NADPH-binding Component of the Respiratory Burst Oxidase System: Studies using Neutrophil Membranes from Patients with Chronic Granulomatous Disease Lacking the β-Subunit of Cytochrome *b*558

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Summary

The NADPH-binding site of the respiratory burst oxidase system of neutrophils has been proposed to be either at a cytosolic component or at the β-subunit of cytochrome *b*558. In this study, affinity labeling of resting and stimulated membranes, the latter having been assembled by all of the oxidase components from both membrane and cytosol, was carried out using [^{32}P]NADPH dialdehyde (oNADPH). Stimulation of human neutrophils with PMA greatly increased O_2^--generating activity and caused considerable translocation of the cytosolic components p47^{phox} and p67^{phox}. Nevertheless, PMA stimulation did not produce a labeled band which included positions at 47, 67, and ~32 kD. The most intense band reflected a molecular mass of 84 kD regardless of the state of activation, but a labeled band was never found near the β-subunit (91 kD) of cytochrome *b*558. This 84-kD protein was further confirmed in neutrophils of 14 patients with gp91^{phox}-deficient X-linked chronic granulomatous disease. These results indicate that the NADPH-binding component is not recruited from the cytosol, and also, that a membranous redox component besides cytochrome *b*558 must be involved in the NADPH oxidase system.

Upon stimulation, phagocytes (neutrophils, monocytes, and eosinophils) generate superoxide anion and other oxygen intermediates to kill microbes (for reviews see references 1–4). This respiratory burst is caused by activation of the O_2^--generating NADPH oxidase system, the components of which are distributed in cell membrane and cytosol in the resting state (5). Chronic granulomatous disease (CGD), in which the respiratory burst is hampered by genetic dysfunction, has contributed greatly to the discovery of the indispensable constituents of this system i.e., determination of the membranous components p22^{phox} (α-subunit) and gp91^{phox} (β-subunit) of cytochrome *b*558 in X-linked CGD and determination of the cytosolic components p47^{phox} and p67^{phox} in autosomal recessive CGD (1, 2, 4). *Rac* p21s, which are regulated by their stimulator and inhibitor for GDP dissociation, have recently been proposed to be involved as modulatory components in this NADPH oxidase system (6, 7). However, CGD caused by their absence has not been identified.

In contrast to the above constituents, the NADPH-binding component of this oxidase system still remains controversial. The NADPH-binding site has been proposed to be located in a cytosolic protein and transferred upon cell stimulation (1, 8, 9). On the other hand, another recent hypothesis proposed that cytochrome *b*558 in membrane, but not in cytosol, is a flavoprotein containing both Flavin Adenine Dinucleotide (FAD)- and NADPH-binding sites, based only on the amino acid homology between p91^{phox} and the putative $NADP^+$- and FAD-binding sites of the ferredoxin reductase (FNR) family (10–12). In one report (11), direct elucidation was attempted by photoaffinity labeling using [^{32}P]2-azido-$NADP^+$; however, binding to gp91^{phox} was extremely difficult to judge. Furthermore, a recent report (13) has demonstrated that a membrane-bound flavoenzyme, in addition to purified cytochrome *b*558, is absolutely required for reconstitution of O_2^--generating activity in a cell-free system.

In this study, [^{32}P]oNADPH-affinity labeling profiles of membranes of resting and stimulated cells were compared, as well as those of normal subjects and gp91^{phox}-deficient CGD patients, to determine whether the NADPH-binding site actually exists in the β-subunit of cytochrome *b*558.

Materials and Methods

Isolation of Neutrophils. Human blood was drawn from normal volunteers and 14 X-linked CGD subjects with their informed

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consent, and was subjected to sequential Ficoll-Paque differential density centrifugation followed by 0.2% (wt/vol) methyl cellulose sedimentation the next day, unless otherwise indicated. The isolated neutrophils were treated with 2 mM diisopropyl fluorophosphate for 15 min at 4°C after hypotonic lysis of erythrocytes, washed, and finally suspended in Ca^{2+}- and Mg^{2+}-free Krebs-Ringer phosphate buffer containing 5 mM glucose (KRPG), pH 7.4.

Cell Stimulation and Subcellular Fractionation. PMA-dependent stimulation was carried out for the indicated times at 37°C in the presence of 100 ng PMA/2 × 10^6 cells/ml of KRPG containing 1.2 mM $MgCl_2$ and 2 mM NaN_3. After being washed with cold KRPG, the cells were disrupted by sonication on ice in buffer A: 100 mM KCl, 3 mM NaCl, 3.5 mM $MgCl_2$, and 10 mM Pipes (pH 7.3) containing 10 μM leupeptin and 1 mM PMSF. The sonicate was spun at 500 *g* for 5 min, and the postnuclear supernatant was then separated into membrane and cytosol fractions by centrifugation at 200,000 *g* for 20 min at 4°C. The membrane fraction was resuspended in an initial volume of buffer A containing the above protease inhibitors. Membrane fraction from resting cells was prepared by the same procedure without PMA stimulation, and solubilized in KRP containing 1.5% (wt/vol) octyl glucoside and 12% (vol/vol) ethylene glycol, when stated. The cytochrome *b*558 concentration of the membrane fraction was determined spectrophotometrically using a reduced-minus-oxidized extinction coefficient of 21.6 mM^{-1} cm^{-1} at 558 nm (14). Protein concentration was determined with BCA protein assay reagent (Pierce, Rockford, IL).

FNR-NADP$^+$. The enzyme from spinach chloroplast, ferredoxin affinity purified at the final step, was a generous gift of Dr. H. Sakurai (Waseda University). Its concentration was spectrophotometrically determined using an extinction coefficient of 85,000 M^{-1} cm^{-1} at 275 nm (15).

Synthesis of [2'-^{32}*P]NADP(H) Dialdehydes.* [^{32}P]NADP$^+$ was first synthesized from NAD$^+$ and γ-[^{32}P]ATP (10 Ci/mmol, New England Nuclear, Boston, MA) using NAD$^+$ kinase essentially according to White and Levy (16) and Takasugi et al. (9) as described previously (17). The synthesis was conducted by incubating 8 mM NAD$^+$, 8 mM ATP (0.10–0.13 mCi), 10 mM $MgCl_2$, and 50 U/ml NAD$^+$ kinase in 100 mM Tris-HCl (pH 7.5) for 90 min at 37°C. The reacted mixture was then adsorbed to a DEAE Sepharose CL-6B column and the synthesized [^{32}P]NADP$^+$ was eluted with a gradient of 50–110 mM ammonium bicarbonate. The [^{32}P]NADP$^+$ was then oxidized with $NaIO_4$ to form [^{32}P]oNADP$^+$. Finally, an aliquot of the oxidized [^{32}P]oNADP$^+$ was enzymatically reduced to [^{32}P]oNADPH by isocitrate dehydrogenase essentially according to the method of Mas and Colman (18) by incubating 1.5 mM [^{32}P]oNADP$^+$, 0.1 mM $MnSO_4$, 4 mM DL-isocitrate, 10% (vol) nonfluorescent glycerol, and 7.5 U/ml isocitrate dehydrogenase for 75 min at 25°C. The reacted mixture was applied to a DEAE-Sepharose CL-6B column and the synthesized [^{32}P]oNADPH was eluted with a gradient of 10–400 mM triethanolamine HCl buffer (pH 8.0).

Affinity Labeling of FNR and Membrane Fraction with [^{32}*P]oNADP(H).* Covalent linkage of [^{32}P]oNADP(H) to NADP(H)-binding sites was performed by incubating FNR and membranes or their solubilized lysate with 0.1 mM [^{32}P]oNADP(H) in 30 mM triethanolamine HCl buffer (pH 8.0) for 60 min on ice, and then adding 10 mM $NaCNBH_3$. After an overnight incubation, labeled proteins were precipitated with a final concentration of 5% (wt/vol) trichloroacetic acid (for FNR precipitation, 50 μg BSA was also added), washed with cold acetone, dissolved in sample buffer, and analyzed by SDS-PAGE. Coomassie blue–stained gels were dried and then exposed to Kodak XAR-5 film at −80°C or to an imaging plate at room temperature followed by development in a bio-image analyzer (Fuji BAS2000, Fuji Photofilm Corp., Tokyo, Japan). The radioactivity stored in the imaging plate was expressed as a photostimulative luminescence (PSL) count.

Immunoblotting. Antisera against the components of the NADPH oxidase system were raised in rabbits by injection of KLH-conjugated synthetic peptides corresponding to gp91^{phox} (residues 536-555), p47^{phox} (residues 376-390), and p67^{phox} (residues 508-526). Membrane and cytosol fractions subjected to SDS-PAGE were transferred to a polyvinylidene difluoride microporous membrane (PVDF) (Millipore Corp., Bedford, MA). The transferred proteins were probed with the above primary antisera, subsequently localized with a donkey ^{125}I-labeled F(ab')$_2$ fragment against rabbit Ig (Amersham Corp., Arlington Heights, IL), and exposed either to film or to an imaging plate. For quantitative analysis of the translocation of p47^{phox} and p67^{phox} from cytosol to membrane, areas of the PVDF sheet which corresponded to the radioactive bands developed on film were excised, and ^{125}I radioactivities of the pieces were counted in a gamma counter (model no. ARC-1000, Aloka, Tokyo, Japan). Percent translocation was expressed as the count in the membrane fraction divided by the sum of the counts in both the membrane and cytosol fractions.

Assay of O_2^--gene Generation. The NADPH oxidase activity of membrane fractions was determined by the rate of superoxide dismutase-inhibitable ferricytochrome *c* reduction with a dual wavelength spectrophotometer (model 557; Hitachi). The reaction mixture consisted of 10 μg membrane protein, 30 μM ferricytochrome *c*, 1 mM $MgCl_2$, 0.1 mM EGTA, 1 mM NaN_3, and 225 U/ml catalase in saline–20 mM Hepes (pH 7.4). The reaction was begun by adding 0.2 mM NADPH. At maximal velocity, 200 U/ml of superoxide dismutase was added to determine the net ferricytochrome *c* reduction.

Results

Quantitative Relationship between NADPH Oxidase Activity and the Translocation of p47^{phox} *and p67*^{phox} *to the Membrane Fraction.* Before identifying the NADPH-binding proteins in the stimulated membranes, which are assembled by all of the constituents of the NADPH oxidase system to generate O_2^- anion using NADPH as an electron source, the translocation of p47^{phox} and p67^{phox} were determined quantitatively. Stimulation of neutrophils with PMA caused a time-dependent increase in both the O_2^--generating activity and the translocation of p47^{phox} and p67^{phox} to membrane with their simultaneous decrease in cytosol (Figs. 1 and 2). Consequently, 24.8% of p47^{phox} and 32.3% of p67^{phox} became membrane-associated after a 5-min incubation period, which is consistent with a previous report (5).

[^{32}*P]NADP(H) Dialdehydes as Affinity-labeling Reagents.* For affinity labeling, [^{32}P]oNADPH was prepared from the synthesized [^{32}P]oNADP$^+$. We first investigated whether or not the synthesized oNADP$^+$ possesses the same stereospecificity as native NADP$^+$ in binding, using FNR, which catalyzes the reduction of NADP$^+$ with reduced ferredoxin. As shown in Fig. 3, the ferredoxin affinity–purified FNR enzyme exhibited a single [^{32}P]oNADP$^+$-binding band at a molecular mass of 40 kD. Compared to this [^{32}P]oNADP$^+$ binding, a significant band was not detected with [^{32}P]oNADPH, reflecting its far lower affinity to NADPH

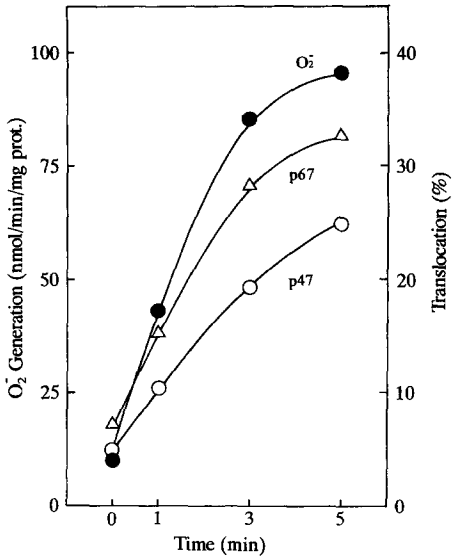

Figure 1. Simultaneous stimulation of the NADPH oxidase activity and translocation of $p47^{phox}$ and $p67^{phox}$ with PMA. Membranes and the corresponding cytosol were fractionated from resting (at 0 min, treated with DMSO) and PMA-stimulated cells for the indicated periods. O_2^- generation (●) was initiated by adding 0.2 mM NADPH to 10 μg membrane fraction. To determine percent translocation of $p47^{phox}$ (○) and $p67^{phox}$ (△), both fractions (2×10^6 cell equivalent) were subjected to SDS-10% PAGE, transferred to a PVDF sheet, and immunoblotted with primary antisera against their synthetic peptides. The components were then revealed on film with a secondary ^{125}I-labeled $F(ab')_2$ fragment against rabbit Ig. ^{125}I radioactivities in excised bands from the PVDF sheet were counted.

than to $NADP^+$ (15). Labeling of the FNR enzyme with [^{32}P]oNADP$^+$ was significantly diminished by the addition of unlabeled $NADP^+$ and NADPH, but not by the addition of NADH (Table 1). This labeling was also considerably inhibited by unlabeled NAD^+, which denotes that the enzyme is specific for $NADP^+$ under physiological conditions, but also recognizes NAD^+ at high concentrations (15).

Next, [^{32}P]oNADP$^+$ was converted to [^{32}P]oNADPH using a strictly NADPH-dependent enzyme, isocitrate dehydrogenase. The integrity of [^{32}P]oNADPH with regard to binding specificity was also confirmed by the finding that unlabeled NADP(H), but not NAD(H), inhibited its binding to an NADPH-binding 84-kD protein (see below, and Table 1). In addition, the same Michaelis constant value (20 μM) for the NADPH oxidase was obtained for both the synthe-

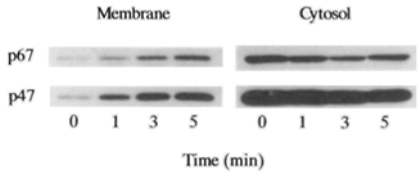

Figure 2. Time course of translocation of $p47^{phox}$ and $p67^{phox}$ to membrane fraction. The protocol is the same as that in Fig. 1. Increase in membrane and decrease in cytosol fraction of $p47^{phox}$ and $p67^{phox}$ with time were revealed on film.

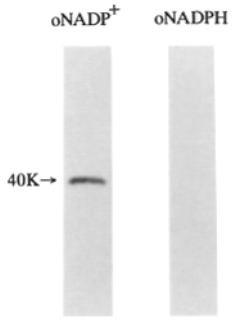

Figure 3. Affinity labeling of FNR with [^{32}P]oNADP(H): FNR (64 pmol) was affinity labeled with 0.1 mM of either [^{32}P]oNADP$^+$ or [^{32}P]oNADPH as described in Materials and Methods, subjected to SDS-12.5% PAGE, exposed to an imaging plate, and then image analyzed.

oxidase was obtained for both the synthesized oNADPH and native NADPH (data not shown). These results provide evidence for stereospecifically identical recognition of oNADP(H) to native NADP(H).

Affinity Labeling of the PMA-stimulated Membrane Fraction with [^{32}P]oNADPH. To examine the hypothesis that a cytosolic NADPH-binding component is conveyed to the cell membrane by cell stimulation (1, 8, 9), we examined whether or not the number of NADPH-binding proteins increased during cell stimulation with PMA. Affinity labeling of membranes from resting cells (at 0 min and treated with DMSO vehicle instead of PMA) with [^{32}P]oNADPH revealed that the most radioactive band had a molecular mass of 84 kD (Fig. 4). The intensity of this 84-kD band decreased with time. Incubating neutrophils up to 5 min may desensitize their ability to bind [^{32}P]oNADPH. Radioactivities of other bands at 57 and 16–24 kD were far less than that of the band at 84 kD. When we used a further purified membrane fraction from porcine neutrophils, the most intense band was detected at a molecular mass of 80 kD without these faint bands, in contrast to the present one prepared from human

Table 1. *Effect of Pyridine Nucleotides on [^{32}P]oNADP(H) Binding to FNR or the 84-kD Membrane Protein*

Pyridine nucleotide (10 mM)	Binding	
	FNR	84-kD protein
	%	
	(oNADP$^+$)	(oNADPH)
None	100	100
NADPH	37.1	25.1
$NADP^+$	10.4	24.0
NADH	101.4	100.9
NAD^+	41.0	78.7

FNR (50 pmol) and membranes (3×10^6 cell equivalent), stimulated with PMA for 5 min at 37°C, were first preincubated with 10 mM unlabeled pyridine nucleotides for 1 h on ice, and then affinity labeled with 0.1 mM of either [^{32}P]oNADP$^+$ or [^{32}P]oNADPH. Counts at 40 (FNR) and 84 kD (membranes) were image analyzed and expressed as percent control. Results shown are means of three representative experiments.

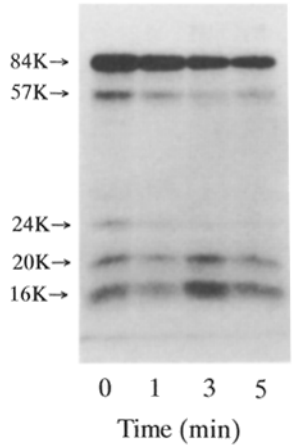

Figure 4. Autoradiograph of [^{32}P]oNADPH-binding proteins in PMA-stimulated membranes. Resting (at 0 min) and PMA-stimulated membranes (3×10^6 cell equivalent) were affinity labeled with 0.1 mM [^{32}P]oNADPH, analyzed by SDS-12.5% PAGE, and then exposed to film. The fractions correspond to those in Fig. 1.

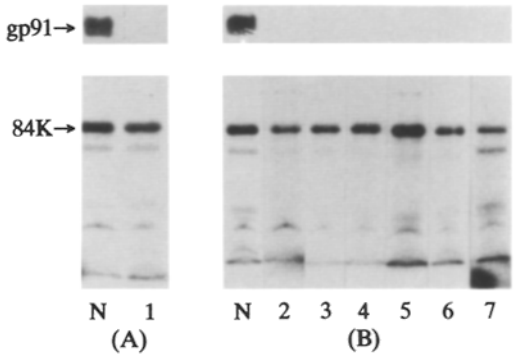

Figure 5. [^{32}P]oNADPH-binding proteins in membranes from gp91^{phox}-deficient CGD patients. Resting membranes fractionated from normal and CGD subjects were solubilized with 1.5% (wt/vol) octyl glucoside. For immunoblotting, the lysates (10^6 cell equivalent) were subjected to SDS-12.5% PAGE. The proteins which had been transferred to a PVDF sheet were probed with antiserum against a synthetic peptide of gp91^{phox}, and then revealed with a secondary ^{125}I-labeled F(ab')$_2$ fragment against rabbit Ig (*top*). The corresponding lysates (3×10^6 cell equivalent) were affinity labeled with 0.1 mM [^{32}P]oNADPH (*bottom*). Both were image analyzed. Figures denote normal volunteers (*N*) and seven examples (lanes *1–7*) of the 14 CGD patients examined. Membrane fractions were prepared immediately (*A*) or 1 d (*B*) after venipuncture.

neutrophils (17). The band at 57 kD is most likely not related to p47^{phox} or p67^{phox} since its radioactivity was not augmented by cell stimulation with PMA. Neither p47^{phox} nor p67^{phox} was recognized, even though 5 min–stimulated membranes were affinity labeled with [^{32}P]oNADPH (Fig. 4). However, a considerable amount of these constituents had been transferred from cytosol (Figs. 1 and 2). Therefore, neither p47^{phox} nor p67^{phox} could be considered the NADPH-binding component. This is consistent with the finding that neither is reflavinated with FAD (19), since, generally, a molecule must contain a flavin moiety if it is to be an electron acceptor from NADPH.

The most important finding here was that the amount of NADPH-binding protein remained constant regardless of the activation state of the membrane, which suggests that the NADPH-binding component is located in the cell membrane and none, including a recently proposed cytosolic ~32-kD protein (20), is recruited from the cytosol.

Affinity Labeling of Resting Membranes from gp91^{phox}-deficient CGD patients with [^{32}P]oNADPH. A molecular mass of 84 kD is very close to that of the β-subunit (91 kD) of cytochrome *b*558. Therefore, affinity labeling of membranes from 14 gp91^{phox}-deficient X-linked CGD patients was performed to determine whether or not the 84-kD protein was actually a band of gp91^{phox}. The neutrophils of all of these patients exhibited neither the ability to generate O_2^- anion nor the heme spectrum of cytochrome *b*558 (data not shown). Complete deletion of the gp91^{phox} polypeptide was confirmed by immunoblotting with antiserum against its synthetic peptide (Fig. 5, *top*). However, the intensities of [^{32}P]oNADPH bound to the 84-kD protein in the 14 CGD patients were comparable to those in normal subjects (Fig. 5, *bottom* shows seven examples). Although the pattern of labeled bands tended to vary in each case (Fig. 5 *B*), since membrane fractions were prepared from each subject's neutrophils 1 d after venipuncture, similar profiles were attained in normal subject and CGD patient when membrane fractions, which had been freshly isolated from neutrophils, were used for affinity labeling (Fig. 5 *A*).

Affinity Labeling of Resting Membranes with [^{32}P]oNADP$^+$. The notion that the β-subunit of cytochrome *b*558 is the NADPH-binding component of the oxidase is based on the proposed sequence similarity of the β-subunit to the NADP$^+$-binding sequences in the FNR flavoprotein family (10–12), which may allow higher affinity labeling of cytochrome *b*558 to NADP$^+$ than to NADPH. Therefore, affinity labeling of the membrane fraction (12.8 pmol heme of cytochrome *b*558) was carried out with [^{32}P]oNADP$^+$ as well as [^{32}P]oNADPH (Fig. 6, *a* and *b*, respectively). Although the labeled patterns differed slightly from each other, the most striking band was similarly detected at the same 84-kD position as that labeled with [^{32}P]oNADPH, but a broad spanning band responsible for the β-subunit (see immunoblot in Fig. 5) did not emerge. Lane *c* in Fig. 6 shows the [^{32}P]oNADP$^+$ binding to 64 pmol of FNR. These 84-kD proteins exhibited comparable binding of [^{32}P]oNADP$^+$ (6,594 PSL) and [^{32}P]oNADPH (5,226 PSL), respectively, unlike the higher affinity of spinach

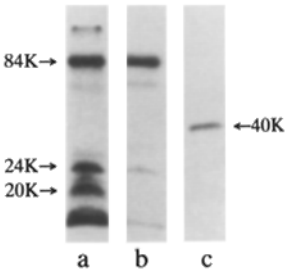

Figure 6. Autoradiograph of [^{32}P]oNADP$^+$-binding proteins. Resting membranes of 1.5×10^6 cell equivalent (12.8 pmol cytochrome *b*558) and FNR (64 pmol) were affinity labeled with 0.1 mM of either [^{32}P]oNADP$^+$ or [^{32}P]oNADPH. After separation by SDS-12.5% PAGE, their binding profiles were analyzed by a bio-image analyzer. (*a*) Membrane with [^{32}P]oNADP$^+$; (*b*) membrane with [^{32}P]oNADPH; (*c*) FNR with [^{32}P]oNADP$^+$.

FNR to [^{32}P]oNADP$^+$ (Fig. 3). Despite that, the amount of cytochrome *b*558 was increased to 25.5 pmol (40% of 64 pmol FNR), [^{32}P]oNADP$^+$ binding to the β-subunit could not be detected (high binding ability of the 84-kD protein to [^{32}P]oNADP$^+$ or its abundance in membrane interfered with further scrutiny using a larger amount of cytochrome *b*558). Taken together, these results suggest that the binding site for accepting an electron from NADPH is unlikely to be the β-subunit of cytochrome *b*558, and is probably located in a membrane protein distinct from it.

Discussion

Cytochrome *b*558 was recently proposed to be the sole redox component of the NADPH oxidase system in the membrane (21, 22). These reports led to the idea that cytochrome *b*558 is a flavocytochrome protein which contains FAD- and NADPH-binding sites within the same molecule (10–12). However, this concept was based only on the similarities between the amino acid sequences of gp91^{phox} and other FNR flavoproteins. One study (11) attempted photoaffinity labeling of gp91^{phox} using [^{32}P]2-azido-NADP$^+$, but both its binding to gp91^{phox} and the difference in binding intensity near the 91-kD position between normal subjects and patients with gp91^{phox}-deficient X-linked CGD were extremely difficult to judge. In this study, affinity labeling of membranes using [^{32}P]oNADPH and [^{32}P]oNADP$^+$ showed that the most radioactive bands had molecular masses of 84 kD in both cases (Figs. 4 and 6). It was further confirmed using neutrophil membranes from gp91^{phox}-deficient CGD patients that the 84-kD protein was a molecule distinct from the β-subunit of cytochrome *b*558. These results agree well with the following previous observations: (*a*) in studies conducted to identify the reductase activity of the NADPH oxidaset system, partially purified fractions always contained FAD, but were free of heme (23, 24); (*b*) conversely, purified cytochrome *b*558 did not contain any FAD (22); and (*c*) reflavination of purified cytochrome *b*558 has been attempted, on the assumption that FAD had been released during purification (10, 11). However, even in a study of mild submembranous fractionation by density-gradient centrifugation, cell membrane–derived cytochrome *b*558, more clearly, specific granule–derived cytochrome *b*558, was not colocalized with flavin. After cell stimulation, NADPH oxidase activity emerged only in the flavin peak which recruited some of cytochrome *b*558 (25).

The molecular mass of 84 kD is inconsistent with the previous reported values of 66 (26) and 67 kD (8) in stimulated and resting membranes, respectively, which were indirectly affinity labeled with oNADPH in combination with [^{3}H]NaCNBH$_3$. However, assuming that they are likely glycoproteins, the different procedures used for membrane fractionation may cause a discrepancy of this type in the molecular masses. It is also well known that membrane-associated flavin enzymes such as cytochrome P-450 reductase have a site at which a portion of the molecule is easily cut off during separation (27). On the other hand, indirect affinity labeling with [^{3}H]NaCNBH$_3$ instead of [^{32}P]oNADPH may cause this discrepancy. In the latter report (8), in which a cytosolic NADPH-binding protein was proposed to translocate to the membrane upon cell stimulation using oNADPH, the authors concluded that the 67-kD protein had no relation to NADPH oxidase, because it was affinity labeled even in resting membranes. However, this proposal is likely to stem from the ambiguous recognition of oNADPH by cytosolic components in a cell-free activation system (17). Since no NADPH-binding protein, including a recently proposed ~32-kD protein (20), was recruited from the cytosol (Fig. 4), it is most likely located in the membrane in situ in the resting state. If this is the case, then there is no discrepancy between references 8 and 26. The most important finding in this and the previous studies is that no labeled protein corresponding to the β-subunit of cytochrome *b*558 was found.

Studies in which the NADPH oxidase activity was reconstituted in a cell-free activation system with purified (6, 10) or recombinant (28) cytochrome *b*558 as the sole membranous component in the presence of recombinant or purified p47^{phox}, p67^{phox}, and small G-proteins, have also greatly contributed to promoting the above flavocytochrome hypothesis. However, this system has worked only in the presence of exogenous FAD (10 μM), at an extremely high, nonphysiological concentration, which may artificially mediate O_2^- generation, as discussed in a recent paper (13). Coelution of FAD with a cytochrome *b*558 fraction in reflavination experiments (10, 11) cannot be a cogent explanation for stereospecifically proper interaction between FAD and cytochrome *b*558, excluding the possibility of electrostatic interaction between them. Whereas purified cytochrome *b*558 always requires the addition of FAD to a cell-free activation system, which in membrane or its solubilized fraction does not (10, 28), which suggests the involvement of physiological reductase of cytochrome *b*558 in neutrophil membranes. In fact, it has been demonstrated that a membrane-bound flavoenzyme can give purified cytochrome *b*558 O_2^--generating capability without any addition of exogenous FAD (13). Along with this previous report (13), the present results suggest that cytochrome *b*558 may have been erroneously believed to be the only redox component in cell membrane because of the lack of a supplemental experiment which combines the flavoprotein-containing flowthrough fraction, a nitroblue tetrazolium (NBT) reductase fraction in (13), with the eluted cytochrome *b*558 fraction of wheat germ agglutinin agarose (21), or heparin-agarose chromatography (22) using a nonexogenous FAD system.

The molecular mass of the 84-kD protein is similar to those of the 77- (29) and 80-kD (30) proteins of NADPH-cytochrome *c* reductase purified from rabbit and porcine neutrophils, respectively, which had been proposed to be the catalytic site of respiratory burst oxidase for NADPH. These proteins contained FAD and FMN in a 1:1 ratio, similarly to cytochrome P-450 reductase. However, whereas neutrophil membranes contain an abundance of FAD, they contain only a negligible amount of FMN (31). In addition, the separated fraction of the NBT reductase contained FAD, but only a negligible amount of FMN, and showed an O_2^--generating activity 100 times greater than that of liver NADPH-cytochrome P-450 reductase in a reconstituted system when

combined with purified cytochrome *b*558 (13). This evidence supports our conclusion that the NADPH-binding site of the oxidase is likely to exist in a molecule distinct from cytochrome *b*558, and probably not in cytochrome P-450 reductase. The actual molecule that changed from having an NADPH diaphorase activity to an O_2^--generating oxidase activity (32) may differ from the foregoing 77-kD protein (29) that was purified by the same group using NADPH-cytochrome *c* reductase activity as an indicator. The 84-kD band may contain two enzymes: an NADPH-specific FAD enzyme and contaminating cytochrome P-450 reductase. We have not yet determined whether the protein at 84 kD is associated with the NADPH oxidase catalytic site. Further studies are needed to clarify this point.

The present study revealed that: (*a*) no NADPH-binding protein is recruited from the cytosol to the cell membrane on cell stimulation, and (*b*) some membranous redox component besides cytochrome *b*558, which should be an NADPH-binding flavoprotein, must be involved in the NADPH oxidase system.

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