γ-^{32}P]ATP (Amersham), 0.5 mM $CaCl_2$, 50 μg/ml phosphatidylserine, 5 μg/ml diacylglycerol and relaxation buffer (pH 7.3) in a total volume of 30 μl at 37°C. After terminating the phosphorylation reactions by the addition of 10 μl of 4X SDS-sample buffer, the samples were subjected to SDS-PAGE using an 8% running gel according to the method of Laemmli (1970). ^{32}P-labeled proteins on the dried gels were detected by autoradiography, and ^{32}P was quantified by excising the labeled bands from the dried gel and measuring their radioactivity using Cerenkov counting.

Preparation of phosphorylated GST-$p47^{phox}$

Phosphorylation of recombinant GST-$p47^{phox}$ was typically carried out as described above except that radioactive ATP was omitted and 10 μg of fusion protein and a reaction volume of 100 μl were employed. Incubations were carried out in Eppendorf tubes for 30 min at 37°C. Each incubation was terminated by the addition of 1.0 ml of ice-cold MTPBS (150 mM NaCl/16 mM Na_2HPO_4/4 mM NaH_2PO_4, pH 7.3) and 100 μl of packed MTPBS-washed GSH-agarose beads. The tubes were then rotated end-over-end for 1 h at 4°C, then spun for a few seconds at maximum speed in an Eppendorf centrifuge to sediment the GSH-agarose beads. After washing the beads with four 1-ml portions of ice-cold MTPBS, the bound phosphorylated $p47^{phox}$ fusion protein was eluted by incubating for 30 min at 4°C with 200 μl of 50 mM Tris·HCl (pH 8.0)/5 mM GSH/0.2 M NaCl. Before use, the eluted fusion protein was dialyzed against relaxation buffer.

Separation of membrane from cytosol after initial incubation

Membranes from the initial incubation were reisolated by layering the incubation mixture over a discontinuous sucrose gradient composed of 1 ml 15% (w/v) sucrose layered over 0.5 ml 50% (w/v) sucrose, both in relaxation buffer, and centrifuging at 105,000×g for 30

min at 4°C. After centrifugation, the contents of the tube were carefully withdrawn from the bottom, discarding the first 250 μl and saving the next 300 μl as the pre-incubated membrane (Park, 1996b).

Cell-free NADPH oxidase assay

The activity of protein kinase-activated NADPH oxidase was measured by chemiluminescence (El Benna *et al.*, 1995). The oxidase was partly activated in an initial incubation carried out in the absence of added phoshorylated GST-p47^{phox}, then fully activated and assayed for activity in a second incubation initiated by adding phosphorylated GST-p47^{phox}, NADPH and the detection system to the initial incubation mixture. A reaction mixture containing 2.5×10^7 cell-equivalent cytosol and 1.5×10^7 cell-equivalent membrane plus 50 μM GTPγS in a total volume of 0.35 ml (the initial incubation) was incubated for 20 min at 37°C. The second incubation was then started by adding the 3 μg phosphorylated GST-p47^{phox} and assay mixture (18 μg horseradish peroxidase, 10 μM luminol and 0.16 mM NADPH in a final volume of 0.5 ml) and measuring chemiluminescence at room temperature in a Monolight 2010 luminometer (Analytical Luminescence Laboratories, San Diego) at successive 10 sec interval.

Electrophoresis and immunoblotting

Protein samples were subjected to SDS-PAGE on 8% polyacrylamide gels using the Laemmli buffer system (Laemmli, 1970). The separated proteins were electrophoretically transferred onto a nitrocellulose sheet (Towbin *et al.*, 1979), which was blocked with dried milk, then probed with the indicated primary antibody; proteins were detected with a 1:2000 dilution of alkaline phosphatase-labeled goat anti-rabbit Ig antibody (Sigma) using 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium as substrate (Bio-Rad).

Results and Discussion

It has been known that p47^{phox}, one of the cytosolic subunits of the leukocyte NADPH oxidase, becomes heavily phosphorylated on serine residues when the oxidase is activated (Hayakawa *et al.*, 1986). More recent studies have shown that the phosphates were confined to the C-terminal quarter of the molecule, identified many of the phosphorylation sites (S304, 305, 315, 320, 328, 345, 348, 359, 370, and 379) (Faust *et al.*, 1995), and suggested that a number of such kinases, including PKC, the p21-activated kinases (PAK kinases), and other kinases yet to be characterized, are potentially responsible for the phosphorylation of p47^{phox} (El Benna *et al.*, 1994; 1996; Knaus *et al.*, 1995). However, the role of phosphorylation of p47^{phox} in the activation of oxidase has not been clearly elucidated.

In a cell-free system, the leukocyte NADPH oxidase has customarily been activated by anionic amphiphiles including arachidonic acid, SDS and phosphatidic acid (Bromberg and Pick, 1984; 1985; Curnutte, 1985; McPhail *et al.*, 1985; Qualliotine-Mann *et al.*, 1993; Park, 1996b). We have recently shown that p47^{phox} phosphorylated by PKC induces the translocation of p47^{phox} itself (Park and Ahn, 1995), which is the essential step for the oxidase activation. The enzyme can be activated in a cell-free system by PKC or by supplementing with p47^{phox} which had been pre-phosphorylated with PKC (El Benna *et al.*, 1995; Park et al., 1997). In the latter condition, it has been suggested that PKC-dependent activation of the leukocyte NADPH oxidase is a multistep process. The initial incubation, which is dependent upon ATP and guanine nucleotides, activates the membrane; the addition of phosphorylated p47^{phox} after activation of the membrane induced activation of oxidase. It is probable that activation of the membrane by the initial incubation may be caused by kinases other than PKC.

Under the *in vitro* phosphorylation conditions used in our experiments, SDS-PAGE and autoradiography revealed that GST-p47^{phox} reached a maximum phospho-

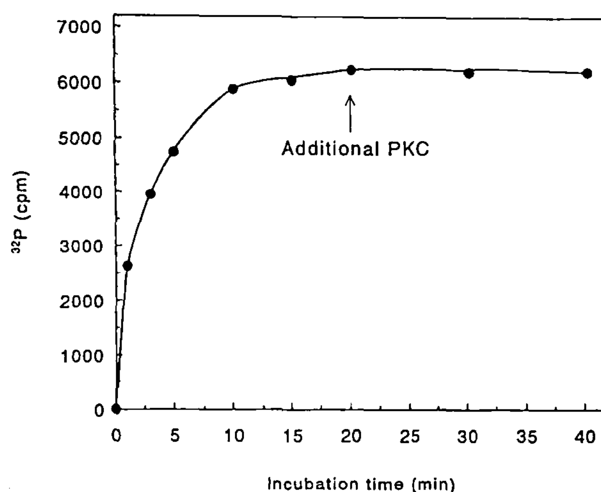

Fig. 1. Phosphorylation of GST-p47^{phox} *in vitro* by PKC. GST-p47^{phox} (10 μg) was phosphorylated with [γ-^{32}P]ATP as described in Materials and Methods, adding 0.5 units of additional PKC at 20 min as indicated. At various times a 30 μl aliquot of reaction mixture containing 1 μg of fusion protein was removed from the reaction mixture, added to 10 μl 4 x Laemmli sample buffer, boiled for 5 min and then subjected to SDS-PAGE followed by autoradiography. The labeled bands were excised from the gel and the radioactivity measured by Cerenkov counting. The values shown are the averages from two separate experiments. At each point, the difference between the two experimental values amounted to ≤ 101 cpm.

rylation by a 10-20 min incubation with PKC as shown in Fig. 1. Addition of 0.5 units of PKC after 20 min incubation did not increase the level of phosphorylation with further incubation. Autoradiography and immunoblotting with anti-p47^{phox} and anti-pan PKC antibodies of the unbound and eluate fractions showed that the phosphorylated GST-p47^{phox} could be separated from PKC during the purification by GSH-agarose chromatography (Fig. 2).

The effect of guanine nucleotides in the initial incubation was investigated by evaluating the incorporation of ^{32}P from [γ-^{32}P]ATP into proteins which were analyzed by gel electrophoresis and autoradiography. The incubation was carried out in the presence of either 50 μM GTPγS or 1 mM GDPβS and the membrane was separated after incubation. The autoradiogram (Fig. 3) showed that in this system there appeared to be a significant difference in phosphoprotein content of membranes from GTPγS- (lane 1) and GDPβS-treated (lane 2) initial incubation mixtures. A previous report showed that the initial incubation using the phosphorylation-dependent cell-free activation of the NADPH oxidase altered the membrane. This process is activated by ATP and GTPγS whereas the oxidase activity was absent when GTPγS was replaced with GDPβS even in the presence of ATP (Park *et al.*, 1997). These findings and present results indicate that the kinase(s) whose activity is regulated by guanine nucleotide binding protein is involved in the activation of the membrane.

An enzyme that seems to be involved in many signal transduction pathways is PI 3-kinase, a heterodimer of 110 kDa and 85 kDa subunits, which catalyzes phosphorylation at the 3 position of the inositol moiety in phosphatidylinositols (converting phosphatidylinositol-4, 5-diphosphate, for example, to phosphatidylinositol-3,4, 5-triphosphate) (Shepherd *et al.*, 1996). PI 3-kinase activity can be stimulated by GTP analogs, and several studies have implicated Ras (Rodriguez-Viciana *et al.*, 1994), Rho (Zhang *et al.*, 1992) and Cdc 42 (Zheng *et al.*, 1994) as regulators of PI 3-kinase. It has also been reported that PI 3-kinase is involved in the activation of several unknown protein kinases in neutrophils (Ding *et al.*, 1995). Therefore, the possibility that PI 3-kinase might be involved in the phosphorylation-dependent activation of the leukocyte NADPH oxidase was examined through the use of two chemically unrelated reagents. Wortmannin and LY294002 are known to be specific inhibitors of PI 3-kinase when used at nanomolar to low micromolar range. Wortmannin, a fungal metabolite, is a covalent, irreversible inhibitor of PI 3-kinase (Powis *et al.*, 1994), whereas LY294002 reversibly inhibits PI 3-kinase by competing with ATP for its substrate binding site (Vlahos *et al.*, 1994). We found that both wortmannin and LY294002 inhibited cell-free oxidase activation if present during the initial incubation but did not if present only after the addition of phosphorylated GST-p47^{phox} (Fig 4). At 100 nM, wortmannin inhibited cell-free activation of the oxidase by 74% when added to the initial incubation mixture. The inhibitory effects of wortmannin and LY294002

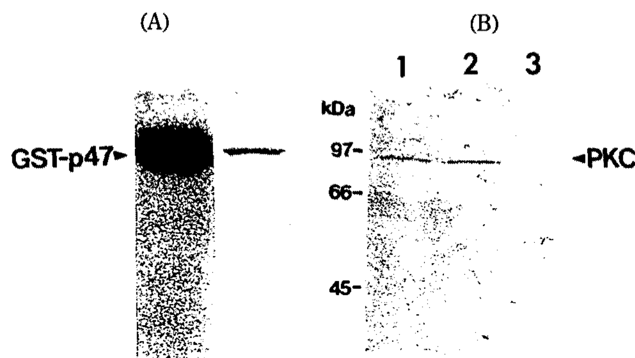

Fig. 2. Affinity purification of phosphorylated GST-p47^{phox}. Recombinant GST-p47^{phox} was phosphorylated by PKC. The resulting phosphorylated GST-p47^{phox} was purified on GSH-agarose as described in the text. The unbound and eluate fractions were analyzed by SDS-PAGE followed by autoradiography or by immunoblotting, detecting with either anti-p47^{phox} (1:10,000 dilution) or anti-pan PKC (5 μg/ml Blotto). (A) autoradiogram (*left*) and anti-p47^{phox} immunoblot (*right*) of eluate from GSH-agarose beads. (B) anti-pan PKC immunoblot of SDS-PAGE electrophotogram. 1. pure PKC, 80 ng; 2, unbound fraction from GSH-agarose beads; 3, GSH eluate from GSH-agarose beads.

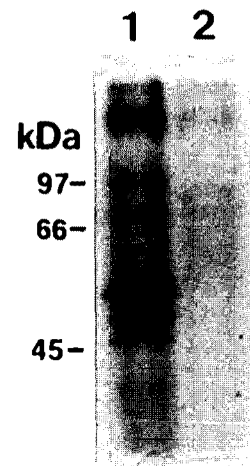

Fig. 3. Effects of guanine nucleotides on the phosphorylation induced by initial incubation. Membranes were reisolated from the initial incubation mixture composed of 5×10^7 cell equivalent cytosol, 10^8 cell equivalent membrane, 50 μM GTPγS, 10 μCi [γ-^{32}P]ATP, and 0.25 μM calyculin A (lane 1), or the same mixture except GTPγS was replaced with 1 mM GDPβS (lane 2) by ultracentrifugation with a sucrose gradient and then each 1×10^7 cell equivalent of membrane was analyzed by SDS-PAGE and autoradiography. Similar results were obtained in two separate experiments.

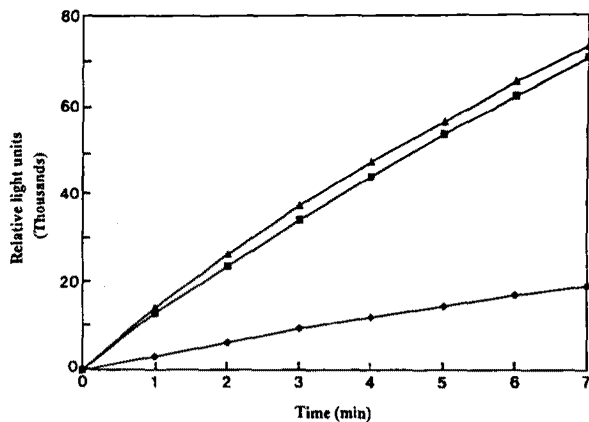

Fig. 4. Effect of wortmannin on the phosphorylation-dependent oxidase activity. Incubations were carried out as described in the text. ▲, no wortmannin; ◆, 0.1 μM wortmannin in the initial incubation mixture; ■, 0.1 μM wortmannin added after initial incubation. Results are representative of three separate experiments.

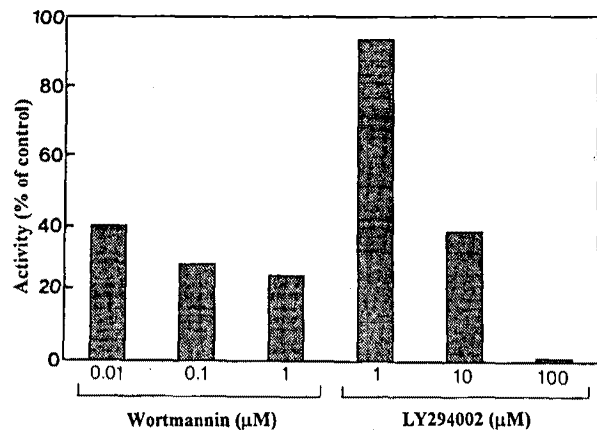

Fig. 5. Concentration-dependent inhibition of wortmannin and LY294002 on the phosphorylation-dependent oxidase activity. Wortmannin or LY294002 was added in the initial incubation mixture and assays were carried out as described in the text. Results are shown as the percent of control activity, where the control activity was the activity seen in the absence of inhibitors. Values shown represent the means of four determinations. At each point, the standard deviation was less than 10%.

were concentration-dependent as shown in Fig. 5. These findings suggest that PI 3-kinase participates in the phosphorylation-dependent activation of the oxidase, and that it exerts its effect during the initial incubation.

In intact cells, it is known that PI 3-kinase interacts with a number of systems that may be of importance in the regulation of O_2^- production by neutrophils. Through the Rho-GAP homologous domain of its regulatory (85 kDa) subunit, PI 3-kinase forms a complex with Rac1 (Zheng *et al.*, 1994), a guanine nucleotide binding protein known to be essential for oxidase activation (Knaus *et al.*, 1992; Pick *et al.*, 1993). This complex appears to be functionally important, because in Swiss 3T3 cells, PI 3-kinase induces the cytoskeletal actions of Rac (Nobes *et al.*, 1995). PI 3-kinase binds to and is activated by the Lyn·Shc (Lyn is a Src-related tyrosine kinase; Shc is an adaptor protein containing an SH2 domain) complex formed when tyrosine residues on the latter two proteins are phosphorylated following the exposure of neutrophils to the chemotactic peptide fMLP (Ptasznik *et al.*, 1995). PI 3-kinase is involved in the fMLP-mediated activation of four neutrophil kinases, all of which can phosphorylate a 34-residue peptide from the C-terminal quarter of $p47^{phox}$ (Ding and Badwey, 1994; Ding *et al.*, 1995).

PI 3-kinase is a dual specific enzyme that exerts both lipid (Whitman *et al.*, 1985) and protein serine kinase activities (Carpenter *et al.*, 1993; Dhand *et al.*, 1994; Lam *et al.*, 1994). In this regard, present observations suggest the following as a possible route for the oxidase activation: 1) a guanine nucleotide binding protein-dependent process activates PI 3-kinase (perhaps the Rac 2-dependent transfer of the lipid kinase to the membrane or the activation of a protein kinase, perhaps the p21-activated kinase, which in turn is able to stimulate a PI 3-kinase) with the end result being the generation of phosphatidylinositol 1,3,4-triphosphate in the neutrophil membrane; 2) the activation of PKC, which phosphorylates $p47^{phox}$; and finally 3) the assembly of the active NADPH oxidase on a membrane that somehow has been rendered capable of supporting the oxidase activation by virtue of its newly acquired phosphatidylinositol-1,3,4-triphosphate. Alternatively, the phosphorylation of a membrane-associated oxidase subunit, or the participation of phosphatidic acid in oxidase activation can also be considered. It is clear, however, that phosphorylation-dependent oxidase activation requires an alteration in the membrane, at least in this system and perhaps in the intact cell as well.

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