A Phosphoprotein of M_r 47,000, Defective in Autosomal Chronic Granulomatous Disease, Copurifies with One of Two Soluble Components Required for NADPH:O_2 Oxidoreductase Activity in Human Neutrophils

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Abstract

The NADPH:O_2 oxidoreductase (NADPH oxidase) of human neutrophils is converted from a dormant to an active state upon stimulation of the cells. We have studied the soluble fraction that is required for NADPH oxidase activation in a cell-free system. Human neutrophils were separated in a membrane-containing and a soluble fraction. The soluble fraction was separated on carboxymethyl (CM) Sepharose in 10 mM 4-morpholino-ethanesulfonic acid buffer of pH 6.8. Reconstitution of the NADPH oxidase activity, measured as O_2 consumption, was only found when the membrane fraction was combined with the flowthrough of the CM Sepharose column as well as with a fraction that eluted at 125 mM NaCl. This result indicates that at least two soluble components are necessary for reconstitution of the NADPH oxidase activity: one that does not bind to CM Sepharose and one that does bind. These components were designated soluble oxidase component (SOC) I and SOC II, respectively. Boiling destroyed the activity in both fractions. In the soluble fraction of human lymphocytes and thrombocytes neither SOC I nor SOC II activity was found.

SOC II copurified with a 47-kD phosphoprotein, previously found defective in patients with the autosomal form of chronic granulomatous disease (CGD). Inactive soluble fractions of cells from autosomal CGD patients were reconstituted with a SOC II fraction from control cells. The result of this experiment indicates that autosomal CGD patients are normal in SOC I but defective in SOC II.

Introduction

Neutrophils and other phagocytic cells contain an NADPH:O_2 oxidoreductase that is dormant in resting cells. Binding of opsonized microorganisms to the plasma membrane of the neutrophil leads to activation of the oxidoreductase (1, 2). As a result, the oxygen consumption by the neutrophils is greatly enhanced, and highly toxic oxygen products are formed (3). After fractionation of activated cells, the NADPH oxidase is found in the plasma-membrane fraction (4). The cascade of events leading to activation of the NADPH oxidase is not well understood. Until recently, activation of the enzyme could only be studied in intact cells. Evidence has been found that a protein of M_r 47,000 is involved (5–7). Segal et al. (5) found a defect in phosphorylation of this protein in cells from patients suffering from the autosomal form of chronic granulomatous disease (CGD).[1] Neutrophils from CGD patients fail to show a respiratory burst after stimulation (8, 9).

Recently, several research groups reported the activation of the NADPH:O_2 oxidoreductase in a cell-free system, either by arachidonate (10, 11) or dodecyl sulfate (12). The cell-free system requires both a membrane fraction and a soluble, presumably cytosolic, fraction (10–13). The specificity of this system was shown by the absence of NADPH oxidase activity in a cell-free system derived from CGD cells upon addition of AA (11, 13). The cell-free system thus obviously reflects the physiological enzyme activity, and offers a way to isolate and characterize the relevant soluble components needed for NADPH:O_2 oxidoreductase activity or activation.

Evidence has been provided that a G protein is involved (14, 15) that is pertussis- and cholera toxin–insensitive (14) and therefore different from the G protein involved in phosphoinositide breakdown. Involvement of a G protein explains the requirement for Mg^{2+} (16) because protein phosphorylation is probably not involved in the activation of the NADPH oxidase in the cell-free system (13).

Curnutte et al. (13) and Gabig et al. (14) found several peaks of cytosolic cofactor activity of different molecular mass after gel permeation chromatography. However, detailed characterization of the soluble component or components has not yet been reported.

In this paper we present evidence for the participation of at least two soluble components, which could be separated by cation exchange chromatography. We named these components soluble oxidase component (SOC) I and SOC II. SOC II activity was shown to coelute with a phosphoprotein of M_r 47,000. Cells from autosomal CGD patients lack SOC-II activity but contain normal SOC-I activity. Both components were found to be absent in lymphocytes and thrombocytes.

Methods

Materials. Phosphatidylcholine and phosphatidylserine, PMSF, acetyl-L-leucyl-L-leucyl-L-arginine (leupeptin), histone III-S, Hepes and nitro-blue tetrazolium (NBT) were obtained from Sigma Chemical

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Received for publication 20 November 1987 and in revised form 10 October 1988.

J. Clin. Invest.

0021-9738/89/03/0757/07 $2.00
Volume 83, March 1989, 757–763

1. *Abbreviations used in this paper:* CGD, chronic granulomatous disease; CM, carboxymethyl; MES, 4-morpholino-ethanesulfonic acid; NBT, nitro blue tetrazolium; SOC, soluble oxidase component.

Co., St. Louis, MO. ATP, guanosine 5'-*O*-(3-thiotriphosphate) (GTP-γ-S), NADPH, and 4-morpholino-ethanesulfonic acid (MES) were purchased from Boehringer, Mannheim, FRG. X-Omat AR and X-Omat S diagnostic films were from Eastman Kodak Co., Rochester, NY. [^{32}P]γ-ATP was from Amersham International, Amersham, UK. Phorbol myristic acetate (PMA) was from Consolidated Midland Corp., Katonah, NY. Carboxymethyl Sepharose and Sephacryl S-300 were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Reagents and molecular weight markers for SDS-PAGE were from Bio-Rad Laboratories, Richmond, CA.

Preparation of neutrophils. Neutrophils were prepared from the buffy coat (leukocyte-rich fraction) of 500 ml fresh, citrated blood. Routinely, up to 15 buffy coats were combined and the neutrophils were isolated according to Lutter et al. (17).

Preparation of lymphocytes and thrombocytes. Human lymphocytes and thrombocytes were isolated by counter-flow centrifugation elutriation with a elutriation rotor (JE-6B; Beckman Instruments, Fullerton, CA) system as described by Figdor et al. (18).

Classification of CGD patients. CGD patients were classified as autosomal when complementation of NBT reductase activity was demonstrated after fusion of their monocytes with monocytes of X-linked CGD patients, as described by Hamers et al. (19).

In vitro phosphorylation of proteins with purified protein kinase C. Protein kinase C was isolated from human platelets by DEAE cellulose chromatography, phenyl-Sepharose and lipid affinity chromatography, essentially according to the procedure described by Kikkawa et al. (20) and Girard et al. (21). The purified kinase appeared as one band on an SDS-PAGE gel after silver staining. The preparations were stored in 0.01% Triton X-100 and 10% glycerol at −70°C. The protein kinase C (specific activity 350 nmol/min per mg protein) was added to a protein fraction in the following mixture: 30 mM Tris-HCl (pH 7.6), 2 mM EGTA, 7.5 mM $MgCl_2$, 10 μM [^{32}P]γ-ATP (2 Ci/mmol), 2.5 μl of protein kinase C (with an average activity of 20 pmol of phosphate transfer per minute as measured with 40 μg of histone III-S), and protein substrate in a total volume of 120 μl. The kinase was stimulated with 3 μg of PS, 1 μg of PC, and 1.0 μg of PMA (presented as a liposome mixture in 25 μl of water after 3 min of sonication at 4°C, 21 kHz frequency and 8 μm peak-to-peak amplitude). The reaction mixture was preincubated for 5 min at 30°C in the absence of ATP. After the addition of ^{32}P-labeled γ-ATP, the incubation was continued for 10 min. The phosphate incorporation was stopped by the addition of sample buffer for SDS-PAGE or two-dimensional gel electrophoresis.

SDS-PAGE. SDS-PAGE was carried out at ambient temperature in 5–15% (wt/vol) gradient slab gels containing 0.1% (wt/vol) SDS. Samples and molecular weight markers were incubated with one quarter volume of sample buffer (125 mM Tris-HCl, pH 6.8) containing 20% (wt/vol) SDS, 10% (wt/vol) β-mercaptoethanol and 10% glycerol, for 15 min at 80°C. Electrophoresis was carried out according to Laemmli (22).

Two-dimensional gel electrophoresis. Two-dimensional gel electrophoresis was performed as described by Vasilov et al. (23). For isoelectric focusing in tube gels, ampholines (LKB Instruments, Bromma, Sweden) were used in the following concentrations: pH 3.5–10, 1% (vol/vol); pH 5–7, 4% (vol/vol) and pH 7–9, 1% (vol/vol). Samples were prepared with sample buffer for SDS-PAGE with omission of bromophenol blue. The isoelectric focusing gels were layered on a SDS-PAGE gel (10% separation gel).

Autoradiography. Gels were dried and covered with an intensifying screen. Both XRP and XAR films were used and exposed for 1–6 d at −70°C.

Fractionation of neutrophils. Neutrophils were fractionated according to Markert et al. (24). Briefly, the procedure was as follows: unstimulated neutrophils were suspended in sonication buffer (0.34 M sucrose; 10 mM Hepes; 1 mM EGTA; 1 mM PMSF; 100 μM leupeptin in PBS, pH 7.0) at a concentration of 3×10^8 cells/ml and were sonicated for three 15-s intervals at 21 kHz frequency and 9 μm peak-to-peak amplitude. The sonicated suspension was diluted three times with sonication buffer and centrifuged for 10 min at 160 *g* to remove unbroken cells and cell nuclei. 5 ml of the postnuclear supernatant was layered on a discontinuous gradient of 5 ml of 30% (wt/vol) sucrose resting on 10 ml of 50% (wt/vol) sucrose. The gradient was centrifuged in a rotor (AH 627; Sorvall) at 100,000 *g* for 60 min at 4°C in a ultracentrifuge (TGA-50; Kontron Analytical, Everett, MA). The application zone, containing soluble oxidase-component activity, was stored at −70°C or used immediately. This fraction is referred to as soluble fraction throughout the text.

The membranes were collected from the interface between the 50 and 30% sucrose layers and diluted to a final concentration of 1×10^8 cell equivalents/ml. The membranes were homogenized with a Dounce homogenator and stored in small aliquots at −70°C. Both membranes and soluble fraction were stored in this way for months without loss of activity.

The fractions were assayed for the following marker enzymes: lactate dehydrogenase (cytosol); alkaline phosphatase (plasma membrane); lysozyme (specific granules and azurophilic granules); vitamin B_{12}-binding protein (specific granules); and β-glucuronidase (azurophilic granules). The soluble fraction contained all the lactate dehydrogenase and was essentially free from markers of plasma membrane, specific granules, and azurophilic granules.

The membrane fraction contained 31±4% (n = 3) of the alkaline phosphatase activity, 75±12% (n = 3) of the vitamin B_{12}-binding activity, and 13±6% of the β-glucuronidase activity. Although this fraction should be considered as a mixture of plasma membranes and specific granules, it is referred to as membrane fraction throughout the text. The remainder of the alkaline phosphatase activity was found in the 30% sucrose layer between the soluble fraction and the interface. However, the amount of NADPH oxidase activity in this fraction, tested in the presence of SDS and soluble fraction, was < 5% compared with the membrane fraction. Therefore, we only used the membrane fraction as the nonsoluble NADPH oxidase component.

Fractionation of the soluble fraction. The fractionation of the soluble fraction was carried out at 4°C. Soluble fraction was dialyzed against elution buffer (10 mM MES 1 mM EGTA and 1 mM PMSF, pH 6.8) and applied onto a column of carboxymethyl Sepharose, which was equilibrated with elution buffer. Per 10^8 cell equivalents, 0.5 ml packed volume of the cation exchanger was used. After the light absorbance at 280 nm had dropped to zero, a linear gradient of 0–0.20 M NaCl in elution buffer was applied. 75–150 μl of the collected fractions was assayed for SOC activity.

Measurement of NADPH:O_2 oxidoreductase activity. NADPH:O_2 oxidoreductase activity was measured as the rate of oxygen consumption with a oxygen electrode (Clark, Yellow Springs Instrument Co., Yellow Springs, OH) at 27°C. The assay mixture (0.65 ml) was composed of: assay buffer (10 mM Hepes/10 mM potassium phosphate; 0.17 M sucrose; 175 mM NaCl; 0.5 mM EGTA; 1 mM $MgCl_2$, pH 7.0); 50–150 μl of soluble fraction or column fractions, and 25–50 μl of membrane fraction (equivalent of $2–4 \times 10^6$ neutrophils). Where indicated, 10 μM GTP-γ-S or 10 μM FAD were added to the reaction mixture. The reaction was initiated by the addition of 25 μl of SDS to a final concentration of 100 μM, as described by Bromberg and Pick (12). The reaction vessel was closed, 3 min later NADPH was added to a final concentration of 200 μM, and the oxygen consumption was recorded.

Results

Characterization of NADPH:O_2 oxidoreductase in a cell-free system. After the addition of SDS, neutrophil homogenate produces superoxide in an NADPH-dependent fashion (12). Because this activity requires both a cell membrane fraction and a soluble cell fraction, we used this in vitro system to study the soluble component(s). We measured oxygen consumption rather than superoxide production, because high background values of cytochrome *c* reduction were found in the soluble

fraction alone. However, ~ 90% of the oxygen consumed by the in vitro system was recovered as superoxide by the measurement of SOD-inhibitable cytochrome *c* reduction. In our hands, a completely reconstituted system consumed 75 nmol O_2/min per 10^7 cells, which is ~ 85% of the oxygen consumption found in intact neutrophils after stimulation with serum-treated zymosan (25). The K_m of the system for NADPH was found to be 32 μM, in good agreement with values reported by others (10, 12, 26). In addition, we found no requirement for added flavins, either in the crude soluble fraction or in partially purified soluble components (see below). Also, we found that ATP is not necessary in this cell-free system, because neither exhaustive dialysis of the soluble fraction nor addition of hexokinase plus glucose affected the rate of O_2 consumption. However, the presence of Mg^{2+} was found to be essential for activity. These results confirm the findings published by Curnutte et al. (13) and Seifert and Schultz (15).

Fractionation of the soluble fraction. We fractionated the soluble fraction on a column of CM Sepharose as described in Methods. The elution profile is shown in Fig. 1. None of the collected fractions could support NADPH oxidase activity in the presence of membranes and SDS. However, when the fractions eluting at increasing salt concentrations were tested in the presence of the flowthrough fraction (fraction 4 in Fig. 1), a peak of activity was recovered that eluted at 125 mM NaCl. Four independent experiments showed a recovery of 68±8% of the soluble component activity applied to the CM-Sepharose column. These results suggest that (at least) two

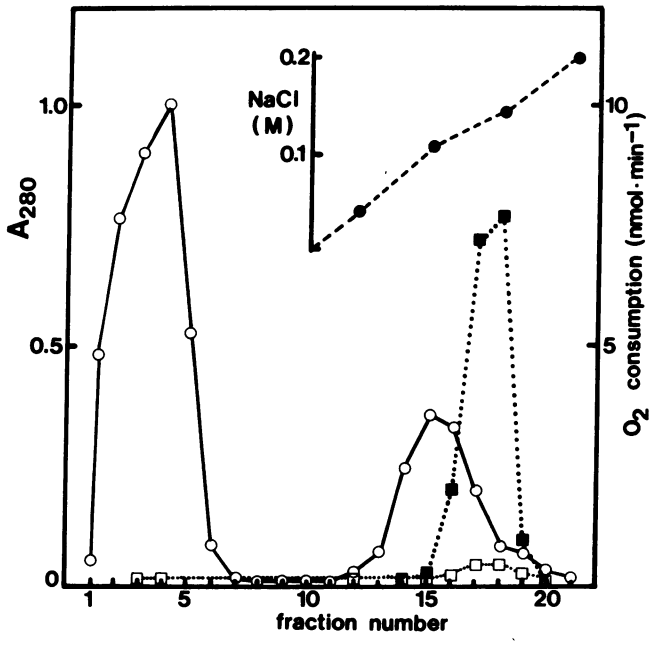

Figure 1. Fractionation of soluble fraction on carboxymethyl Sepharose. Soluble fraction (equivalent of 3×10^9 neutrophils) was dialyzed against elution buffer and applied to CM Sepharose equilibrated with elution buffer. A linear gradient of 0–200 mM NaCl (2 × 50 ml) was applied. Fractions of 8.8 ml were collected. Fractions were assayed for soluble oxidase-component activity with a membrane fraction (3.5×10^6 cell equivalents) as described in Methods. ○, light absorbance at 280 nm; ●, NaCl gradient; □, NADPH-dependent O_2 consumption of 75 μl of the fractions; ■, NADPH-dependent O_2 consumption of 75 μl of the fractions in the presence of 75 μl of fraction 4.

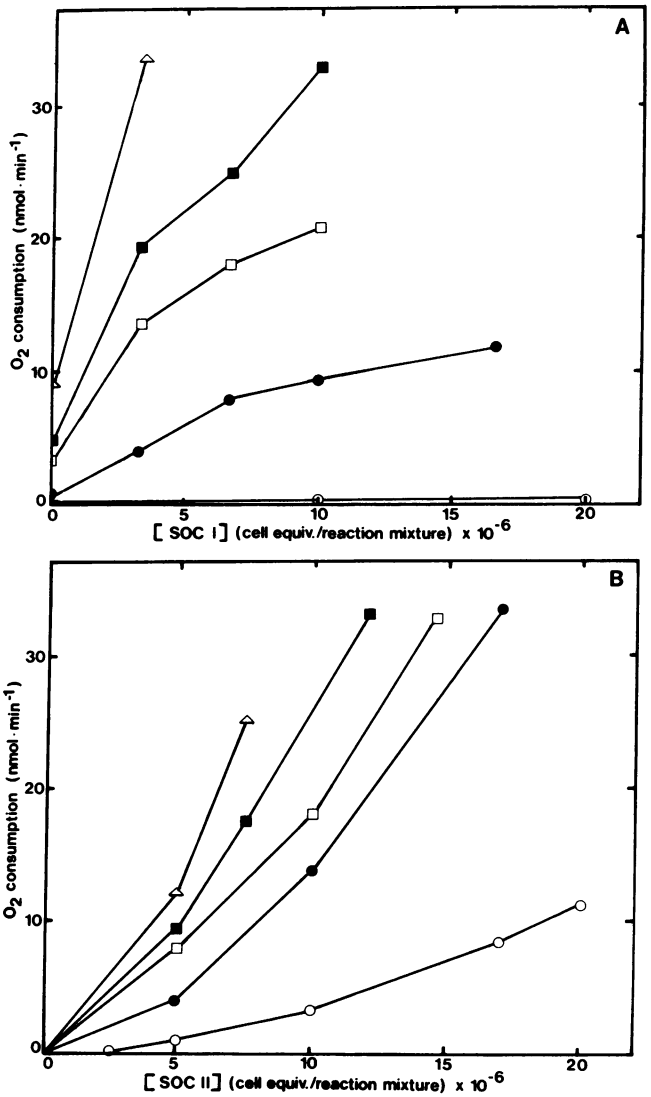

Figure 2. The effect of the concentration of SOC I and SOC II on the rate of O_2 consumption. O_2 consumption was measured with membranes from 4×10^6 cell equivalents as described in Methods in the presence of 10 μM GTP-γ-S. The amount of SOC II was recalculated to cell equivalents by the degree of dilution observed on the CM Sepharose column. (*A*) The effect of increasing concentrations of SOC I at various concentrations of SOC II. ○, no SOC II; ●, 5×10^6 cell equivalents of SOC II; □, 10^7 cell equivalents of SOC II; ■, 1.2×10^7 cell equivalents of SOC II; △, 1.7×10^7 cell equivalents of SOC II. (*B*) The effect of increasing concentrations of SOC II at various concentrations of SOC I. ○, no SOC I; ●, 3×10^6 cell equivalents of SOC I; □, 6×10^6 cell equivalents of SOC I; ■, 10^7 cell equivalents of SOC I; △, 1.7×10^7 cell equivalents of SOC I.

soluble components are required for oxidase activity, one that does not bind to CM Sepharose and one that does bind to CM Sepharose. For convenience we termed these components SOC I and II, respectively.

The inability of SOC I and II to support O_2 consumption when added separately could have been due to a quantitative effect. It has been shown that O_2^- production in a cell-free system shows a sigmoidal dependence on the amount of cytosol added (27). Therefore, titration experiments were performed. Fig. 2 *A* shows that SOC I alone failed to support O_2 consumption even at concentrations as high as 2×10^7 cell

equivalents. In contrast, SOC II did support some O_2 consumption without the presence of SOC I (Fig. 2 *B*). However, with 10^7 cell equivalents of SOC II in the reaction mixture, the rate of O_2 consumption was eightfold increased by the addition of 10^7 cell equivalents SOC I. The activity found with SOC II alone was mediated by the respiratory burst oxidase, because a similar experiment with membrane fractions from neutrophils of three X-linked cytochrome b_{558}-negative CGD patients, showed no O_2 consumption. This experiment clearly shows that at least two different soluble components act in concert for full activity.

Both components were found to lose activity upon heating during 10 min at 100°C, suggesting that both are proteins. When the fractions were stored for 2 wk at 4°C, 60% loss of activity was observed. However, when 10 μM GTP-γ-S was added to the assay mixture, the activity was restored to 95% of the initial value. The addition of GTP-γ-S to either SOC I or SOC II alone did not generate O_2 consumption. These results indicate that GTP is an essential factor in the NADPH oxidase system as reported previously (14, 15).

SDS-PAGE analysis of SOC I and SOC II after in vitro phosphorylation with purified protein kinase C. It has been described that a protein of 47 kD is involved in the activation mechanism of the NADPH:O_2 oxidoreductase of neutrophils and macrophages (5–7). Upon cell stimulation, this protein becomes phosphorylated. This phosphorylation is not observed in cells from patients suffering from the common autosomal form of CGD (5). Previously, we found this protein to be a substrate of protein kinase C in vitro (28). To investigate whether one of the soluble components contained this phosphoprotein, we assayed the CM Sepharose fractions for phosphoproteins by phosphorylation with purified protein kinase C in the presence of [^{32}P]γ-ATP. The autoradiograph of the SDS-PAGE gel is shown in Fig. 3 *A*. The fractions containing SOC I contain many phosphorylated proteins but no band is observed at the position of 47 kD. In contrast, it is clear that a 47-kD phosphoprotein coelutes with SOC-II activity. In four separate experiments this coelution of SOC-II activity and 47-kD phosphorylation was observed. Also, on size exclusion chromatography with Sephacryl S-300 after CM-Sepharose fractionation, SOC-II activity coeluted with the 47-kD phosphoprotein at a position that correlates with a M_r of ~ 50,000 (result not shown).

Fractionation of neutrophils from an autosomal CGD patient. It has been described that in neutrophils from autosomal CGD patients the phosphorylation of a 47-kD protein is missing (5). We tested this for five unrelated autosomal CGD patients, and found no phosphorylation at the 47-kD level in cell lysates derived from any of these patients (28).

We used the soluble fraction of neutrophils from one of these autosomal CGD patients as starting material on CM Sepharose in parallel to a control separation experiment. The elution profiles of the light absorbance at 280 nm were similar to the one shown for the control experiment in Fig. 1, but none of the collected fractions from the CGD patient cells showed oxidase activity in the presence of the flowthrough fraction derived from either the control experiment or patient's cells. In contrast, the SOC I fraction from the CGD cells was able to support O_2 consumption in the presence of the SOC II fraction from control cells (5.8 and 4.2 nmol O_2 consumed/min for the control SOC I and the patient SOC I, respectively, when tested with 10^6 cell equivalents).

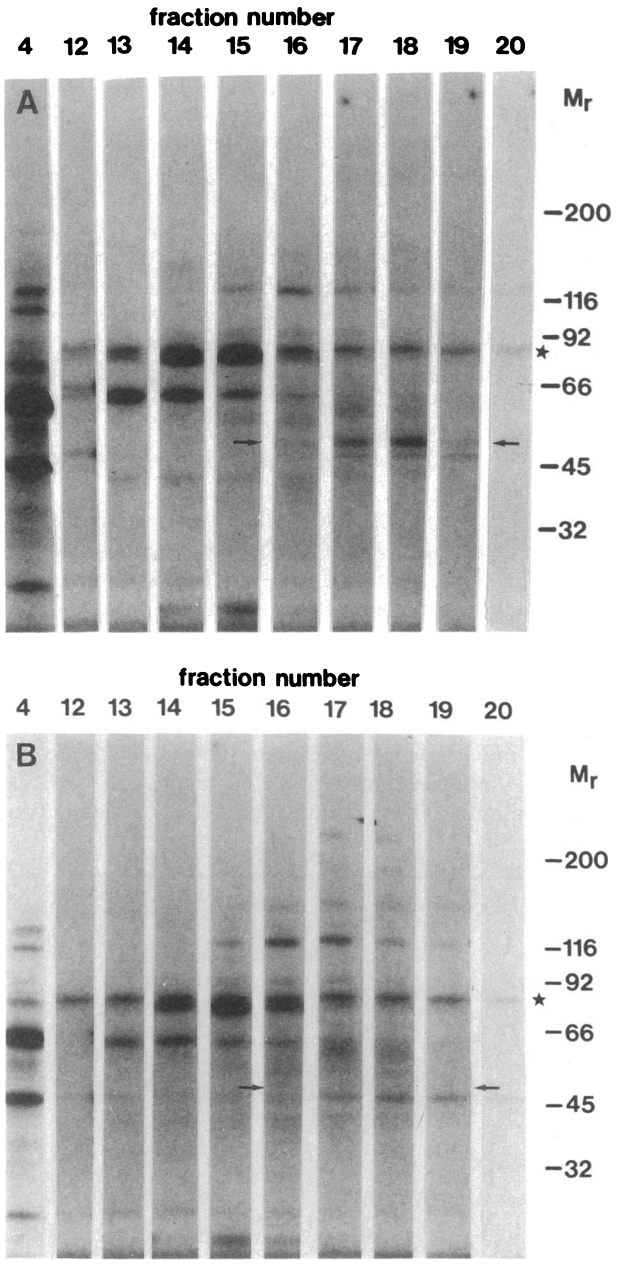

Figure 3. (*A*) Autoradiograph of SDS-PAGE gel of CM-Sepharose fractions. Samples of 50 μl from the CM Sepharose fractionation (Fig. 1) were phosphorylated in vitro with purified protein kinase C as described in Methods. SDS-PAGE was run in a 5–15% polyacrylamide gel. (*B*) Autoradiograph of SDS-PAGE gel of CM-Sepharose fractionation of the soluble fraction from autosomal CGD neutrophils. Soluble fraction of autosomal CGD neutrophils (120×10^6 cell equivalents) was separated on CM Sepharose in parallel to a control experiment as described in Fig. 1. A gradient of 0–200 mM NaCl (0–12.5 ml) was applied, and fractions of 2.2 ml were collected. 100 μl of the fractions was phosphorylated in vitro with protein kinase C as described in Methods. SDS-PAGE was run in a 5–15% polyacrylamide gel, followed by autoradiography of the gel. The numbers of the fractions correspond to the control experiment in Fig. 1 and Fig. 3 *A*. The arrows show the level of M_r 47,000. The asterisks indicate protein kinase C autophosphorylation.

The autoradiograph after phosphorylation of the collected patient fractions is shown in Fig. 3 *B*. The pattern of phosphoproteins was identical to the control experiment, except that in

the fractions normally containing SOC-II activity phosphorylation at the 47-kD level was absent. This result indicates that during the separation on the CM-Sepharose column no 47-kD phosphoprotein is generated from another protein unrelated to the respiratory burst. The phosphorylated band at 45 kD is, although to a lesser extent, also present in the control experiment.

To investigate further whether the 47-kD protein that coelutes with SOC-II activity is the relevant phosphoprotein for respiratory burst activity, we subjected a peak fraction of the CM-Sepharose chromatography to a two-dimensional gel electrophoresis analysis. It has been described by several groups (28, 29) that heterogeneity is observed in this protein with respect to the isoelectric point after phosphorylation. Three (29) and five (28) spots have been observed, ranging in pI from 6.6 to 8.2. Fig. 4 shows the pattern of phosphoproteins in a CM-Sepharose SOC II peak fraction analyzed on a two-dimensional gel. Three major phosphoproteins with M_r of 47,000 are observed (Fig. 4 *A*), which again are absent in the two-dimensional analysis of the corresponding autosomal CGD fraction (Fig. 4 *B*).

Complementation of cell fractions from patients with autosomal CGD. The experiment described in the previous section showed that only SOC II was defective in the neutrophils of an autosomal CGD patient. To test whether this is a general phenomenon, we obtained a soluble and a membrane fraction from the neutrophils of three patients, classified as autosomal CGD. (The amount of isolated neutrophils was too small to perform a CM Sepharose fractionation.) None of the soluble fractions of these patient cells supported NADPH-dependent oxygen consumption after reconstitution with a membrane fraction derived from control cells (Table I). When SOC I from control cells was added as well, there was still no detectable oxygen consumption in the reconstituted system of either of the three patients. However, when SOC II was added, all three soluble fractions from CGD neutrophils showed O_2 consumption comparable to the values found in the control experiment.

Complementation studies with soluble fractions from lymphocytes and thrombocytes. SOC activity has been reported to be absent in lymphocytes, placenta, brain, and liver (30). To investigate whether this accounts for both soluble components, we sonicated lymphocytes and thrombocytes and separated the sonicates in a soluble fraction and a membrane fraction. The soluble fraction was tested for NADPH oxidase activity with a membrane fraction from neutrophils. No oxygen consumption was detected when soluble fractions from lymphocytes or thrombocytes were used. Moreover, no oxygen consumption was generated by the addition of either SOC I or SOC II. In fact, the soluble fraction of both lymphocytes and thrombocytes had a slightly inhibitory effect on the activity of a reconstituted neutrophil system (consisting of SOC I, SOC II, and membranes).

It has been described that the NADPH oxidase is regulated by guanine nucleotides in neutrophils (14) and DMSO-induced HL-60 cells (15). Therefore, the experiments with fractionated lymphocytes and thrombocytes were repeated in the presence of 10 μM GTP-γ-S. However, similar results as described above were obtained. These results suggest that SOC I and II are specific for neutrophils and probably also for other cells expressing NADPH:O_2 oxidoreductase activity.

Table I. Effect of SOC I and SOC II on NADPH-dependent O_2 Consumption in a Reconstituted System of Soluble Fractions from Autosomal CGD Neutrophils and Normal Membranes

Source of soluble fraction	Addition	NADPH-dependent O_2 consumption
	2×10^6 cell eq.	*nmol O_2/min per 2×10^6 cell eq.*
Control neutrophils	None	8.8±0.7
Autosomal CGD neutrophils	None	0.1±0.1
Autosomal CGD neutrophils	SOC I	0.4±0.2
Autosomal CGD neutrophils	SOC II	7.7±0.8
None	SOC I	0.1±0.1
None	2 × SOC I	0.1±0.1
None	SOC II	0.4±0.2
None	2 × SOC II	0.9±0.2

NADPH:O_2 oxidoreductase was measured as the rate of O_2 consumption as described in Methods, in the presence of 10 μM GTP-γ-S. The reaction mixture contained membrane fraction from 2×10^6 control cells and soluble fraction from 2×10^6 control cells or autosomal CGD cells. The amount of added SOC I or SOC II was the equivalent of either 2 or 4×10^6 neutrophils. The data represent the mean±SD of three unrelated donors and three unrelated patients. When 2×10^6 cell equivalents of SOC I or SOC II were added to control membranes with control soluble fraction, the rate of O_2 consumption was 14.5±0.3 and 12.3±0.3 nmol O_2/min, respectively. This enhancement is caused by the deliberate limitation of the assay system by the amount of soluble components. The membrane fractions of the CGD patients showed normal values when reconstituted with 2×10^6 cell equivalents of control soluble fraction.

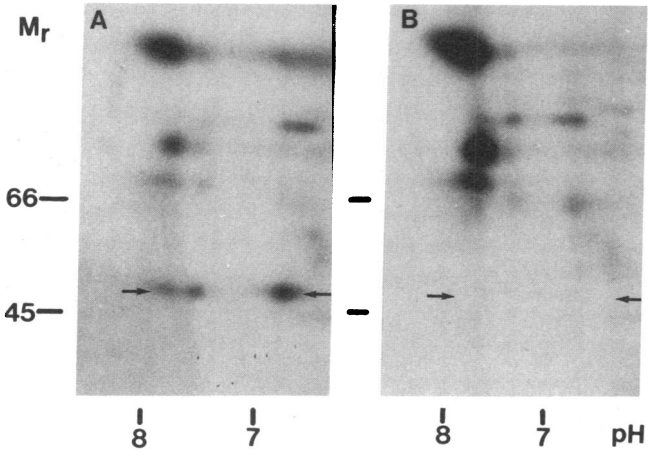

Figure 4. Autoradiograph of SOC II peak fractions from a CM-Sepharose fractionation,after in vitro phosphorylation with purified protein kinase C and two-dimensional gel electrophoresis. Samples were prepared and run as described in Methods. The arrows indicate the position of the 47-kD phosphoproteins. (*A*) Control SOC II fraction; (*B*) SOC II fraction from autosomal CGD cells.

Discussion

Since it has been reported that phagocyte NADPH:O_2 oxidoreductase can be stimulated in a cell-free system by arachidonate (10, 11) or dodecyl sulfate (12), the components necessary for activation of the enzyme and the enzyme itself can be

studied in a more direct way than in intact cells. Although it is not yet clear whether the cell-free system fully represents the pathway of activation in intact cells, all the components necessary for activity of the oxidase in intact cells are also required in the cell-free system. First, a membrane and a soluble fraction are necessary. Second, cells from patients suffering from CGD do not show O_2 consumption (this report) or O_2^- production (11, 13, 31) in the cell-free system. In the autosomal, cytochrome b_{558}-positive form of the disease, the defect is found in the soluble fraction (Table I and reference 31), whereas in the X-linked form and in the autosomal, cytochrome b_{558}-negative form the defect is membrane-bound (11, 13). Therefore, it is generally believed that the autosomal form is defective in the activation mechanism, whereas in the cytochrome *b*-negative forms the defect lies in the enzyme itself.

The cell-free system offers prospects to study separately the different components of the NADPH oxidase. We focused our attention on the soluble components of human neutrophils. By the use of CM Sepharose we have separated two soluble components both necessary for activity. For convenience we named these components SOC I and SOC II. We were able to show that these components act synergistically in the generation of the NADPH:O_2 oxidoreductase activity in a cell-free system. First, the difference in behavior on the cation-exchange chromatography column indicates a molecular difference between the two components. Second, SOC I was completely unable to support oxygen consumption in the absence of SOC II. In contrast, high levels of SOC II were able to generate low levels of oxygen consumption in the absence of SOC I. Presumably, this is due to contamination of the membrane fraction with SOC I. Third, the differences in shape of the titration curves of SOC I and SOC II (Fig. 2) indicates a difference in the way these two components act in the generation of the respiratory burst activity. The sigmoidal shape of the titration curves of SOC II (Fig. 2 *B*) suggests that SOC II acts in a stoichiometry of more than one with respect to SOC I and the membrane component(s). Alternatively, SOC II could be composed of two or more different components.

One of the components (SOC II) coelutes with the 47-kD protein that becomes phosphorylated upon stimulation of phagocytes from normal individuals, but not in those from patients with autosomal cytochrome b_{558}-positive CGD. Whether indeed phosphorylation of the 47-kD protein is required for activation of the respiratory burst in intact cells, is not quite established. It has been observed that inhibition of this phosphorylation does not inhibit the respiratory burst induced in intact neutrophils by FMLP (32, 33). However, in the cell-free system, phosphorylation does not play a role in the activation of the oxidase because ATP is not required (this paper and reference 13). The normal 47-kD protein (SOC II) can be phosphorylated in vitro (28). This 47-kD phosphorylation is not observed in corresponding CM Sepharose fractions of cytosol derived from cells from autosomal, cytochrome b_{558}-positive CGD patients. This result strongly suggests that the 47-kD protein itself is essential for O_2^- production, although the precise role of this protein remains to be elucidated.

In two-dimensional electrophoresis of stimulated neutrophils (29) or of cell lysates after in vitro phosphorylation (28) a heterogeneity is observed in the 47-kD protein with respect to its isoelectric point. This heterogeneity was also observed in a two-dimensional gel analysis of a SOC II peak fraction (Fig. 4). If this heterogeneity would also exist in the unphosphorylated protein, multiple peaks would be expected upon ion-exchange chromatography. However, we only observed a single peak of both SOC II activity and the 47-kD protein. Most likely therefore, the different isoelectric points of this protein observed after neutrophil stimulation or in vitro phosphorylation, reflect different degrees of phosphorylation of a single protein.

The identity of the other component (SOC I) is not yet clear. Probably, it is a protein (or proteins), because boiling destroys its activity. Moreover, we showed that this component is not present in lymphocytes and thrombocytes. In contrast to SOC II, SOC I is normal in cells from the autosomal, cytochrome b_{558}-positive CGD patients we tested. However, it is possible that a variant form of CGD exists in which this component is absent or defective. Nunoi et al. have studied complementation studies with autosomal, cytochrome b_{558}-positive CGD patients in the cell-free system (34). They have observed that the neutrophil cytosol of one autosomal, cytochrome b_{558}-positive CGD patient could support O_2^- production when mixed with the cytosol derived from other autosomal CGD patients. We are currently screening CGD patients for a defect in SOC I activity.

We observed, as did other investigators (14, 15), that GTP is essential for activation of the NADPH:O_2 oxidoreductase in vitro. When we stored our active fractions for several days at 4°C, NADPH-oxidase activity was diminished. However, addition of GTP-γ-S completely restored the activity. This indicates that GTP is present in the supernatant fraction but is hydrolyzed in solution during storage. It is conceivable that one of the oxidase components is a G protein. We are testing this hypothesis at present.

Our finding of two soluble components is in conflict with reports by Curnutte et al. (13) and Gabig et al. (14) who found soluble oxidase-component activity in single fractions after gel permeation chromatography. Both investigators found multiple peaks of activity (two in reference 13 and three in reference 14). However, recovery of activity in the cytosol fractions was low (10–30% in reference 13). It is conceivable that during gel permeation, complexes of SOC I and SOC II of different stoichiometry are separated, which does not occur during ion exchange chromatography. Alternatively, the membrane fractions used in references 13 and 14 might be contaminated with either SOC I or SOC II or both. We, too, have observed small activities elicited by SOC II alone. Possibly, in vitro phosphorylation, with purified protein kinase C, could show the presence of the 47-kD protein in these active fractions after gel filtration.

The synergism of two, possibly three, soluble components from pig neutrophils was reported by Fujita et al. (35). On gel permeation chromatography these components eluted at positions of M_r 300,000 and 50,000. Although we have no indications about the molecular weight of SOC I, this result suggests that the M_r 50,000 component in reference 34 correlates with SOC II (the 47-kD phosphoprotein), because SOC II elutes around M_r 50,000 on size exclusion chromatography. This would indicate that the 47-kD protein exists as a monomer.

Acknowledgments

We like to thank Dr. R. L. Van der Bend of the Netherlands Cancer Institute in Amsterdam for his help in the two-dimensional gel electrophoresis experiments.

This study was supported by a grant from the Foundation for Medical Research (MEDIGON), which is subsidized by the Netherlands Organization for the Advancement of Pure Research (ZWO) (grant 900-503-091).

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