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ISOLATION FROM NEUTROPHIL MEMBRANES OF A COMPLEX CONTAINING ACTIVE NADPH OXIDASE AND CYTOCHROME b-245

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NADPH-dependent O_2^- forming activity was extracted with deoxycholate from subcellular particles of guinea-pig neutrophils following stimulation with phorbol myristate acetate. The solubilized enzyme was purified by chromatography on Ultrogel AcA22, by isopycnic glycerol density gradient centrifugation and by treatment with 0.4 M NaCl. This procedure yielded a high-molecular-weight complex containing phospholipids, cytochrome b-245 and NADPH oxidase activity. Cytochrome b was found to be purified to the same extent as NADPH oxidase activity. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of the various purification fractions showed a progressive enrichment of a band whose molecular weight is $3.2 \cdot 10^4$. The enrichment of this protein band paralleled those of NADPH oxidase activity and of cytochrome b, indicating that it is a component of the oxidase system. The possibility that this band corresponds to either cytochrome b or a flavoprotein/cytochrome b complex is considered.

Introduction

During phagocytosis or perturbation of the plasma membrane by soluble stimuli, neutrophils undergo a cyanide-insensitive increase in oxygen consumption which is due to the activation of a membrane-bound NADPH oxidase dormant in resting cells. The enzyme catalyzes the one-electron reduction of oxygen by the following reaction [1–5]:

$$2O_2 + NADPH \rightarrow 2O_2^- + NADP^+ + H^+$$

An electron transport chain has been proposed to be responsible for the oxygen consumption [6–10]. A role for a flavoprotein was proposed on the basis of the stimulatory effect exerted by exogenous FAD added to NADPH oxidase solubilized

with Triton X-100 [6,11] and of the total inhibition of its activity by 5-carba-5-deazaFAD, which is competent only for two-electron transfers [12]. The activity seems to require phospholipids [13]. A b-type cytochrome was found to be located in the plasma membrane and in the specific granule fraction of neutrophils [14,15] and to be incorporated into the phagocytic vacuoles [15]. The involvement of this cytochrome in the O_2^- generating system of neutrophils has been suggested following its observed reduction in cells activated under anaerobic conditions [15] and by abnormalities observed in cells from patients with chronic granulomatous disease [15–17]. Ubiquinone-50 has been suggested to be responsible for carrying electrons between components of such a multicomponent electron transport chain [18].

Various attempts at solubilization and purification of the NADPH oxidase activity from human and guinea-pig neutrophils have been made

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Abbreviation: PMSF, phenylmethylsulfonyl fluoride.

[6,10,12,19,20]. The instability of the enzyme, however, has been a major problem during the purification procedure.

In a previous report from this laboratory [10] we have described a method for extracting with deoxycholate a very active NADPH oxidase from membranes of guinea-pig neutrophils. In the present paper we describe an improved procedure for isolating NADPH oxidase from activated neutrophils of guinea pig. The sodium dodecyl sulfate polyacrylamide gel electrophoresis of the fractions obtained during the purification procedure is also reported.

Materials and Methods

Reagents. Cytochrome *c* type III, NADPH type III, phorbol myristate acetate, deoxycholic acid and phospholipid standards were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The deoxycholic acid was recrystallized by the method of MacLennan [21] and stored as a 10% (w/v) solution. Human copper/zinc superoxide dismutase was prepared from outdated blood [22]. All other reagents were of the highest grade available.

Cell preparation and activation. Neutrophils were raised in guinea pigs following intraperitoneal injection of sterile 0.9% NaCl containing 1% sodium caseinate and collected after 12 h with two peritoneal lavages. The cell suspension was filtered through gauze and centrifuged for 10 min at 500 $\times g$. Contaminating erythrocytes were removed by hypotonic lysis. Neutrophils were finally suspended in Krebs-Ringer phosphate buffer (pH 7.4)/5 mM glucose/0.5 mM CaCl$_2$. Neutrophils preparations were activated by incubation at 37°C for 90 s with 0.2 μg/ml of phorbol myristate acetate [10] and then centrifuged at 1000 $\times g$ for 8 min. The cell pellet was resuspended in 10 mM Tris-HCl buffer (pH 7.0)/340 mM sucrose/2 mM phenylmethylsulphonyl fluoride at 4 · 10^8/ml neutrophil concentration.

Preparation of subcellular particles and solubilization of NADPH oxidase. All procedures were carried out at 0–4°C. The cell suspension was disrupted with a Potter-type homogenizer and unbroken cells and nuclei were sedimented by centrifugation at 250 $\times g$ for 8 min. The homo-genate was centrifuged at 100000 $\times g$ for 30 min and the resulting pellet containing the subcellular particles (fraction 1) was resuspended in 10 mM potassium phosphate buffer (pH 8.0)/20% (v/v) glycerol/1 mM EGTA to a protein concentration of about 5 mg/ml. Recrystallized deoxycholate was then added to 0.3% (w/v) final concentration. The suspension was kept for 20 min under magnetic stirring and then centrifuged at 100000 $\times g$ for 60 min. The resulting supernatant (fraction 2) was either used immediately for the next purification step or stored at −70°C.

Gel filtration on Ultrogel AcA22. The solubilized extract (10–16 mg protein) was applied to an Ultrogel AcA22 column (3.8 × 19 cm) equilibrated with 10 mM potassium phosphate buffer (pH 8.0)/20% (v/v) glycerol/1 mM EGTA. Fractions of 3 ml were collected at a flow rate of 0.8 ml/min.

Glycerol density gradient centrifugation. Pooled fractions from Ultrogel AcA22 column containing NADPH oxidase and cytochrome *b* (fraction 3) were concentrated, on an Amicon XM 300 membrane, to protein concentration of about 0.6 mg/ml, and 2 ml of the concentrate were layered on top of 14 ml linear density gradient (25–35% glycerol (v/v) in 10 mM potassium phosphate buffer (pH 8.0)/1 mM EGTA). The gradient was centrifuged for 14 h at 131000 $\times g$ in a Beckman SW 28 rotor. 1-ml fractions were collected by puncturing the bottom of the tube. The portion of the gradient containing the bulk of the NADPH oxidase and cytochrome *b* activity was pooled (fraction 4). To fraction 4 was added 1 volume of 10 mM potassium phosphate buffer (pH 8.0)/1 mM EGTA/0.8 M NaCl. After stirring for 20 min, the suspension was centrifuged at 100000 $\times g$ for 4 h. The resulting pellet (fraction 5) was suspended in 10 mM potassium phosphate buffer (pH 8.0)/1 mM EGTA/20% (v/v) glycerol.

Assays. NADPH oxidase activity was quantitated as O$_2^-$ production by measuring the superoxide dismutase inhibited reduction of cytochrome *c* [23]. Assays were carried out with a Perkin-Elmer 576 double-beam spectrophotometer at 22°C. Reference and sample cuvette contained 50 mM Hepes (pH 7.0)/110 mM KCl/25 mM NaCl/1 mM diethylenetriaminepentaacetic acid/2 mM NaN$_3$/0.1 mM cytochrome *c*/0.150 mM NADPH. The final volume was 1 ml. The reference cuvette also

contained 30 μg superoxide dismutase. The reaction was started by addition of enzyme preparation to both cuvettes. The absorption change at 550 nm was monitored. The reduction of cytochrome c was calculated using an extinction coefficient of 21.1 $mM^{-1} \cdot cm^{-1}$ [24].

Cytochrome b was measured by reduced minus oxidized difference spectroscopy using $E_{559-540} = 21.6$ $mM^{-1} \cdot cm^{-1}$ [25].

Protein concentration was measured after trichloroacetic acid precipitation by the method of Lowry et al. [26]. Phospholipid determinations were carried out following extraction with chloroform/methanol (2:1). Phosphate was determined by the method of Fiske and SubbaRow [27]. Separation of the phospholipids was carried out by high-performance thin-layer chromatography [28].

Sodium dodecyl sulfate polyacrylamide gradient gel electrophoresis. Slab gels (8×8 cm) consisting of a linearly increasing polyacrylamide concentration (4–30%) were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). The running buffer was 40 mM Tris-HCl (pH 8.0)/20 mM sodium acetate/2 mM EDTA/1% SDS. A preelectrophoresis at 20°C and at a constant voltage of 70 V was conducted for approx. 1 h. Samples to be loaded into the gels were treated with 1% SDS and incubated at room temperature for 4–5 h. Electrophoresis was conducted at constant voltage of 150 V for 2 h. Gels were stained according to Fairbanks et al. [29] and scanned with an Ultroscan laser Densitometer 2202 (LKB, Bromma, Sweden).

Results

Solubilization of NADPH oxidase activity

Subcellular particles isolated from phorbol myristate acetate-activated guinea-pig neutrophils had NADPH oxidase specific activity of 72.06 ± 27.2 nmol O_2^-/min per mg protein. Treatment of subcellular particles with 0.3% deoxycholate resulted in solubilization of 48.7% protein, 41.0% of NADPH oxidase activity and 37.9% cytochrome b (Table I). The specific activity of NADPH oxidase in solubilized extract slightly decreased relative to subcellular particles and a similar decrease was obtained for cytochrome b specific content.

Ultrogel AcA22 gel filtration

Previously [10] we reported the stabilization of the solubilized NADPH oxidase activity by dilution of the deoxycholate concentration from 0.3 to 0.05% (i.e., under the critical micellar concentration). In the present work this step was omitted, as the solubilized extract was immediately subjected to Ultrogel AcA22 chromatography. Ultrogel AcA22 was performed with a short column so that the chromatographic procedure was accomplished in 1–2 h instead of 30 h as previously described [10]. Under such conditions the majority of NADPH oxidase (70–80%) emerged from the column as a symmetrical peak of activity in the void volume (Fig. 1), whilst approx. 70% of the protein was retained. Cytochrome b determination was also made on the column fractions. Its elution

TABLE I

SOLUBILIZATION OF PROTEIN, NADPH OXIDASE AND CYTOCHROME b FROM SUBCELLULAR PARTICLES OF PHORBOL MYRISTATE ACETATE-STIMULATED GUINEA-PIG NEUTROPHILS

NADPH oxidase activity, cytochrome b and protein content were determined as described under Materials and Methods. Subcellular particles were assayed for NADPH oxidase activity in the presence of 0.3% deoxycholate.

	Protein (mg obtained from $2.5 \cdot 10^8$ cells) [a]	NADPH oxidase		Cytochrome b	
		Specific activity (nmol O_2^-/min per mg protein) [a]	Recovery (%)	Specific content (pmol/mg protein) [a]	Recovery (%)
Subcellular particles	4.1 ± 0.22	72.0 ± 15.7	100	147.1 ± 11.2	100
Solubilized extract	2.0 ± 0.15	60.6 ± 22.4	41.0	114.3 ± 2.3	37.9

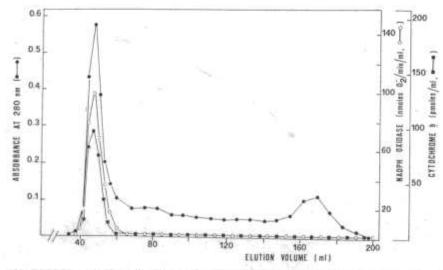[a] These values are mean ± S.E. of three experiments.

Fig. 1. Gel filtration of solubilized NADPH oxidase. 14 mg protein (95.0 nmol O_2^-/min per mg; 118 pmol cytochrome b/mg) were chromatographed on Ultrogel AcA22 column. Operating procedures, NADPH oxidase activity, cytochrome b and protein determinations are described under Materials and Methods.

TABLE II

PURIFICATION OF NADPH OXIDASE AND CYTOCHROME b FROM GUINEA-PIG PHORBOL MYRISTATE ACETATE-ACTIVATED NEUTROPHILS

NADPH oxidase activity, cytochrome b and protein content were determined as described under Materials and Methods.

Fractions	Protein (mg obtained from $2.5 \cdot 10^8$ cells) [a]	NADPH oxidase			Cytochrome b	
		Specific activity (nmol O_2^-/min per mg protein) [a]	Purification ratio	Purification ratio corrected for spontaneous decay	Specific content (pmol/mg protein) [a]	Purification ratio
Solubilized extract (Fr. 2)	2.0 ± 0.15	60.6 ± 22.4	1	–	114.3 ± 2.2	1
Pooled Ultrogel AcA22 fractions (Fr. 3)	0.5	179.8	2.9	–	300.5	2.6
Fraction 3 concentrated and stored at 0°C for 14 h	0.4 ± 0.07	122.4 ± 15.3	2.0	2.9	301.1 ± 46.3	2.6
Glycerol density gradient peak (Fr. 4)	0.05 ± 0.003	209.3 ± 25.0	3.5	5.0	655.6 ± 70.2	5.7

[a] These values are mean $\pm$ S.E. of three experiments.

profile perfectly corresponded to that of NADPH oxidase activity. Such chromatographic procedure resulted in a purification factor for NADPH oxidase and cytochrome b of 2.9 and 2.6, respectively (Table II). Since the exclusion limit of Ultrogel AcA22 resin is about $1.2 \cdot 10^6$ Da, with this procedure NADPH oxidase activity and cytochrome b are eluted as high molecular weight complex.

Glycerol density gradient centrifugation

The complexes eluted from Ultrogel AcA22 chromatography were sedimented through a linear glycerol density gradient. Fig. 2 shows the profile of the distribution of protein, NADPH oxidase activity and of cytochrome b in a 25–35% glycerol gradient. The NADPH oxidase together with cytochrome b, moved to the center of the gradient and represented about 50% of the activities in the initial amount loaded, whilst 50% sedimented to the bottom of the tube. A low-density protein peak

without NADPH oxidase activity and cytochrome b was also obtained (Fig. 2). The specific activity of the most active pooled glycerol gradient fractions (fraction 4) increased 3.5-fold with respect to that of solubilized particles (Table II). However, if the specific activity of glycerol gradient fractions were corrected for the decay observed during the concentration procedure and the time span needed for the centrifugation through the glycerol gradient, a purification ratio of 5.0 instead of 3.5 was obtained. The purification ratio obtained for cytochrome b was 5.7, which was similar to that obtained for NADPH oxidase activity

In an attempt to purify further the oxidase activity, the enzyme preparation obtained from glycerol gradient was treated with dissociating agents such as 8 M urea, 1% Lubrol, 1% Triton X-100 or 1 M KCl. All these procedures, however, resulted in complete loss of NADPH oxidase activity. With a mild treatment with NaCl a better result was obtained. The pooled active fractions of
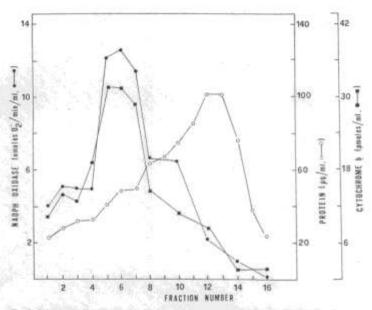

Fig. 2. Behavior of NADPH oxidase and cytochrome b on a glycerol density gradient, 25–35% (v/v). 1.25 mg protein (125 nmol O_2^-/min per mg and 290 pmol cytochrome b/mg) were fractionated and the fractions were assayed for NADPH oxidase activity, cytochrome b and protein content as described under Materials and Methods.

glycerol gradient (fraction 4) were treated with 0.4 M NaCl (see Methods). Under these conditions only 20–30% of NADPH oxidase activity was lost. The pellet obtained by centrifugation at 100 000 × g for 4 h (fraction 5) and containing 65% of protein and virtually all the NADPH oxidase activity and cytochrome b was subjected to electrophoresis.

Electrophoresis

During the course of purification, protein samples were analyzed by SDS-polyacrylamide gel electrophoresis and the results are given in Fig. 3. A progressive decrease of the number of polypeptide bands in the samples was obtained during the various purification steps. The intensity of most of the bands does not increase in the various purification fractions, whilst a band with a molecular weight around $3.2 \cdot 10^4$ (arrow) shows a progressive enrichment. The enrichment of this band is particularly evident in the lane 4, which is the electrophoretic pattern of the peak of the glycerol gradient (fraction 4) containing the highest NADPH oxidase activity and cytochrome b. It is worthy of note that this band is absent in Fig. 3, lane 5, which is the low-density protein peak of the glycerol gradient, devoid of NADPH oxidase activity and of cytochrome b (see Fig. 2). The final pellet obtained after treatment of the active frac-
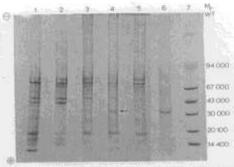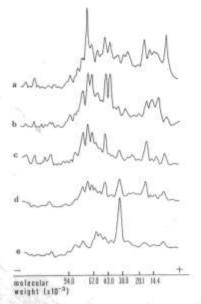

Fig. 4. Densitometric scaning of SDS-polyacrylamide gel electrophoresis. Trace a: fraction 1. Trace b: fraction 2. Trace c: fraction 3. Trace d: fraction 4. Trace e: fraction 5.

Fig. 3. SDS-polyacrylamide gel electrophoresis of fractions obtained during the purification procedure of NADPH oxidase. Lane 1: fraction 1. Lane 2: fraction 2. Lane 3: fraction 3. Lane 4: fraction 4. Lane 5: low density peak from glycerol density gradient. Lane 6: fraction 5. Lane 7: protein standards. All samples contained 25 µg protein.

tions of the glycerol gradient with 0.4 M NaCl (fraction 5) resulted in a further enrichment of the band at $3.2 \cdot 10^4$ and in the disappearance or decrease of the other protein bands (Fig. 3, lane 6). A more precise estimation of the enrichment of this band has been performed by densitometric scanning of stained gels (Fig. 4). In the subcellular particles and in the deoxycholate extract it comprised about 2% of total protein (Fig. 4, a,b) and increased to 5% in pooled fractions from Ultrogel AcA22 chromatography (Fig. 4, c) and to 10% in the peak fraction of glycerol gradient (Fig. 4, d). A comparison between the enrichment of this band and that of NADPH oxidase specific activity and cytochrome b specific content in the corresponding fractions (Table II), shows that its enrichment strictly parallels that of NADPH oxidase and cytochrome b. The $3.2 \cdot 10^4$ band is by far the major component remaining after extraction with 0.4 M NaCl (Fig. 4, e). The electrophoretic analysis of all the preparations, including that obtained with

NaCl treatment of the glycerol gradient peak, was performed five times, and in all the experiments the $3.2 \cdot 10^4$ band emerged as the major component of the active complex.

In order to rule out the possibility that the electrophoretic pattern of the purified enzyme was affected by artifact due to proteolytic activity during the electrophoresis step, an experiment was carried out in which fraction 5 was submitted to various treatments inhibiting proteinase activity. As can be seen from Fig. 5, neither the addition of 2 mM PMSF alone, nor its combination with other proteinase inhibitors, nor treatment at 100°C, changed the electrophoretic profile of fraction 5 with respect to untreated sample.
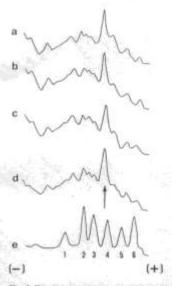

Fig. 5. Densitometric scanning of SDS-polyacrylamide gel electrophoresis in the absence and in the presence of proteinase inhibitors. Fraction 5 (20 μg protein) was treated with 1% SDS for 4 h at room temperature (trace a); for 4 h at room temperature in the presence of 1 mM PMSF (trace b); for 4 h at room temperature in the presence of 1 mM PMSF, 20 μg/ml pepstatin, 10 μg/ml leupeptin, 1 mM diisopropylfluorophosphate (trace c); for 5 min at 100°C in the presence of the same inhibitors of trace c (trace d). Trace e (protein standards): 1, phosphorylase b (94000); 2, albumin (67000); 3, ovalbumin (43000); 4, carbonic anhydrase (30000); 5, trypsin inhibitor (20100); 6, α-lactalbumin (14400). The arrow indicates the $3.2 \cdot 10^4$ Da protein band.

Phospholipid content.

The data so far presented indicate that NADPH oxidase activity and cytochrome *b* are associated with high-molecular-weight complexes which are sedimentable by centrifugation at $100\,000 \times g$ for 4 h. These complexes contain a great amount of phospholipids and the phospholipid/protein ratio increases during purification. The phospholipid content of the subcellular particles (fraction 1) is 301.1 ± 32.3 nmol phospholipid/mg protein, that of the final pellet obtained after extraction with 0.4 M NaCl (fraction 5) is 1769.7 ± 295.7 nmol phospholipid/mg protein. The phospholipids were found to be in the following increasing concentration: phosphatidic acid < phosphatidylinositol < phosphatidylserine < sphingomyelin < phosphatidylcholine < phosphatidylethanolamine. The relative concentration of each phospholipid did not change during the purification steps.

Discussion

An improved procedure is described for the purification of NADPH oxidase activity from phorbol myristate acetate-activated guinea-pig neutrophils. The procedure consists of gel-filtration chromatography on Ultrogel AcA22 of deoxycholate-extracted subcellular particles, followed by isopycnic glycerol density gradient centrifugation and treatment with 0.4 M NaCl. The final preparation is a high-molecular-weight complex containing the original phospholipid content of cell membrane and has a 5.0-fold increase in the specific NADPH oxidase activity. This procedure constitutes the first report on the purification of solubilized NADPH oxidase activity from neutrophils, since previous attempts have been hampered by extremely rapid loss in enzymatic activity following detergent solubilization [6,12,18,19]. The detergents are probably causing the loss of phospholipids which appear to be essential for activity of many membrane-bound enzymes [30] and also of NADPH oxidase [13]. Under our conditions, the enzyme activity was found to be more stable, since the detergent was removed during gel filtration resulting in the preservation of the phospholipid content.

Spectroscopic analysis of the solubilized preparations showed the presence of cytochrome *b*.

During the course of NADPH oxidase purification, the profiles of the enzymatic activity, eluted from Ultrogel AcA22 column and sedimented through the glycerol density gradient, coincided with the profiles of this cytochrome (Figs. 1 and 2). Moreover, the increase of NADPH oxidase specific activity in the various purification steps was accompanied by a parallel increase in the specific content of cytochrome b (Table II). It is worth pointing out that this cytochrome has characteristics similar to those reported for cytochrome b of human neutrophils [8]. It has an $E_{m,7.0}$ of -245 mV and binds CO to about 30%, and its reduced form is reoxidized by air [31].

The analysis of SDS-polyacrylamide gel electrophoresis of various fractions obtained during the purification procedure shows that only one protein band with apparent molecular weight of $3.2 \cdot 10^4$ was greatly enriched in the purified enzyme preparation (Figs. 3 and 4), thus suggesting that this band is a component of the oxidase system. This conclusion is also supported by the fact that this band is absent in the low-density protein peak of the glycerol gradient which is devoid of NADPH oxidase activity. This electrophoretic pattern is not due to artifact induced by proteolysis, as shown by the experiment made in the presence of various proteinase inhibitors (Fig. 5).

The component(s) of the NADPH oxidase corresponding to the enriched band remain(s) to be identified. At first sight, this band seems to be cytochrome b-245, because its percent increase relative to total protein parallels the purification ratio of the cytochrome. However, since the enrichment of the $3.2 \cdot 10^4$ Da band parallels the purification ratio of the NADPH oxidase activity, the identification of the band with cytochrome b alone requires the assumption that this hemoprotein be, as a matter of fact, the oxidase itself. On the other hand, other components, such as a flavoprotein, have been proposed as participants in the transfer of electrons from NADPH to oxygen [6,12,31,32]. If this is the case, the enriched band could correspond to both flavoprotein and cytochrome b, with molecular weights so similar that they cannot be separated by the electrophoretic system used in the present study. Further investigations are in progress in our laboratory to discriminate these two alternative hypotheses.

The SDS-polyacrylamide gel electrophoresis, even of the most purified active complex, showed the presence of other light protein-staining bands. The fact that none of these bands is enriched during the various purification steps, but that, on the contrary, they progressively decrease, suggests that they are contaminants still linked to the phospholipid-protein complexes and not components of the NADPH oxidase system.

Acknowledgements

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