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Articles

Purification and Characterization of an Oxidase Activating Factor of 63 Kilodaltons from Bovine Neutrophils†

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ABSTRACT: A 63-kDa protein, which behaves as an oxidase activating factor in bovine neutrophils, has been purified to electrophoretic homogeneity. The protein was isolated from the cytosol of resting bovine neutrophils after several steps, including ammonium sulfate precipitation and chromatography on AcA44, DE-52 cellulose, Mono Q, and Superose 12 in the presence of dithiothreitol. The oxidase activating potency of the protein was assayed with a cell-free system consisting of neutrophil membranes, GTPγS, arachidonic acid, and a complementary cytosolic fraction. The purification factor was 200 and the yield 3%. During the course of gel filtration on calibrated Superose 12, the 63-kDa protein eluted as a dimer. Its isoelectric point was 6.4 ± 0.1. Antibodies raised in rabbits against the 63-kDa protein reacted with a protein of similar size in human neutrophils and in HL60 promyelocytic cells induced to differentiate into granulocytes. No immune reaction was observed in cytosol from undifferentiated HL60 cells, in extracts from bovine skeletal muscle, liver, and brain, or in cytosol prepared from neutrophils derived from a patient with an autosomal cytochrome *b* positive form of chronic granulomatous disease lacking the 67-kDa oxidase activating factor. Immunoblotting with the 63-kDa bovine protein antiserum demonstrated that activation of bovine neutrophil oxidase by phorbol myristate acetate induced the translocation of the 63-kDa protein from cytosol to the membrane.

Resting neutrophils do not generate significant amounts of the superoxide anion $O_2^{\cdot -}$. However, when they are challenged by appropriate soluble or particulate stimuli, their $O_2^{\cdot -}$ production is markedly enhanced. This abrupt enhancement in $O_2^{\cdot -}$ generation, known as the respiratory burst, together with degranulation, contributes to the microbicidal function of neutrophils during phagocytosis. The respiratory burst is catalyzed by an activated plasma membrane-bound NADPH oxidase, which is believed to consist of an NADPH-dependent flavoprotein and a low-potential *b*-type cytochrome [for reviews, see Rossi (1986), Bellavite (1988), and Segal (1990)]. The molecular approach to the mechanism of oxidase activation has largely benefited from the use of a cell-free system

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of oxidase activation originally developed with guinea pig macrophages (Bromberg & Pick, 1984) and horse neutrophils (Heyneman & Vercauteren, 1984). This system has been improved and extended to phagocytes of different species (Curnutte, 1985; Mc Phail et al., 1985; Clark et al., 1987; Gabig et al., 1987; Seifert & Schultz, 1987; Ligeti et al., 1988; Tanaka et al., 1988). Its components are a membrane fraction enriched in plasma membrane, a cell homogenate supernatant called cytosol, a long-chain unsaturated fatty acid, and GTP or GTPγS. Cytosol contains activating factors which are translocated to the membrane during the course of oxidase activation (Doussière et al., 1988; Ligeti et al., 1989). The use of the cell-free system combined with the identification of molecular defects responsible for lack of oxidase activation in patients with the autosomal recessive form of chronic granulomatous disease (CGD)[1] (Curnutte et al., 1985, 1988; Caldwell et al., 1988; Kramer et al., 1988; Nunoi et al., 1988; Volpp et al., 1988, 1989; Lomax et al., 1989) has led to the demonstration that at least two soluble activating proteins of ~67 and ~47 kDa termed cytosolic factors are required for oxidase activation to occur. In the case of bovine neutrophils, using a cytosol preparation in which proteins were randomly derivatized by [^{14}C]phenyl isothiocyanate in such a way that the activating potency was only partially altered, it was found that oxidase activation was accompanied by the transfer to the membrane of a limited number of water-soluble labeled proteins, including protein species with molecular masses equal or close to those mentioned above (Doussière et al., 1990). In the present paper, we report the purification and characterization of a 63-kDa cytosolic protein competent in oxidase activation in a cell-free system and probably equivalent to the 67-kDa human cytosolic factor recently cloned (Leto et al., 1990). The bovine neutrophil 63-kDa cytosolic factor of oxidase activation was specific of phagocytic cells, and its translocation to the membrane depended on the activation of the oxidase complex.

Materials and Methods

Materials. Arachidonic acid, ferricytochrome *c* (horse heart, grade VI), dibutyryl cyclic AMP (AMPc), diisopropyl fluorophosphate (DFP), EDTA, EGTA, dithiothreitol (DTT), and PMSF were purchased from Sigma; NADPH, superoxide dismutase, GTPγS, and leupeptin from Boehringer; DEAE-(DE-52) cellulose from Whatman (U.K.); ^{125}I-labeled protein A and colored molecular weight markers from Amersham; Percoll, Mono Q column (HR 5/5), Mono S column (HR 5/5), and molecular weight markers from Pharmacia; Ultrogel AcA44 from IBF; bicinchoninic acid (BCA reagent) from Pierce. Arachidonic acid was dissolved in absolute ethanol and stored at −80 °C under N_2 until used. Phosphate-buffered saline (PBS), used to suspend neutrophils, consisted of 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, and 1.5 mM KH_2PO_4, pH 7.4.

Biological Preparations. Human neutrophils and bovine neutrophils were prepared as described (Morel et al., 1985; Doussière & Vignais, 1985). HL60 promyelocytic cells were grown in suspension culture in RPMI 1640 medium (Boehringer) supplemented with 10% heat-inactivated fetal calf serum, 100 μM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere of 90% air and 10% CO_2. The cells were subcultured every 4–5 days. They were induced to differentiate into granulocytes by growth in the presence of 500 μM dibutyryl-AMPc for 3 days. Microscopic examination of differentiated HL60 cells stained with the May–Grunwald–Giemsa mixture revealed between 70 and 80% of mature cells. Cytosols from neutrophils and HL60 cells were obtained by high-speed centrifugation of homogenate preparations obtained by sonication for 4 × 15 s at 40-W output at 2–4 °C. The homogenates were centrifuged at 10000*g* for 10 min. The supernatants were resolved into a membrane fraction and a high-speed supernatant referred to as cytosol by centrifugation at 140000*g* for 1 h. Human neutrophil cytosol from two patients with autosomal cytochrome *b* positive CGD was provided by Drs. D. Roos and A. J. Verhoeven (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam). The cytosol of one patient was deficient in the 67-kDa factor, and that of the other was deficient in the 47-kDa factor. The identification of the defect was made by Western blot with the B1 antiserum raised by Volpp et al. (1988). The 67-kDa protein deficiency was diagnosed in the Department of Dr. R. Seger (Children's Hospital, Zurich) and the 47-kDa protein deficiency in the Department of Dr. R. S. Weening (Department of Pediatrics, Academic Medical Center, Amsterdam).

Cytosols from bovine skeletal muscle, brain, and liver were obtained as follows: 10 g of tissue in 100 mL of PBS was homogenized in a Potter–Elvejhem apparatus, and the homogenates were subjected to differential centrifugation in order to obtain cytosol and membrane fractions.

Measurement of Protein Concentration. Protein was determined with the BCA reagent from Pierce. Absorbance was read at 562 nm after 30 min of incubation with the reagent at 37 °C, using bovine serum albumin as the standard. The protein content of purified cytosolic fractions was assayed by the method of Bradford (1976).

Electrophoresis. SDS–PAGE was carried out by the method of Laemmli and Favre (1973), with a 4% stacking gel and a 12% resolving gel. The gels were stained with Coomassie Blue. Two-dimensional gel electrophoresis was performed by the method of O'Farrell (1975) with slight modifications (Stasia et al., 1990). The first-dimension isoelectric focusing gel consisted of 5% acrylamide supplemented with 8.4 M urea and 2% ampholines (pH range 4–8). The gel was prefocused for 1 h before the protein sample was loaded. The sample was diluted with an equal volume of lysis buffer consisting of 6.7 M urea, 1.5% Triton X-100, and 3% mercaptoethanol. Focusing was allowed to proceed for 2 h at 1500 V. The gel was then equilibrated in 2% SDS, 0.06 M Tris-HCl, pH 7.0, 5% mercaptoethanol, and 15% glycerol for migration in the second dimension by SDS–PAGE with a separating gel of 12% acrylamide and a stacking gel of 4% acrylamide. Molecular weight standards were run in a well that was fashioned on one side of the second-dimension gel. The gel was stained with silver nitrate (Wray et al., 1981). For determination of the isoelectric point (p*I*), only the first dimension was used. the pH gradient was determined by cutting the 1-D gel into 5-mm slices. Each slice was soaked in degassed and deionized water, and the pH was measured.

Activation of the Membrane-Bound Oxidase in a Cell-Free System. The bovine neutrophil cell-free system also referred to as the reconstituted oxidase system was described by Ligeti et al. (1988) and was used with some modifications. It con-

[1] Abbreviations: PBS, phosphate saline buffer; PBS–T, PBS supplemented with 0.05% Tween 20; CGD, chronic granulomatous disease; PBS–ED, PBS supplemented with 1 mM EGTA and 200 μM DTT; Tris–ED, Tris supplemented with 1 mM EGTA and 200 μM DTT; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PMA, phorbol myristate acetate; PMSF, phenylmethanesulfonyl fluoride; DFP, diisopropyl fluorophosphate; DTT, dithiothreitol; NBT, nitroblue tetrazolium; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PMN, polymorphonuclear leukocytes or neutrophils.

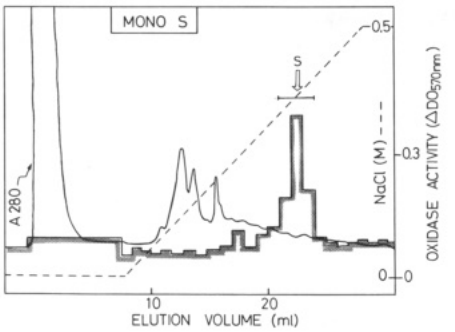

FIGURE 1: Preparation of a cytosolic fraction containing factor(s) complementary to the 63-kDa cytosolic factor. One milliliter of cytosol, corresponding to 10–15 mg of protein, from resting bovine neutrophils (cf. Materials and Methods) was diluted 2-fold in 50 mM Hepes buffer and injected on a 1-mL Mono S HR 5/5 column equilibrated with the same buffer. The pass-through fraction was recovered. Then a linear 0–0.5 M NaCl gradient in Hepes buffer was applied to the column, and 1-mL fractions were collected. The oxidase activating potency of the eluates was tested on 25-μL aliquots as described under Materials and Methods in the presence of 50 μL of the pass-through fraction. Three active fractions detected by reduction of NBT were eluted by NaCl at concentrations ranging between 240 and 360 mM and pooled (pool S indicated by an arrow). An aliquot (routinely 50 μL) was used to complement the 63-kDa protein during the course of its purification. In the absence of the pass-through fraction, the oxidase activating potency of pool S was approximately 20% of that determined in the presence of the pass-through fraction.

sisted of 30–100 μg of membrane protein, 100–150 μg of cytosolic protein, 10 μM GTPγS, 10 mM $MgSO_4$, and arachidonic acid in a final volume of 100–200 μL of PBS. The added amount of arachidonic acid was adjusted with respect to the amount of membrane and cytosolic proteins and also to the ionic strength of the medium to provide optimal conditions for oxidase activation (Pilloud et al., 1989a). The incubation was for 10 min at room temperature, followed by addition to the photometric cuvette for the assay of the elicited oxidase activity.

Activation of the Membrane-Bound Oxidase in Intact Neutrophils. Bovine neutrophils were suspended at a concentration of 10^8 cells/mL in PBS supplemented with 20 mM glucose. The suspension was brought to 37 °C by a 2-min incubation in a water bath. PMA in DMSO (2 mg/mL) was added at a concentration of 50 ng/10^9 cells, corresponding to 5 ng/mL, and incubation was allowed to proceed for 5 min at 37 °C. Neutrophils were diluted 10-fold with cold PBS and washed twice to remove PMA. The cells were disrupted by sonication for 4 × 15 s at 40-W output, at 2–4 °C. A membrane fraction and a high-speed supernatant referred to as cytosol were prepared by differential centrifugation of the homogenate.

Assay of the Oxidative Activating Potency of Purified Cytosolic Factors. As several cytosolic factors participate in a synergistic manner to the activation of the resting oxidase in the cell-free system and are ineffective when separated (Bolscher et al., 1988; Nunoi et al., 1988; Volpp et al., 1988; Pilloud et al., 1989b; Doussière et al., 1990), the activity of a given cytosolic factor in the course of its purification had to be followed in the presence of the complementary factor(s). The activating potency of the 63-kDa factor whose purification is described thereafter was assayed in the presence of a cytosolic fraction, referred to as fraction S, which contained factor(s) complementary to the 63-kDa protein (Figure 1). The S fraction per se had only a small oxidase activating potency which was deducted from the oxidase activity elicited by the combined effects of the 63-kDa protein and fraction S.

For a rapid assessment of the oxidase activity in fractions purified from bovine neutrophil cytosol, a rapid screening assay based on reduction of nitroblue tetrazolium (NBT) was used. In a preincubation step corresponding to oxidase activation, aliquots of chromatographic fractions were mixed with PBS containing 60 μg of membrane protein, 10 mM $MgSO_4$, 10 μM GTPγS, an optimal amount of arachidonic acid ranging between 60 and 80 nmol, and 50 μL of the complementary S fraction obtained as described in Figure 1, the final volume being 150 mL. After a 10-min incubation at room temperature, the elicited oxidase activity was determined. One milliliter of PBS containing 2.5 mM $MgSO_4$, 200 μM NADPH, and 200 μM NBT was added. After 5 min of incubation at room temperature, the reaction was stopped by addition of 100 μL of 10% SDS, and the absorbance was read at 570 nm. Active eluates were pooled, and their oxidase activating potency was accurately measured by assessment of the elicited oxidase activity by the superoxide dismutase inhibitable reduction of cytochrome *c*. After a 10-min preincubation during which the membrane-bound oxidase was activated in the presence of the cytosolic fractions as described above, the elicited oxidase was assayed by using a medium consisting of 2 mL of PBS supplemented with 100 μM ferricytochrome *c*, 2.5 mM $MgSO_4$, and 100 μM NADPH in the absence and presence of SOD. Reduction of ferricytochrome *c* was measured spectrophotometrically at 550 nm. When membranes from PMA-activated neutrophils were used to generate $O_2^{\bullet-}$ deoxycholate was added at the concentration of 0.05% in the photometric cuvette to permeabilize the membrane vesicles. In the case of intact neutrophils, 20 mM glucose was used as a substrate instead of NADPH.

Rabbit Immunization. Before immunization, serum was collected from rabbits and used as control. Polyclonal antibodies against the bovine neutrophil 63-kDa protein were raised as follows. The purified cytosolic protein factor (50 μg in 500 μL of PBS) was mixed with complete Freund's adjuvant and injected subcutaneously in the neck region of a rabbit. At 3-2 week intervals, second and third injections were made with the purified protein emulsified in incomplete Freund's adjuvant. Ten days later, blood was collected from an ear vein. The immune serum was assayed for specific antibodies by comparison with the preimmune serum collected prior to the immunization. The ability of the antiserum to react with the 63-kDa protein was tested by ELISA, using microtitration polystyrene plates. The plate was coated with the purified protein at a concentration of 2 μg/mL. After reaction with diluted antiserum for 3 h at room temperature, and washing with PBS supplemented with 0.05% Tween 20 (PBS–T), the antibody–antigen complex was incubated for 2 h in the presence of peroxidase-conjugated A protein diluted 2000-fold with PBS–T supplemented with 3% BSA. The bound peroxidase was detected with 3,3′,5,5′-tetramethylbenzidine diluted 100-fold with 0.1 M sodium acetate adjusted to pH 6.0 with citric acid, and supplemented with 0.015% H_2O_2. The yellow color obtained after addition of H_2SO_4 was quantified at 450 nm with an automatic reader (Titertek Multiskan Plus, Flow Laboratories). The two rabbit antisera assayed by ELISA gave maximal responses at a dilution higher than 1000-fold.

Western Immunoblotting. After SDS–PAGE of cytosolic proteins, the gels were washed with the transfer medium consisting of the Laemmli electrophoresis buffer supplemented with 20% methanol. The protein bands were transferred electrophoretically to nitrocellulose sheets (Towbin et al., 1979). Colored molecular weight markers were used and could

be visualized on nitrocellulose after transfer. The nitrocellulose sheet was saturated for 1 h at room temperature with 3% BSA in PBS and 0.05% Tween 20 (PBS–T). This was followed by incubation overnight at 4 °C with the antiserum diluted 500-fold in the same buffer. After being washed once with the saturation buffer and twice with PBS–T, the nitrocellulose sheet was placed for 3 h at room temperature in contact with the ^{125}I-labeled protein A diluted 500-fold in the saturation buffer. The nitrocellulose sheet was washed twice with PBS–T and finally twice with PBS. The sheet was dried and then exposed to a Fuji RX film.

Immunoprecipitation of the Cytosolic Factor by the Staphylococcus aureus–Antibody Complex. The antiserum raised against the bovine neutrophil 63-kDa factor was incubated for 1 h at room temperature with a 10% suspension of *Staphylococcus aureus* previously washed with PBS and 0.01% Triton X-100. The suspension of the *S. aureus* coated with antibodies was washed 3 times with PBS. Increasing amounts of this suspension were allowed to react with a fixed amount of cytosol (500 μg) for 2 h at 4 °C to allow binding equilibrium of the 63-kDa cytosolic factor present in neutrophil cytosol with the *S. aureus*–immunoglobulin complex. The suspension was centrifuged, and the oxidase activating potency remaining in the supernatant was determined. A parallel assay was performed with preimmune serum.

Purification of a Cytosolic Factor of Oxidase Activation. Routine preparations were carried out at 2–4 °C with 5×10^9 to 1×10^{10} resting neutrophils obtained from 10 L of fresh bovine blood. The neutrophils were suspended in 35–40 mL of PBS supplemented with 1 mM DFP, 1 mM PMSF, 1 mM EDTA, 1 mM EGTA, and 20 μM leupeptin, as anti-proteases. They were disrupted by sonication for 4 × 15 s at 40-W output at 2–4 °C in a Branson sonifier. The nondisrupted cells, the lysosomal granules, and nuclei were sedimented by a 10-min centrifugation at 10000*g* in a Sorvall rotor. The supernatant was fractionated by centrifugation in a Beckman 40 rotor at 140000*g* for 60 min into a membrane fraction enriched in plasma membrane (Morel et al., 1985) and a supernatant. The pellet containing the membranes was resuspended by gentle sonication in PBS supplemented with 1 mM EGTA to give a final concentration of 5–10 mg of protein. The supernatant was further spun in a Beckman 60 Ti rotor at 300000*g* for 60 min, to discard any membranous particles. It was referred to as cytosol and contained 10–15 mg of protein/mL. The membrane suspension was distributed in 1-mL fractions, which were frozen at −80 °C. These membrane fractions were still efficient in reconstitution assays after 1 month of storage. Crude cytosol could be directly used for purification of cytosolic factors, or kept at 4 °C for 2 or 3 days, or frozen for several weeks, without noticeable loss of activity.

The purification procedure comprised a fractionation by ammonium sulfate and four chromatographic steps. All operations were performed at 2–4 °C. To the cytosolic fraction (in routine operations 30 mL containing about 500 mg of protein) was added powdered ammonium sulfate with gentle stirring to 60% saturation. The suspension was stirred for 4–5 h and the precipitate collected by centrifugation at 20000*g* for 15 min. The pellet was dissolved in 10 mL of PBS supplemented with 1 mM EGTA and 200 μM DTT (PBS–ED). Any insoluble residue was removed by a 10-min centrifugation at 20000*g*. The solubilized protein was applied to an AcA44 column (100 × 2.6 cm) equilibrated with PBS–ED. The column was eluted with the same buffer at the rate of 40 mL/h. Ten-milliliter fractions were recovered. Aliquots of 25 μL were withdrawn to screen the eluted oxidase activating potency by the NBT assay method mentioned above. Dextran blue was used to determine the flow-through volume of the column. Cytochrome *c*, ovalbumin, and bovine serum albumin were used to calibrate the column. Protein elution was followed by monitoring the absorbance at 280 nm. Two peaks of oxidase activating potency were obtained: a large one with an apparent molecular mass of 120 kDa and a small one of 60–65 kDa. Seven fractions corresponding to the large peak of activity were combined to give a volume of 70 mL, which was brought to 100 mL by addition of 10 mM Tris, pH 7.5, 1 mM EGTA, and 200 μM DTT (Tris–ED) to decrease the salt concentration.

The pool of fractions was further purified by fractionation on a DE-52 cellulose column (15 cm × 1 cm) equilibrated with Tris–ED. The flow-through fraction and that obtained by washing the column with 20 mL of Tris–ED were collected and concentrated by ultrafiltration on an Amicon PM10 membrane to a volume of 10 mL with slow stirring. A linear 75–250 mM gradient of NaCl made in the Tris–ED buffer was then applied to the column, and 5-mL fractions were collected. Two parallel assays of oxidase activating potency were run, using the reconstitution medium described above, with 25-μL aliquots withdrawn from the eluates. One of the assays was run in the presence of a complementary fraction referred to as fraction S, issued from chromatography of crude cytosol on Mono S, to be described below. The other assay was performed without complementation. Only in the presence of the S fraction (which per se showed a relatively low activity) was an oxidase activating potency peak revealed, corresponding to fractions eluted between 125 and 150 mM NaCl. As will be discussed, this was due to the fact that the cytosolic factor eluted from the DE-52 cellulose column was freed of complementary factor(s) and to the synergistic effect of complementary factor(s) present in the S fraction on oxidase activation.

The next step was Mono Q chromatography of the bioactive fractions recovered from the DE-52 cellulose column. A 1-mL Mono Q HR 5/5 column was equilibrated with Tris–ED, pH 7.5. The flow-through fractions, as well as the fractions eluted by a 150 mM NaCl wash, were pooled separately and concentrated by ultrafiltration on a PM10 Amicon membrane. They exhibited no oxidase activating potency, either alone or recombined with the S fraction. The column was then eluted with a linear NaCl gradient (150–225 mM) made in Tris–ED, pH 7.5. Five hundred microliter fractions were collected, from which 5-μL aliquots were withdrawn to test the oxidase activating potency in the presence of the reconstitution medium and 50 μL of fraction S, as described above. In parallel, SDS–PAGE was performed on 25-μL aliquots of the eluates. The bioactive fractions contained predominantly a 63-kDa protein, which coeluted with a 55-kDa protein in fractions 34–39 and with a 40-kDa protein in fractions 40–45. Fractions 34–39 and 40–45 were combined in two pools termed MQ1 and MQ2, respectively. The MQ pools were concentrated to a final volume of 0.2 mL by ultrafiltration on a YM10 Amicon membrane, and applied separately to FPLC Superose 12 gel filtration columns, equilibrated with PBS–ED flowing at 0.25 mL/min. Proteins were eluted in 0.2-mL fractions, 5 μL of which was used for determination of the oxidase activating potency in the presence of 50 μL of the S fraction, as described above. Twenty-microliter aliquots were used for SDS–PAGE.

Preparation of the Complementary S Fraction. Crude cytosol (10 mg of protein in 1 mL of PBS) was applied to a 1-mL column of Mono S HR 5/5 equilibrated with 50 mM Hepes, pH 7.5. Bound proteins were eluted in 1-mL fractions

Table I: Purification of the 63-kDa Cytosolic Factor

step	fraction	vol (mL)	protein (mg)	elicited oxidase[a] act. (total) (nmol of $O_2^{\cdot-}$/min)	elicited sp oxidase act. (nmol of $O_2^{\cdot-}$/min^{-1} mg^{-1})	purification (x-fold)
I	cytosol	40	532	44000	83	
II	$(NH_4)_2SO_4$ ppt	10	306	40000	131	1.6
III	AcA44	55	53	23000	434	5.2
IV	DE-52 cellulose	10	16	8600 (2400)	537	6.5
V	Mono Q (1)	1.5	0.25	2500 (400)	10000	120
	Mono Q (2)	1.5	0.25	3680 (480)	14720	177
VI	Superose 12 (1)	0.6	0.10	1200 (80)	12000	144
	Superose 12 (2)	0.6	0.0065	1250 (130)	19230	231

[a] In the first three steps, addition of the S fraction containing factor(s) complementary to the 63-kDa protein (cf. Materials and Methods) did not influence the elicited oxidase activity of the reconstituted system. In the last two steps, addition of fraction S was required to complement the purified 63-kDa protein. The values of elicited oxidase activities given in the table were corrected for the oxidase activity elicited in the presence of the S fraction added alone. Values in parentheses are those found in the absence of the complementary fraction S.

with a linear gradient of NaCl increasing from 0 to 0.5 M in 50 mM Hepes, pH 7.5. Fractions eluted with 0.24–0.36 M NaCl contained factor(s) complementing the cytosolic factor to be purified (see above).

Results

Purification of a 63-kDa Oxidase Activating Cytosolic Factor from Resting Bovine Neutrophil Cytosol. The purification of the 63-kDa cytosolic factor (cf. Materials and Methods) required optimized conditions for the assay of the elicited oxidase activity, in particular an optimal amount of arachidonic acid with respect to membrane and cytosolic proteins (Pilloud et al., 1989a; Doussière et al., 1990), and appropriate protection against adverse effects inherent in the possible presence of lysosomal enzymes in the medium, at least during the first steps of the purification.

Oxidase activation in the cell-free system requires several cytosolic factors that interact synergically. In other words, a cytosolic factor in its purified form cannot promote oxidase activation per se, unless the medium is supplemented with the complementary factor(s). The 63-kDa protein whose purification is described in this paper required, for expression of its oxidase activating potency, complementary factor(s) present in a fraction obtained by Mono S chromatography of crude cytosol and designated as fraction S. In the experiment illustrated in Figure 1, crude cytosol in PBS was fractionated by Mono S chromatography, using a linear NaCl gradient in 50 mM Hepes, pH 7.5. The fraction eluted with 0.24–0.36 M NaCl exhibited per se a small oxidase activating potency. However, when combined with the purified 63-kDa cytosolic factor, marked oxidase activating potency was evoked.

All fractionation operations were performed at 2–4 °C since the 63-kDa factor in crude cytosol suffered at room temperature a significant loss of activity amounting to 30–40% in 5–6 h. A mixture of anti-proteases consisting of DFP, PMSF, EDTA, EGTA, and leupeptin was systematically added (cf. Materials and Methods) to the homogenization medium prior to disruption of neutrophils by sonication. In the subsequent steps of the purification procedure, only EGTA was maintained at the final concentration of 1 mM. Addition of DTT further stabilized the 63-kDa factor. All elutions of chromatographic columns were therefore performed with solutions supplemented with 200 μM DTT. Due to the small size of the samples withdrawn for $O_2^{\cdot-}$ assay, interference of residual DTT with the reduction of NBT or cytochrome c in the $O_2^{\cdot-}$ assay was negligible.

The successive steps of the purification of the 63-kDa cytosolic factor from bovine neutrophil cytosol are documented in Table I. From 532 mg of cytosolic protein, 65 μg of a homogeneous protein was recovered. The purification factor

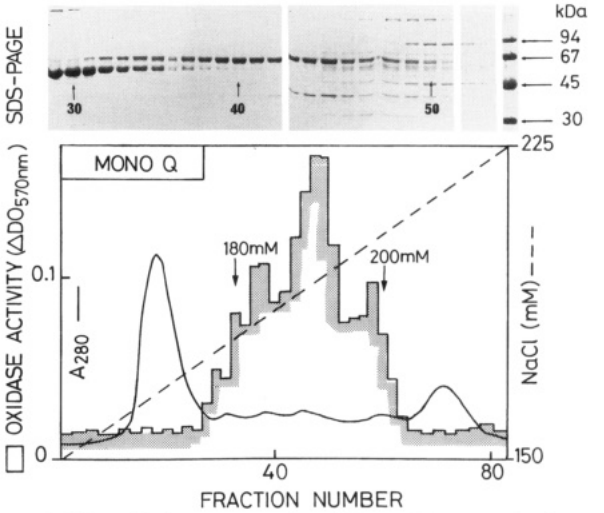

FIGURE 2: Mono Q chromatography of the 63-kDa cytosolic factor. The active fraction issued from DE52 chromatography (see Materials and Methods) was loaded on the Mono Q column equilibrated in 10 mM Tris-HCl, pH 7.5, 1 mM EGTA, and 200 μM DTT (Tris–ED). The column was perfused with 150 mM NaCl in Tris–ED. Then a linear 150–225 mM NaCl gradient in Tris–ED was applied. Fractions of 0.5 mL were collected, from which 5- and 25-μL samples were withdrawn for assay of the oxidase activating potency and SDS–PAGE, respectively. The active fractions eluted between 180 and 200 mM NaCl contained a predominant protein of 63 kDa. Fractions 34–39 and 40–45 were assembled to form pools MQ1 and MQ2, respectively.

was close to 200 and the yield about 3%. During the first two steps corresponding to ammonium sulfate precipitation and AcA44 chromatography, the oxidase activating potency was not substantially enhanced upon addition of fraction S, whereas in the subsequent steps, fraction S was required for elicitation of oxidase activity. This might be explained by the fact that during the initial steps of the purification sequence, the active peak contained all cytosolic factors probably arranged in a complex, whereas at a later stage cytosolic factors were resolved in separate entities. In all cases, the small activity brought by the S fraction was deduced from the elicited oxidase activity observed in the complete system. Correlation of the extent of oxidase activating potency of the eluted fractions from Mono Q and Superose 12 with the presence of a 63-kDa protein in the eluates as assessed by SDS–PAGE is illustrated in Figures 2 and 3.

Chemical and Immunological Properties of the 63-kDa Cytosolic Factor. On gel filtration with Superose 12, the single active peak revealed by the recombination assay with fraction S corresponded to a molecular mass of 120 kDa by reference to standard proteins (Figure 3). However, a molecular mass of 63 kDa was determined by SDS–PAGE of this fraction, using either 8% or 12% acrylamide (not shown). This sug-

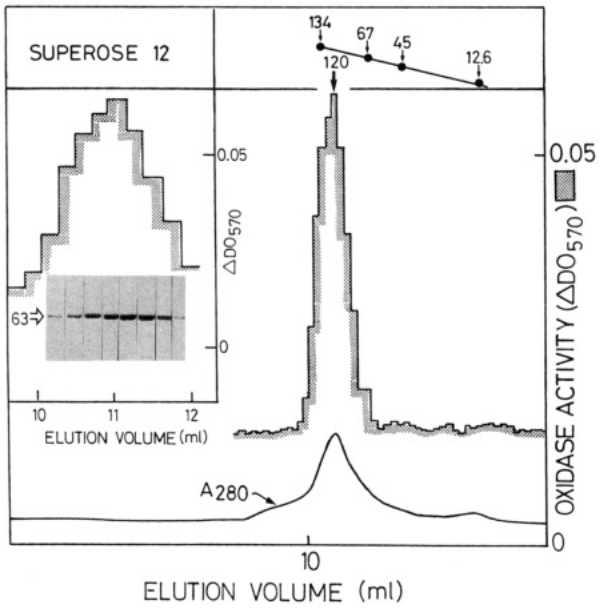

FIGURE 3: Gel filtration on Superose 12 of the 63-kDa cytosolic factor. Fractions 40–45 from the preceding step (MQ chromatography, Figure 2) corresponding to pool MQ2 were concentrated on Centricon 10 to a volume of 0.2 mL. The concentrate was loaded on a FPLC Pharmacia Superose 12 column equilibrated in PBS, 1 mM EGTA, and 200 μM DTT (PBS–ED). The column was eluted with the same medium at a rate of 0.25 mL/min. Two hundred microliter fractions were collected; 5-μL aliquots were assayed for oxidase activating potency. For determination of molecular weight, the column was further calibrated with the dimer of bovine serum albumin (134 kDa), serum albumin (67 kDa), ovalbumin (45 kDa), and cytochrome *c* (12.6 kDa). A peak of maximal activity was found to correspond to a molecular mass of 120 kDa. Insert: SDS–PAGE of eluted fractions. The gel was stained with Coomassie Blue.

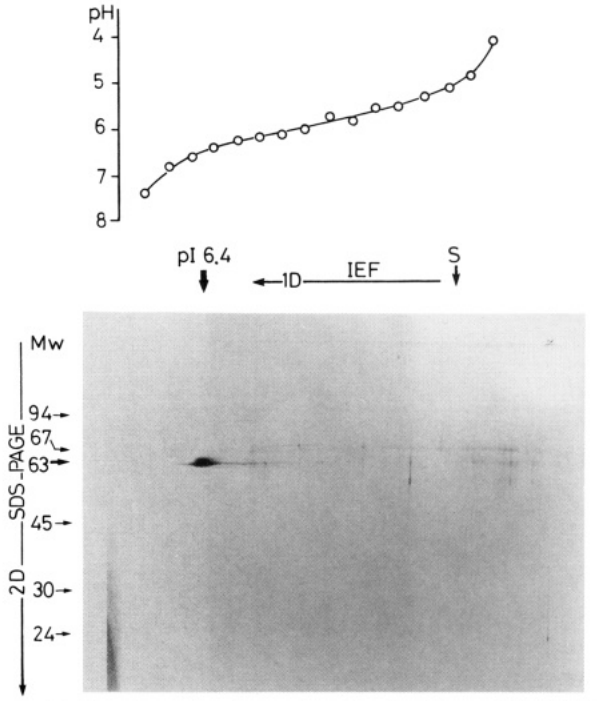

FIGURE 4: Two-dimensional gel electrophoresis of the purified 63-kDa cytosolic factor. Ten micrograms of the purified protein was subjected to isoelectric focusing followed by SDS–PAGE, as detailed under Materials and Methods. The gel was stained with silver nitrate.

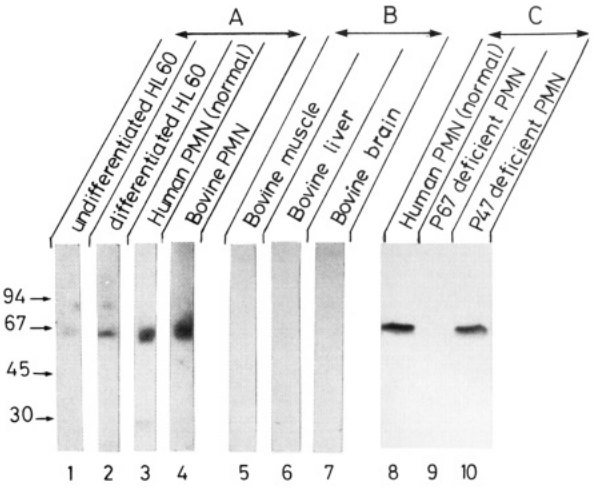

FIGURE 5: Immunodetection of the 63-kDa cytosolic factor in phagocytic and nonphagocytic cells by Western blotting. Cytosols were subjected to SDS–PAGE. After immunoblotting, the immunocomplexes were revealed with radiolabeled protein A. (A) Cytosols (200 μg of protein) from nondifferentiated and differentiated HL60 cells, human neutrophils (PMN), and bovine neutrophils (PMN); (B) cytosols (200 μg of protein) from nonphagocytic cells (skeletal muscle, liver, brain); (C) cytosols (160 μg) from normal human neutrophils and autosomal CGD neutrophils defective in the 47-kDa or the 67-kDa oxidase activating factor.

gested that the purified cytosolic factor was organized as a dimer.

The purified 63-kDa protein was also characterized by 2-D PAGE, corresponding to isoelectric focusing followed by SDS–PAGE. One single protein spot with a p*I* of 6.4 ± 0.1 and a molecular mass of 63 kDa was visualized on the silver nitrate stained gel (Figure 4).

Competency of the 63-kDa protein in oxidase activation was ascertained by the disappearance of activating potency in bovine neutrophil cytosol incubated with a suspension of *S. aureus* coated with 63-kDa cytosolic factor antibodies (cf. Materials and Methods). Preimmune serum was ineffective.

Tissue and Species Specificity. Western blot analysis was used to explore the localization of the bovine neutrophil 63-kDa protein in different bovine tissues, namely, skeletal muscle, liver, and brain, and in phagocytic cells from human origin (Figure 5). Antibodies raised against the bovine 63-kDa protein reacted with a protein of similar size present in the cytosol of human neutrophils and HL60 cells differentiated by growth in the presence of dibutyryl-AMPc (Figure 5, lanes 2 and 3). Only a faint reaction was observed with undifferentiated HL60 cells (Figure 5, lane 1). Emergence of the oxidase activity in the HL60 cells during the course of differentiation was well correlated with the appearance of the 63-kDa protein (not shown). On the other hand, the 63-kDa protein, strongly immunoreactive in bovine neutrophils (Figure 5, lane 4), could not be immunodetected in the cytosols of different bovine tissues tested (Figure 5, lanes 5–7), or in the corresponding membrane fractions (not shown), indicating specificity in the localization of this protein in phagocytic cells.

Immunological Study of Cytosol from Autosomal CGD Neutrophils. Two autosomal cytochrome b_{558} positive forms of CGD have been identified. The most common form is correlated with the lack of a cytosolic 47-kDa protein, while the other form is accompanied by a deficiency in a cytosolic 67-kDa protein. The complementary cDNAs encoding the 67-kDa protein (Leto et al., 1990) and the 47-kDa protein (Volpp et al., 1989; Lomax et al., 1989) have been cloned. It was interesting to determine whether the human neutrophil protein, which was found to immunoreact with rabbit antibodies raised against the isolated bovine cytosolic protein of 63-kDa, was related to the 67-kDa cytosolic protein whose defect is responsible for an autosomal form of CGD. We

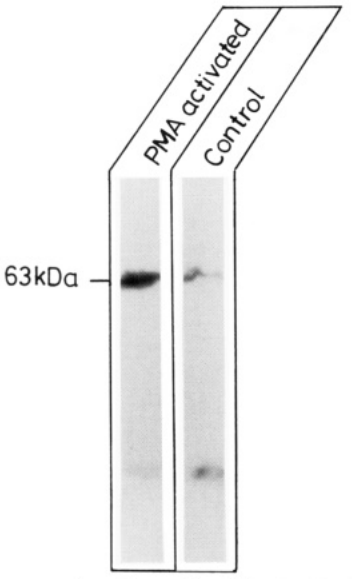

FIGURE 6: Demonstration of transfer of the 63-kDa cytosolic factor to the membrane during PMA activation of bovine neutrophils. Neutrophils were activated by PMA as described under Materials and Methods and then disrupted by sonication. The membrane fraction recovered by differential centrifugation between 10000*g* and 140000*g* was dissolved in the Laemmli buffer, and an aliquot of 200 μg of protein was subjected to SDS–PAGE. The presence of the 63-kDa protein was immunodetected by Western blot (cf. Materials and Methods). A parallel experiment was carried out with resting neutrophils (control).

therefore took neutrophils from a patient with the autosomal form of CGD defective in the 67-kDa protein and tested the immunoreactivity of the cytosol from these neutrophils toward the antiserum directed against the bovine 63-kDa protein (cf. Materials and Methods). Cytosol from CGD neutrophil lacking the 47-kDa factor, but containing the 67-kDa factor, and cytosol from normal human neutrophils containing both factors were used as controls. An immunoreactive band was revealed by immunoblotting in the cytosols from normal neutrophils and neutrophils lacking the 47-kDa factor (Figure 5, lanes 8 and 10), but not in the cytosol from neutrophils lacking the 67-kDa factor (Figure 5, lane 9). This result suggests that the 67-kDa human protein which is lacking in autosomal CGD and the 63-kDa bovine protein have similar epitopes.

Transfer of the 63-kDa Factor to the Membrane in PMA-Activated Bovine Neutrophils. $O_2^{\bullet-}$ production can be elicited upon addition of PMA to intact neutrophils incubated in the presence of glucose. Bovine neutrophils treated with PMA as described under Materials and Methods were able to produce $O_2^{\bullet-}$ at a rate of 15 nmol/10^7 cells, after a lag of 1–2 min. The membrane fraction obtained by differential centrifugation of the homogenate of activated cells was able to generate $O_2^{\bullet-}$ at a rate of 70 nmol/mg of protein. The localization of the 63-kDa factor in this membrane fraction was determined by immunoreaction with the 63-kDa protein antiserum. As shown in the Western blot of Figure 6, a substantial amount of the 63-kDa protein was present in the membrane fraction derived from activated cells. A control carried out with membranes obtained from resting neutrophils showed a negligible oxidase activity and the virtual absence of transfer of the 63-kDa protein to the membrane.

Discussion

The understanding of the mechanism of oxidase activation has benefited from both clinical and biochemical studies. Clinical studies have led to the recognition of several forms of CGD characterized by specific molecular defects. In the case of autosomal CGD, two forms of CGD have been identified. These forms are characterized by neutrophils whose cytosols are defective in soluble proteins of 67 and 47 kDa, respectively, the latter protein having a strong propensity to be phosphorylated upon activation of the neutrophils. Biochemical studies have led to the elaboration of a cell-free system of oxidase activation, opening the way to the purification of the components of the oxidase complex. Due to the low concentration of the cytosolic factors in neutrophils, large amounts of blood are required as starting material for purification of these factors. For this reason, bovine neutrophils are used in our laboratory. The bovine 63-kDa protein, whose purification and properties are described in this paper, is, to our knowledge, the first cytosolic factor purified to electrophoretic homogeneity from resting neutrophils and shown to be competent in a cell-free system of oxidase activation. A recombinant 67-kDa cytosolic factor of human origin was recently obtained from the corresponding complementary DNA expressed in *Escherichia coli* (Leto et al., 1990). However, the recombinant 67-kDa protein showed a limited ability to elicit oxidase activity in a cell-free system containing the complementary 47-kDa cytosolic protein. The 47-kDa cytosolic protein was purified in its phosphorylated form from ^{32}P-labeled human neutrophils by chromatography on ion-exchange resins and hydroxylapatite (Teahan et al., 1990). Its identity with the cytosolic 47-kDa protein was confirmed by the demonstration that the protein was defective in some patients with autosomal CGD and that a partial sequence of the isolated protein was similar to that predicted from the structure of cDNA encoding the 47-kDa protein (Lomax et al., 1989; Volpp et al., 1989). However, no reference was made to the competency of the purified 47-kDa protein in an oxidase activation assay.

The antibodies raised against the isolated bovine 63-kDa protein reacted with a cytosolic protein of similar size present in normal human neutrophils and differentiated HL60 cells; the absence of reactivity of these antibodies with neutrophil cytosol from a patient with the autosomal form of CGD lacking the 67-kDa protein is consistent with the idea that the 63-kDa bovine protein is probably analogous to the 67-kDa human protein.

Evidence for the requirement of more than one cytosolic factor for oxidase activation in a cell-free system prepared from human neutrophils was clearly provided by Bolscher et al. (1989), who described synergism between two fractions from a human neutrophil cytosol separated by carboxylmethyl-Sepharose chromatography. Although synergism between several cytosolic factors is now well documented, experimental conditions leading to apparent synergism should be critically examined. Caution should be exerted in assessing the conditions of optimization of oxidase activation, especially the optimal concentration of unsaturated fatty acid in the cell-free system. For example, oxidase activation is inhibited by an excess of arachidonic acid (Pilloud et al., 1989a). This inhibition might be relieved by addition of unspecific proteins which possibly bind arachidonic acid and thereby decrease its concentration in cytosol, leading to the erroneous interpretation that the added proteins stimulate oxidase activation. The amount of arachidonic acid to be used in the reconstituted system must be optimized not only with respect to the amount of cytosolic protein but also with respect to the ionic strength of the medium (Pilloud et al., 1989a). By use of these optimized conditions, synergism between two separate fractions from bovine cytosol was demonstrated (Pilloud et al., 1989b).

In the present study, the oxidase activating potency specific of the 63-kDa protein was always assayed under optimized conditions, in the presence of fraction S that supplied soluble factor(s) complementary to the 63-kDa protein.

Translocation of the 63-kDa factor from cytosol to membrane was demonstrated in PMA-activated bovine neutrophils, after recovery of the membrane fraction from the neutrophil homogenate by differential centrifugation and detection of the 63-kDa protein by immunoblotting. Translocation of the phosphorylated 47-kDa cytosolic protein was also demonstrated in human neutrophils treated by PMA (Ambruso et al., 1990). Using a polyclonal antiserum that recognizes the 47- and 67-kDa cytosolic proteins from human neutrophils, Clark et al. (1990) also demonstrated transfer of these two proteins to the membrane, concomitant to oxidase activation.

Two possible functions can be imagined for the cytosolic factors of oxidase activation. The cytosolic factors might react with the redox components of the oxidase complex, resulting in modification of their conformation and enhancement of their catalytic efficiency. Alternatively, they may be redox components of the oxidase complex delocalized from the membrane in resting neutrophils, and transferred back to the membrane in activated neutrophils. This view was first advocated by Sha'ag and Pick (1988). Later Smith et al. (1988) and Takusagi et al. (1989) found that labeling by 2',3'-dialdehyde NADPH (dial-NADPH) of a presumed NADPH binding protein located in neutrophil cytosol resulted in loss of the oxidase activating potency of cytosol. Along this line, it was reported that cytosolic proteins of 45, 55, and 66–70 kDa are labeled by dial-NADPH during the course of oxidase activation (Takusagi et al., 1989). The 63-kDa cytosolic factor purified from bovine neutrophils has a size closely related to the 65-kDa NADPH binding protein purified from the plasma membrane of activated bovine neutrophils (Doussière et al., 1985). Preliminary assays have so far not provided evidence that the two protein species are identical.

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Registry No. NADPH oxidase, 9032-22-8.

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