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ELECTROPHORETIC ISOLATION OF A MEMBRANE-BOUND NADPH OXIDASE FROM GUINEA-PIG POLYMORPHONUCLEAR LEUKOCYTES

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Electrophoretic isolation of a membrane-bound NADPH oxidase of guinea-pig polymorphonuclear leukocytes was attempted with the O_2^--generating membranes of cells unstimulated or stimulated with C3b-zymosan or sodium dodecyl sulfate, and also with the phagosomes isolated from the phorbol myristate acetate-coated latex particle-phagocytosing cells. When these vesicles were subjected to discontinuous polyacrylamide gel electrophoresis in the presence of Triton X-100 and then assayed for NADPH-Nitroblue tetrazolium reducing activity, the activity was detected by the appearance of a single, blue band of the reduced dye on the gel, independent of the source of vesicles. In addition, the enzyme was able to generate O_2^- and its activity was significantly augmented with the homologous liver microsomal cytochrome b_5. Its activity was heat-labile and inactivated by *N*-ethylmaleimide and *p*-chloromercuribenzene sulfonate. The enzyme, with an apparent molecular weight of 150 000, in the phagosomes was easily susceptible to limited proteolysis by trypsin and formed an active fragment with a molecular weight of 70 000, accompanying the loss of O_2^--generating activity of the vesicles.

Introduction

When leukocytes encounter appropriate stimuli such as complement-activated zymosan (C3b-zymosan) and sodium dodecyl sulfate, superoxide anion (O_2^-)-generation is promoted, attendant on the respiratory burst [1–5]. There is much information indicating that the O_2^--generating enzyme involved therein is an NADPH oxidase which is embedded in the plasma membrane [6–10]. Although the NADPH oxidase recently was obtained in its soluble form by the detergent-treatment of O_2^--generating vesicles such as the plasma membrane stimulated with C3b-zymosan, and the phagosomes [11–15], biochemical characterization of the enzyme has not been successful. In addition, recent studies [16–25] suggest that the NADPH oxidase system responsible for the respiratory burst is composed of at least two components: the so-called NADPH oxidase (flavoprotein) and cytochrome *b*-245 or certain quinones in the plasma membrane.

In the course of a study on biochemical properties of guinea-pig polymorphonuclear leukocyte NADPH oxidase, we found that a membrane-bound NADPH oxidase, which was capable of reducing Nitroblue tetrazolium as well as molecular oxygen at the expense of NADPH, was isolated by disc polyacrylamide gel electrophoresis in the presence of Triton X-100. Biochemical characterization of the enzyme is reported in this paper. In addition, a possible role of the enzyme in the respiratory burst is discussed.

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Materials and Methods

Polymorphonuclear leukocytes. Guinea-pig polymorphonuclear leukocytes were isolated from the peritoneal cavity of animals intraperitoneally injected with casein. The procedure for isolation of the leukocytes was that described in previous papers from our laboratory [5,26].

Preparation of O_2^--generating membrane fractions.

Leukocytes ($(3–9) \cdot 10^8$ cells) were activated by incubating the cells with 0.9 mg/ml of C3b-zymosan or 0.3 mM sodium dodecyl sulfate in 50–150 ml of Krebs-Ringer phosphate buffer, pH 7.4 (buffer 1), supplemented with 5.6 mM D-glucose and 0.25% bovine serum albumin, for 10 min at 37°C [5,26]. After incubation, the cells were washed twice with the same buffer.

The O_2^--generating membranes were prepared from the stimulated or non-stimulated leukocytes by the method described by Gabig et al. [11], but with some modification [26]. The leukocytes ($(3–6) \cdot 10^8$ cell) were suspended in 6–8 ml of cold 0.15 M NaCl/0.34 M sucrose (pH 7.4) and disrupted by sonication at 0°C. Immediately after sonication, diisopropyl fluorophosphate was added to the sonicates at a final concentration of 1 mM. The sonicates were centrifuged at $400 \times g$ for 10 min, and the supernatants (post-nuclear supernatants) were further centrifuged at $100\,000 \times g$ for 30 min at 4°C. The membrane fractions thus sedimented were washed twice with 0.34 M sucrose/0.15 M NaCl (pH 7.4) and suspended in 2–3 ml of the same medium. After addition of 2–3 ml of 20 mM glycine buffer (pH 8.4)/2 mM NaN_3/1.6 μM $CaCl_2$/40% glycerol, the membrane fractions were sonicated and used for experiments.

Isolation of plasma membrane fractions.

The plasma membrane fractions of C3b-zymosan-stimulated and non-stimulated leukocytes were isolated by the method described by Remold-O'Donnell [27]. The leukocytes ($3 \cdot 10^8$ cells) were suspended in 6 ml of 5 mM Tris-HCl buffer (pH 8.0)/75 mM sucrose/ 1 mM diisopropyl fluorophosphate, and homogenized in a Dounce-type homogenizer in an ice bath. After addition of 1.5 ml of 0.95 M sucrose/20 mM Tris-HCl (pH 7.4)/26 mM $MgCl_2$/1.5 mM EDTA, the homogenate was centrifuged at $500 \times g$ for 10 min.

The post-nuclear supernatant (2 ml), adjusted to a sucrose concentration of 40.5% (w/w) by addition of 2.5 ml of 2.3 M sucrose/10 mM Tris-HCl (pH 7.4)/5.3 mM $MgCl_2$/0.3 mM EDTA/30 mM NaCl/30 mM KCl, was poured into the bottom of a centrifuge tube containing 6.4 ml of a linearly increasing sucrose density gradient (sucrose concentration 34–40%) and then a 20% (w/w) sucrose solution was layered over the gradient. The sucrose solutions used were prepared by dissolving sucrose in 10 mM Tris-HCl (pH 7.4)/5.3 mM $MgCl_2$/0.3 mM EDTA/30 mM NaCl/30 mM KCl. After centrifugation at $130\,000 \times g$ for 10 h at 4°C, 1-ml fractions were collected from the bottom of the gradient, and aliquots of each fraction were assayed for NADPH oxidase and marker enzymes.

As marker enzymes of plasma membrane and lysosomes, activities of alkaline phosphodiesterase-I and β-glucuronidase were measured, using thymidine 5′-monophosphate *p*-nitrophenyl ester [28] and phenolphthalein-glucuronidate [29], respectively.

Isolation of phagosomes. Leukocytes ($4 \cdot 10^8$ cells) were incubated with latex particles ($1 \cdot 10^{10}$ particles, 0.81 μm) coated with phorbol myristate acetate in 5 ml of buffer 1 containing 5.6 mM D-glucose and 0.25% bovine serum albumin, as described by Bellavite et al. [30]. After incubation for 3 min at 37°C, the cells which had engulfed the particles were washed twice with the same medium at 4°C and suspended in 3 ml of 10 mM Tris-HCl buffer (pH 7.5) supplemented with 11% (w/w) sucrose. The cells were homogenized in a Dounce-type homogenizer in an ice bath, and diisopropyl fluorophosphate was added to the homogenate at final concentration of 1 mM.

The isolation of phagosomes was performed by flotation on a sucrose gradient [30]. The homogenate (3 ml) of phagocytosing leukocytes, adjusted to a sucrose concentration of 46% (w/w) by addition of 66% (w/w) sucrose, was placed at the bottom of a centrifuge tube and layered with 3 ml of 33% (w/w) sucrose and 2 ml of 11% (w/w) sucrose solutions. The tube was centrifuged for 30 min at 17 500 rpm (max., $25\,000 \times g$; min., $12\,500 \times g$). The materials at the interface between 11% and 33% (band 1) and between 33% and 46%

sucrose (band 2) were collected and used for experiments. The pellet at the bottom of the tube was suspended in 10 mM Tris-HCl buffer (pH 7.5) and centrifuged for 30 min at $100000 \times g$. The pellet sedimented was resuspended in 11% sucrose dissolved in Tris-HCl buffer (pH 7.5) and used for experiments.

Aliquots of each fraction were assayed for NADPH oxidase and marker enzymes. As marker enzymes of plasma membrane, lysosomes and microsomes, activities of alkaline phosphodiesterase-I, β-glucuronidase and D-glucose-6-phosphatase were measured, using thymidine 5′-monophosphate *p*-nitrophenyl ester [28], phenolphthalein-glucuronidate [29] and D-glucose 6-phosphate [31], respectively.

Assay of O_2^--generating activities of membrane fractions and NADPH oxidase. The O_2^--generating activities of membrane fractions and the NADPH oxidase isolated by gel electrophoresis were assayed by reduction of ferricytochrome *c* in the absence or presence of superoxide dismutase. For this purpose, a difference spectrum which developed between a pair of reaction mixtures containing a test sample together with cytochrome *c* incubated at 25°C in the absence or presence of 50 μg of superoxide dismutase, was determined [26]. The reaction mixtures used contained the membrane fraction or enzyme, 80 μM ferricytochrome *c*, 0.1 mM NADPH, 0.02% Triton X-100, 0.1 mM FAD and 0.1 mM NaN_3 in 1.0 ml of 12.5 mM potassium phosphate buffer (pH 7.4). The amount of O_2^- formed was calculated from the difference spectrum at 550 nm measured by the use of a Hitachi double-beam recording spectrophotometer, model 200-20, on the basis of an absorption coefficient of 21.1 $mM^{-1} \cdot cm^{-1}$ at 550 nm (reduced − oxidized) [32].

Gel electrophoresis of membrane fractions and assay of NADPH oxidase. The NADPH oxidase in the membrane fractions of leukocytes was separated by disc polyacrylamide gel electrophoresis and subsequently assayed for its activity on the gel by developing the blue band due to the reduction of Nitroblue tetrazolium. Gel electrophoresis was performed by the method described by Dulaney and Touster [33], with some modifications. Stacking gel and separating gel were polymerized after addition of 0.2% Triton X-100 to the gel solution. The gel columns (5% polyacrylamide, 5 × 60 mm) were pretreated by application of an electric current of 4 mA per column for 10 min with 0.38 M Tris-HCl buffer (pH 8.9)/0.2% Triton X-100, to remove ammonium persulfate remaining. The membrane fractions were dissolved in 70–100 μl of 0.16 M Tris-HCl buffer (pH 6.8) containing 0.2% Triton X-100, 21.1% glycerol and 130 μg/ml of Pyronin G, and then applied to the gel column after sonication. The solubilized membrane fractions were subjected to disc gel electrophoresis with 5 mM Tris/38 mM glycine buffer (pH 8.6)/0.2% Triton X-100. The electrophoresis was terminated when Pyronin G added as a tracking dye had moved 50 mm.

To assay NADPH-Nitroblue tetrazolium reductase, the gels were placed into 50 mM potassium phosphate buffer (pH 7.4)/0.5 mM NADPH/0.01 mM FAD/0.3 mM Nitroblue tetrazolium, and incubated with gentle shaking at 37°C for 10 min. After incubation, the gels were placed into 10% acetic acid at 0°C to terminate the reaction. The gels were scanned using a Shimazu dual-wavelength TLC scanner, CS-900 ($\lambda_S = 535$ nm, $\lambda_R = 450$ nm). The amounts of reduced Nitroblue tetrazolium were expressed in terms of area of the blue band thus scanned (cm^2).

Measurement of the molecular weight of NADPH oxidase. Estimation of molecular weights of NADPH oxidase and its active, tryptic fragment was performed by the method described by Hedrick and Smith [34], with some modifications. The stacking and separating gels containing 0.2% Triton X-100 were prepared by the method described by Dulaney and Touster [33]. The polyacrylamide concentration of the separating gel was varied from 5.25 to 12.75%.

Disc gel electrophoresis of test samples and standard proteins of known molecular weights (soybean trypsin inhibitor, 21500; bovine serum albumin, 68000; monomeric, dimeric and trimeric transferrin, 78000, 156000 and 234000; factor B of complement, 100000) was performed with 5 mM Tris/38 mM glycine buffer (pH 8.6)/0.2% Triton X-100, as described for the electrophoresis of membrane fractions in the preceding section.

After electrophoresis, a band of NADPH oxidase or its active fragment was stained by its Nitroblue tetrazolium reducing activity, and other

bands of standard proteins were detected by staining with Coomassie brilliant blue. When the log of protein mobility relative to Pyronin G was plotted versus acrylamide gel concentration of the separating gel, the plots of each protein gave a straight line with a constant slope depending on its molecular weight. On the basis of molecular weight-slope relation obtained with standard proteins, the molecular weights of NADPH oxidase and its active fragment were estimated.

Preparation of liver microsomal cytochrome b_5. Guinea-pig liver microsomal cytochrome b_5 was highly purified by Sakane and Takahashi of our laboratory, using the method described by Omura and Takesue [35]. In brief, the livers were homogenized, and the microsomes were collected and digested with trypsin. Cytochrome b_5 released from the microsomes was purified by successive applications of gel filtration on Sephadex G-100 and DEAE-cellulose chromatography. The preparation of cytochrome b_5, finally purified by rechromatography on a DEAE-cellulose column, showed spectral properties which were almost identical with those of rat cytochrome b_5 reported by Omura and Takesue [35]; the oxidized form had absorption maxima at 412, 530 and 560 nm, and the reduced form showed absorption peaks at 422, 524 and 554 nm.

Determination of protein concentration. Protein concentration of membrane fractions and phagosomes was determined by the protein assay reagent (Bio-Rad Laboratories), using bovine IgG as a standard protein.

Materials. Zymosan, bovine serum albumin, thymidine 5′-monophosphate *p*-nitrophenyl ester, phenolphthalein-glucuronidate, bovine superoxide dismutase, equine ferricytochrome *c*, NADPH, NADH, FAD, *p*-chloromercuribenzene sulfonate, trypsin, soybean trypsin inhibitor and human transferrin were purchased from Sigma Chemical Co. Nitroblue tetrazolium, *N*-ethylmaleimide and Triton X-100 were products of Wako Pure Chemical Industries, LTD. Diisopropyl fluorophosphate, phorbol myristate actetate, latex particles and D-glucose 6-phosphate were purchased from Kishida Chemical Co., Funakoshi Chemical, Co., Difco Laboratories and Boehringer Mannheim Co., respectively. Human factor B of complement was highly purified in our laboratory.

Results

Electrophoretic separation of an NADPH oxidase in membrane fractions

The NADPH oxidase of polymorphonuclear leukocytes responsible for the respiratory burst is located on the plasma membrane and solubilized with detergents [11–15]. To investigate biochemical properties of the enzyme, it was attempted to separate electrophoretically the enzyme, using the membrane fractions of C3b-zymosan- and sodium dodecyl sulfate-stimulated leukocytes [5,26]. For this purpose, the membrane fractions of the sonicated cells was prepared by centrifugation of the post-nuclear supernatants containing 1 mM diisopropyl fluorophosphate at $100\,000 \times g$, the inhibitor being added to avoid modification as well as inactivation of the NADPH oxidase by some coexisting proteases, as will be described later. The membrane fractions were solubilized with 0.2% Triton X-100 and applied to disc gel electrophoresis in the presence of the same concentration of the detergent. The activity of NADPH oxidase on the gel was assayed by measuring the reduction of Nitroblue tetrazolium, since leukocytes reduce the water-soluble dye to an almost insoluble formazan, the degree of reduction depending on their O_2^--generating activity [36–39].

When the gel, after electrophoresis, was placed into a solution containing 0.5 mM NADPH, 0.01 mM FAD and 0.3 mM Nitroblue tetrazolium, blue bands were found to appear on the gel: a broad, faint band with a mobility (R_m) of 0.2–0.3 relative to that of Pyronin G added as a tracking dye, and a sharp, main band with an R_m of 0.5. The scanning of the blue band with an R_m of 0.5 showed that the enzyme involved was heat-labile, since the heat-treatment of the membrane fractions for 10 min at 90°C, prior to electrophoresis, eliminated entirely the formation of the blue band. When incubation period was varied, the rate of reduction of Nitroblue tetrazolium was constant up to 20 min and then gradually decreased. The enzyme activity, therefore, was estimated by measuring the amount of dye reduced for 10–15 min at 37°C.

A linear relationship was obtained between the amount of the membrane fractions (0–0.5 mg protein) applied and that of reduced Nitroblue tetra-

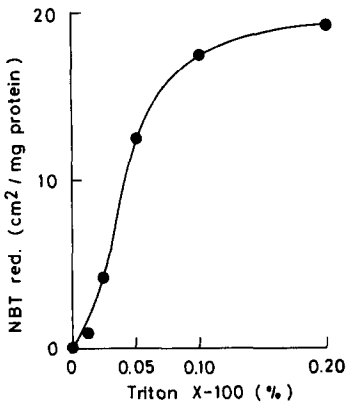

Fig. 1. *Effect of concentration of Triton X-100 on solubilization of the NADPH oxidase.* Constant amounts of the membrane fraction of sodium dodecyl sulfate-stimulated leukocytes (0.11 mg protein) were electrophoresed in varying concentrations of Triton X-100 and assayed for the blue band with R_m 0.5 by the gel scanning. NBT, Nitroblue tetrazolium.

zolium. Furthermore, the enzyme recovered in the position with R_m of 0.5 was dependent upon the concentration of Triton X-100 used, when electrophoresis was performed in various concentrations of the detergent (Fig. 1). This result confirms the membrane-bound nature of the enzyme, and also indicates that more than 0.1% of Triton X-100 is required for complete solubilization of the enzyme.

Biochemical properties of the isolated enzyme

Biochemical properties of the enzyme with R_m 0.5, thus separated, were further studied by scanning the intensity of the blue band. The enzyme was specific to NADPH and the replacement of NADPH by NADH decreased markedly the intensity of the blue band. Omission of FAD from the reaction medium made a slight decrease in the reduction of Nitroblue tetrazolium.

The enzyme activity with R_m 0.5 was not inhibited with 1 mM KCN and NaN_3. The effect of sulfhydryl reagents on the enzyme activity also was studied, since these reagents are known to inhibit the O_2^--generation from the stimulated membranes of leukocytes [12]. The treatment of the membrane fraction with 1 mM *p*-chloromercuribenzene sulfonate or 1 mM *N*-ethylmaleimide, prior to electrophoresis, eliminated almost completely the formation of a blue band due to the enzyme. This result indicates that the enzyme is a thiol-enzyme, its activity being inhibited by irreversible binding of the sulfhydryl reagents to some thiol residue(s) of the enzyme.

To elucidate whether the enzyme is able to generate O_2^- or not, the membrane fraction was electrophoresed on a large gel (7 × 50 mm), and the gel was bisected in the direction of electrophoresis. One segment was stained with Nitroblue tetrazolium in the usual manner, and another was further divided into sections each 3 mm wide and each section was extracted with 1 ml of 0.15 M NaCl/0.2% Triton X-100/0.34 M sucrose. The extracts were assayed for the O_2^--generating activity which was determined by the superoxide dismutase-inhibitable cytochrome *c* reduction, as is the case of membrane fractions. The O_2^--generating activity was definitely demonstrated to be exactly located in the position at which the reduction of Nitroblue tetrazolium occurred. In this case, 70–80% of total cytochrome *c* reduction by the enzyme was inhibited by addition of superoxide dismutase. These results indicate that the enzyme with R_m 0.5 is able to catalyze not only reduction of Nitroblue tetrazolium, but also that of molecular oxygen to O_2^- at the expense of NADPH. On the basis of these findings, the enzyme was tentatively designated to NADPH oxidase.

No O_2^- generation, on the other hand, occurred in the position with R_m of 0.2–0.3, where Nitroblue tetrazolium reduction was slightly observed. At present, whether the formation of a blue band in this position is caused enzymatically or not, is not clear.

Effect of stimulation of leukocytes on the NADPH oxidase activity

It is known that the membrane fractions of leukocytes stimulated with appropriate stimuli show a markedly higher O_2^--generating activity than that of non-stimulated, resting leukocytes. The effect of stimulation of leukocytes on the NADPH oxidase activity in membrane fractions, therefore, was studied by the Nitroblue tetrazolium-staining method.

The stimulations with both C3b-zymosan and sodium dodecyl sulfate augmented the O_2^--generating activity of the membrane fractions (Table I). The NADPH oxidase activity in these stimulated membrane fractions also increased, but slightly. In

TABLE I

THE NADPH OXIDASE ACTIVITY OF VARIOUS O_2^--GENERATING MEMBRANES

Membrane	NADPH oxidase [a]	O_2^--generating activity [b]
C3b-zymosan-stimul.	36	2.0
Non-stimul.	25	0.6
SDS-stimul.	15	4.0
Non-stimul.	12	1.9

[a] cm^2 of reduced Nitroblue tetrazolium/15 min per mg protein.

[b] nmol of O_2^-/min per mg protein.

particular, the stimulation with sodium dodecyl sulfate did not significantly enhance the activity. To study this difference in the ratio of the O_2^--generating activity to the NADPH oxidase activity between the stimulated and non-stimulated membranes, the effect of stimulation of leukocytes on the affinity of the enzyme to NADPH was determined with the sodium dodecyl sulfate-stimulated and non-stimulated membrane fractions. In both the cases, the amount of reduced dye increased, dependently upon the concentration of NADPH in the reaction medium (Fig. 2). The dose-dependency of the NADPH oxidase in the stimulated membrane fraction was identical to that of its counterpart in the non-stimulated membrane fraction. The K_m value calculated from a Lineweaver-Burk plot based on these curves was 62.5 μM, and almost equal to those estimated from the O_2^--generating activities of the membrane fractions used (56 μM for both the stimulated and non-stimulated membrane fractions, data not shown).

Subcellular localization of the NADPH oxidase

The NADPH oxidase in the respiratory burst is demonstrated to be located in the plasma membrane by the sucrose or Percoll density gradient fractionation of the homogenized leukocytes [10]. We therefore attempted to determine the subcellular localization of the NADPH oxidase detected by the Nitroblue tetrazolium-staining method. The sucrose density gradient fractionation showed that the enzyme was consistently accompanied by a plasma membrane marker enzyme, alkaline phosphodiesterase-I (Fig. 3). Stimulation of leukocytes with C3b-zymosan did not seem to vary significantly the distribution of the enzyme among various vesicles. This result suggests that the NADPH

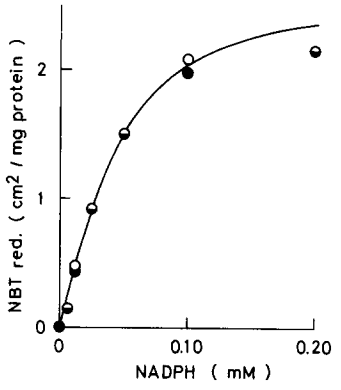

Fig. 2. Dependency of the NADPH oxidase activity on NADPH concentration. Constant amounts (0.23 mg protein) of the membrane fraction of sodium dodecyl sufate-stimulated (○) or non-stimulated (●) leukocytes were electrophoresed and the gels were incubated with the reaction medium containing various concentrations of NADPH. The amounts of reduced Nitroblue tetrazolium (NBT) were determined by the gel scanning.

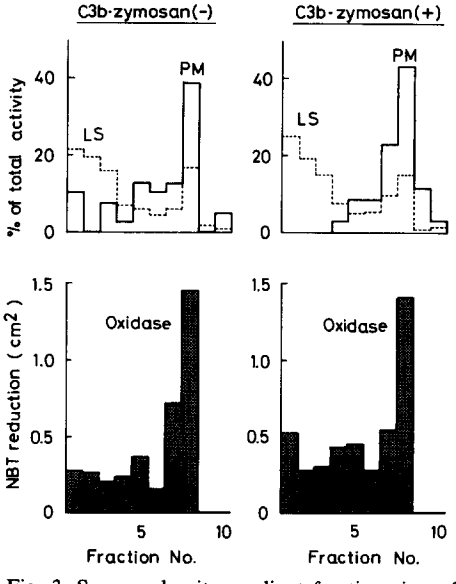

Fig. 3. Sucrose density gradient fractionation of post-nuclear supernatants of the homogenized leukocytes. The supernatants used were those of the C3b-zymosan-stimulated and non-stimulated leukocytes. Oxidase: NADPH oxidase assayed by the Nitroblue tetrazolium staining method; PM, alkaline phosphodiesterase-I; LS, β-glucuronidase.

oxidase is a plasma membrane-bound enzyme, as is the O_2^--generating NADPH oxidase in the respiratory burst.

Sucrose density gradient fractionation, however, could not separate the plasma membrane from the microsomes. To confirm the plasma membrane-bound nature of the NADPH oxidase detected by the Nitroblue tetrazolium-staining method, the O_2^--generating phagosomes were isolated from the phorbol myristate acetate-coated latex particle-treated leukocytes [30] and assayed for the enzyme.

The homogenate of the phagocytosing leukocytes was fractionated by discontinuous sucrose density centrifugation. The fraction obtained at the interface between 11% and 33% sucrose (band 1) was composed mainly of phagosomes containing latex particles with very low contamination by microsomes; the specific activity of alkaline phosphodiesterase-I was very high, whereas that of glucose-6-phosphatase was low (Table II). The non-phagosomal fraction (band 2), on the other hand, was heterogeneous with respect to the marker enzymes, suggesting that it consists of various vesicles including the plasma membrane, microsomes and lysosomes, as reported by Bellavite et al. [30].

The specific activity of O_2^--generation was markedly higher in band 1 than in band 2, indicating that the NADPH oxidase in the respiratory burst is activated by phagocytosis. The specific activity of the NADPH oxidase determined by the Nitroblue tetrazolium-staining method was higher in band 1 than in band 2, but only 1.5-fold. With regard to subcellular distribution, the enzyme was found to be located in the plasma membrane rather than in the microsomes, since the ratio of the enzyme activity to glucose-6-phosphatase activity was very higher in band 1 than in band 2.

Effect of microsomal cytochrome b_5

Recent studies by others [16–26] suggest that some cofactors such as a *b*-type cytochrome and quinones in the plasma membrane of leukocytes either play a role in activation of a so-called NADPH oxidase for O_2^--generation or are directly involved in reduction of molecular oxygen with the enzyme. Requirement of the electrophoretically isolated NADPH oxidase for any cofactor, therefore, was examined, using homologous, microsomal cytochrome b_5. The O_2^--generating activity of the enzyme was found to be augmented dose-dependently by addition of cytochrome b_5 (Fig. 4). In addition, the enhancement with cytochrome b_5 occurred, independently of whether the enzyme was isolated from the stimulated or non-stimulated membrane fraction.

Effect of trypsin digestion

Babior et al. [9] reported that the O_2^--generating activity of phagosomes was almost entirely eliminated by trypsin digestion of the vesicles. The phagosomes (band 1, 28 μg protein) and non-phagosomal membrane fraction (band 2, 30 μg protein) were digested with trypsin (110 μg) for 3 min at 25°C, and the reaction was terminated by addition of soybean trypsin inhibitor (165 μg). We also found that the O_2^--generating activity of the

TABLE II

DISTRIBUTIONS OF NADPH OXIDASE, O_2^--GENERATING ACTIVITY AND MARKER ENZYMES AMONG THE PHAGOSOMES (BAND 1) AND NON-PHAGOSOMAL FRACTION (BAND 2)

Numbers in parentheses indicate percentages of total activity of the homogenate.

Fraction	NADPH oxidase [a]	O_2^--generation [b]	PM [c]	MS [d]	LS [e]
Band 1	3.3 (5)	43 (15)	9.1 (14)	0.09 (0.5)	5.2 (3.3)
Band 2	2.0 (44)	3.6 (24)	2.0 (59)	0.65 (52)	5.0 (60)
Pellet	4.0 (52)	14 (52)	1.3 (24)	0.97 (47)	3.2 (23)

[a] NADPH oxidase estimated by the Nitroblue tetrazolium-staining method, cm^2/15 min per mg protein.
[b] O_2^--generating activity of unsolubilized membrane, nmol O_2^-/5 min per mg protein.
[c] Marker enzyme of plasma membrane, A_{400}/3 h per mg protein.
[d] Marker enzyme of microsomes, μmol P_i/30 min per mg protein.
[e] Marker enzyme of lysosomes, A_{550}/h per mg protein.

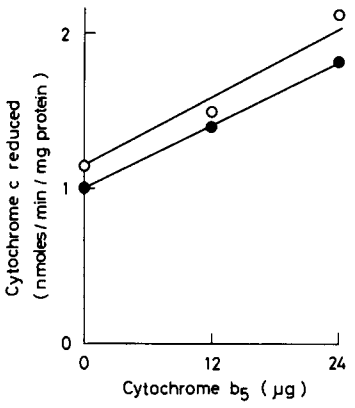

Fig. 4. Enhancement of O_2^--generating activity of NADPH oxidase with cytochrome b_5. The NADPH oxidase was extracted from the gel after electrophoresis of the membrane fraction of sodium dodecyl sulfate-stimulated (○) or non-stimulated (●) leukocytes. The O_2^--generating activity was determined, using the reaction medium containing various concentrations of cytochrome b_5.

phagosomes was markedly lowered to 11% of the original activity by trypsin digestion, while 80% of the activity was retained with the non-phagosomal membrane fraction even after the digestion.

When the trypsin-digested phagosomes were electrophoresed and assayed by the Nitroblue tetrazolium-staining method, the blue band with R_m 0.5 (the activity for non-treated phagosomes, 6.2 cm^2/mg protein applied) was found to disappear, accompanying the appearance of a new band with R_m 1.0 (the activity, 4.9 cm^2/mg protein applied). This change in electrophoretic mobility of the NADPH oxidase may be reflected by certain limited proteolysis of the enzyme by trypsin. In fact, the measurement of molecular weights of the intact enzyme and its active, tryptic fragment by the method of Hedrick and Smith [34] with soybean trypsin inhibitor, bovine serum albumin, transferrin, factor B of complement gave values of 150 000 and 70 000, respectively.

When the O_2^--generating membrane fractions as well as the phagosomes were prepared without addition of diisopropyl fluorophosphate, the Nitroblue tetrazolium-staining method gave a blue band with R_m of 1.0, besides a main band of R_m of 0.5 (data not shown). Certain coexisting proteases in the leukocyte homogenates, therefore, may cleave the NADPH oxidase, producing an active fragment, as in the case of tryptic digestion.

Discussion

The results obtained by the Nitroblue tetrazolium-staining method combined with disc gel electrophoresis in the presence of Triton X-100 demonstrated the existence of an NADPH oxidase in the plasma membrane as well as phagosomes of leukocytes. The enzyme thus isolated was found to reduce not only the dye, but also molecular oxygen at the expense of NADPH as an electron donor. With regard to the following biochemical properties, the enzyme resembles the NADPH oxidase in the respiratory burst which has been studied with the non-solubilized plasma membrane and phagosomes: NADPH-dependency, K_m for NADPH, susceptibilities to inhibition by some sulfhydryl reagents and tryptic digestion, and localization in the plasma membrane and phagosomes.

Recent studies by others [16–24] reveal the existence of cytochrome *b*-245 in the plasma membrane of leukocytes. Furthermore, it is proposed that this cytochrome participates in the enhanced O_2^--generation by stimulated leukocytes [18,19,21,23,24]. For instance, Light et al. [19] suggest that cytochrome *b*-245 either has a role in the activation of the enzyme responsible for O_2^--generation or, alternatively, is directly involved in the one-electron reduction of molecular oxygen. A certain quinone also is proposed to operate as an electron carrier in the NADPH oxidase system in the respiratory burst [25]. It is not clear whether the NADPH oxidase isolated by us contains these cofactors or not. It, however, is probable that the NADPH oxidase requires some cofactor for elicitation of its full activity expression, since microsomal cytochrome b_5 enhances the O_2^--generating activity of the enzyme.

It should be mentioned that the enhancing effect of cytochrome b_5 on the O_2^--generating activity of the isolated enzyme was not so high as expected. The microsomal cytochrome b_5 used in this study differs from leukocyte cytochrome *b*-245 in that the rate of oxidation of reduced cytochrome *b*-245 by molecular oxygen is 2000-fold greater than that of reduced cytochrome b_5, and also that the oxidation-reduction potential of cytochrome *b*-245 is markedly lower than that of cytochrome b_5 [18,19]. Therefore, if cytochrome *b*-245 were used in the place of cytochrome b_5, the

enhancing effect on O_2^--generation by the isolated enzyme would become greater.

The NADPH oxidase in the membrane fraction of leukocytes was found to be unstable upon solubilization of the membrane with 0.2% Triton X-100, as reported by Babior et al. [9] (data not shown). It seems probable that the inactivation of the membrane-bound NADPH oxidase with the detergent is caused by removal of certain cofactor such as quinone and cytochrome *b*-245 rather than by its inherent instability. Babior et al. [9] also reported evidence suggesting that the O_2^--generating NADPH oxidase of human neutrophils is rearranged within the plasma membrane upon the exposure of the cells to stimuli. We observed that the O_2^--generation by the phagosomes was almost entirely eliminated by trypsin digestion, though that by non-stimulated membranes was not substantially affected. In addition, we found that limited hydrolysis of the NADPH oxidase in the phagosomes occurred with a change in molecular weight of the enzyme. In the case of non-activated membrane fractions, similar proteolysis of the enzyme took place when trypsin digestion was performed in the presence of Triton X-100 (data not shown). Certain rearrangement of the enzyme, therefore, may occur within the plasma membrane, attendant on the stimulation of leukocytes. It seems likely that this rearrangement makes the cooperation of certain cofactors with the NADPH oxidase more effective, resulting in the enhanced O_2^--generation. The loss of the O_2^--generating activity of the phagosomes by trypsin digestion seems to be explained by the dissociation of the NADPH oxidase from some cofactor(s) due to the modification of the enzyme by trypsin.

At present, we have no direct evidence indicating that the NADPH oxidase demonstrated in this study is an enzyme primarily responsible for the respiratory burst. The possibility that leukocytes may contain an O_2^--generating enzyme other than the isolated NADPH oxidase, which cannot move into the gel or is inactivated during the isolation procedure used in this study, and that it may play a role in the respiratory burst, also has not been excluded. To solve these problems, further studies, in particular purification of the NADPH oxidase and cytochrome *b*-245, will be required. The study on biochemical properties of the NADPH oxidase isolated is now in progress.

With regard to molecular weight of the NADPH oxidase isolated, a value of 150000 was obtained. However, when a highly purified preparation of liver microsomal NADPH-cytochrome *c* (cytochrome *P*-450) reductase with a molecular weight of 77000 [40] was analyzed for its molecular weight, the method used in this study gave a value of 150000. Nakamura and Okamura of our laboratory also observed that the sodium dodecyl sulfate-polyacrylamide gel electrophoresis gave a value of 80000 for the molecular weight of purified NADPH-cytochrome *c* reductase. It also is probable, therefore, that the value of 150000 for the NADPH oxidase of leukocytes may be an overestimate of its molecular weight due to either dimerization or the effect of conjugation with lipids or carbohydrates, which are not dissociable even in 0.2% Triton X-100 [34].

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