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Respiratory burst oxidase from human neutrophils: Purification and some properties

(phagocytes/superoxide/oxygen radicals/host defense mechanisms)

MICHELE MARKERT*, G. ALLISON GLASS, AND BERNARD M. BABIOR

Blood Research Laboratory and Department of Medicine, Tufts-New England Medical Center, Boston, MA 02111

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ABSTRACT **The respiratory burst oxidase of human neutrophils was purified by "dye-affinity" chromatography over a red agarose column. Electrophoresis of the purified enzyme on NaDodSO$_4$ gel showed a single major band at 64,000–66,000 daltons, together with some minor contaminants. On a nondenaturing gel, the enzyme ran as two closely spaced bands, the faster of which contained flavin. When these two bands were rerun separately on a NaDodSO$_4$ gel, they gave identical patterns, each showing a major band at ca. 65,000 daltons. The specific activity (mean ± SEM) of the purified enzyme was 8.8 ± 3.5 μmol of O_2^- per min/mg of protein.**

When exposed to appropriate stimuli, neutrophils are induced to express the "respiratory burst," a profound alteration in oxygen metabolism whose purpose is the generation of microbicidal oxidants by the partial reduction of oxygen. This alteration results from the activation of a membrane-bound oxidase, dormant in resting cells, that catalyzes the reduction of oxygen to O_2^- at the expense of NADPH (1–11):

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

The O_2^- is then converted in a series of secondary reactions to OCl^- and reactive oxidizing radicals (including OH·), the proximate microbicidal oxidants. The respiratory burst and other aspects of the oxygen-dependent microbicidal mechanisms of phagocytes have recently been reviewed (12–16).

The respiratory burst oxidase was first solubilized from the plasma membranes of activated human neutrophils in 1978 (17). We now report the purification of this oxidase and describe some of the properties of the purified enzyme.

MATERIALS AND METHODS

Human neutrophils were prepared by dextran sedimentation and differential centrifugation as described (18). Zymosan (Sigma) was boiled for 10 min in 1 M NaOH, then washed three times with Hanks' balanced salt solution (GIBCO), and finally opsonized as described elsewhere (3). Superoxide dismutase, horse heart cytochrome *c* (type VI), phosphatidylethanolamine (ovine brain), Lubrol PX, sodium deoxycholate, phenylmethylsulfonyl fluoride, leupeptin, pepstatin, FAD, and NADPH were purchased from Sigma. The sodium deoxycholate was purified by recrystallization from absolute ethanol. Red Sepharose CL-6B (agarose coupled to reactive red 120) was obtained from Pharmacia. The concentration of dye in the lots purchased from Pharmacia ranged from 2.0 to 3.0 μmol/ml of packed gel. Other reagents were the best grade commercially available and were used without further purification.

Gel Electrophoresis. Electrophoresis on 7.5% nondenaturing slab gels was performed by a modification of the method of Davis (19) in which the solution for preparing the lower gel contained 4% glycerol plus twice the usual concentration of Tris, that for preparing the upper gel contained half the usual concentration of bisacrylamide, and polymerization was accomplished with 0.035% (wt/vol) ammonium persulfate, which was removed by preelectrophoresis for 1 hr before the samples were applied to the slab. Nondenaturing gel electrophoresis was carried out at 4°C. NaDodSO$_4$/polyacrylamide gel electrophoresis (NaDodSO$_4$/PAGE) was performed by the method of Laemmli (20) using 9% slab gels. Where necessary, samples were concentrated before electrophoresis in CF 25A ultrafiltration cones (Amicon) and then centrifuged for 30 min at 100,000 × *g* to remove aggregated proteins. Proteins were visualized with Coomassie blue R-250 or with a silver stain kit purchased from Bio-Rad.

Localization of Flavin in the Nondenaturing Gel. The location of flavin in the nondenaturing gel was determined fluorimetrically. The track of interest was excised from the gel, and the region between the top of the running gel and the tracking dye band was cut into 1-mm slices. Each slice was homogenized by hand (6–8 strokes in a Potter–Elvehjem homogenizer) in 3 ml of elution buffer without detergent (see below). The fluorescence spectrum of each homogenate was obtained with a Perkin-Elmer model MPF-3 spectrofluorimeter, exciting at 470 ± 5 nm and measuring the emission between 480 and 540 nm. An excitation spectrum was obtained on the sample showing maximum fluorescence; for this, the excitation wavelength was varied between 380 and 480 nm, and emission was measured at 525 nm. The measurement of flavin fluorescence was not affected by the presence of gel fragments in the sample.

Assays. Superoxide production was measured as described (21), except that FAD was added to a final concentration of 20 μM, and incubations were carried out for only 5 min. Initial rates were calculated by dividing the 5-min value for O_2^- production by 3, a figure that was determined experimentally by comparing initial rates of O_2^- production as measured in a continuous assay (22) with the 5-min values obtained with the same enzyme preparations. Protein concentrations of the membrane suspensions were measured by using the Bradford reagent (Bio-Rad). Protein concentrations of detergent-containing preparations were determined by the method of Schaffner and Weissmann (23). Bovine serum albumin was used as standard for both methods.

RESULTS

Purification of the Oxidase. The oxidase was purified by solubilizing the O_2^--forming activity from the plasma membranes of zymosan-activated neutrophils and then chromato-

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*Present address: Laboratoire Central de Chimie Clinique, CHUV, Lausanne, Switzerland.

graphing the solubilized material over a column of red agarose.

Preparation of neutrophil membranes. Neutrophils were isolated from 300 ml of blood. The cells [resting, or activated with opsonized zymosan (8) with or without a 10-min preincubation with 0.1 μM fMet-Leu-Phe (24)] were suspended at a concentration of 5×10^7 cells per ml in 0.34 M sucrose containing 0.5 mM phenylmethylsulfonyl fluoride, 2 μM pepstatin, and 2 μM leupeptin. Six milliliters of this suspension was placed in a 100-ml glass beaker and sonicated at full power for two 30-sec intervals 1 min apart at 0°C in a Heat Systems model W220F sonifier fitted with a cup horn. The sonicate was centrifuged at 160 × *g* for 5 min at 4°C to remove whole cells and nuclei, and the entire supernatant was layered over 10 ml of 30% (wt/vol) sucrose, which, in turn, rested on 20 ml of 50% (wt/vol) sucrose in a 2.5 × 8.9 cm polyallomer tube. This discontinuous gradient was centrifuged at 140,000 × *g* for 45 min at 4°C using a Spinco SW 28 head in a Beckman model L3-50 preparative ultracentrifuge. The membranes sedimented to the interface between the two sucrose layers. This interface was carefully aspirated with a Pasteur pipet, diluted with 3 vol of distilled water, and centrifuged in a Sorvall RC-5 high-speed centrifuge at 27,000 × *g* for 30 minutes at 4°C. The resulting membrane pellet was suspended in 1.0 ml of 0.34 M sucrose and assayed for protein and O_2^--forming activity.

Solubilization of the oxidase. The membrane suspension was mixed with an equal volume of extraction buffer [2% (wt/vol) sodium deoxycholate in 20 mM sodium glycinate buffer (pH 8.0) containing 1 mM NaN_3, 1.7 μM $CaCl_2$, and 50% (vol/vol) glycerol]. This mixture was incubated on ice for 30 min with occasional gentle agitation on a Vortex mixer. The extract was centrifuged at 100,000 × *g* for 30 min at 4°C (Spinco fixed-angle Ti 60 head). The supernatant was then assayed for protein and for O_2^--forming activity. The protein concentration in the extract averaged 2.3 ± 0.4 mg/ml (mean ± SEM; $n = 5$). Thirty to 40% of the original O_2^--forming activity could routinely be recovered in the extract.

Chromatography over red agarose. Chromatography was carried out at 4°C. A 3-ml (packed volume) column of red agarose was equilibrated with a 1:1 (vol/vol) mixture of 0.34 M sucrose and equilibration buffer [0.25% (wt/vol) Lubrol PX plus 0.25% (wt/vol) sodium deoxycholate in 20 mM sodium glycinate buffer (pH 8.0) containing 1 mM NaN_3, 1.7 μM $CaCl_2$, and 50% (vol/vol) glycerol]. Nine-tenths milliliter of the solubilized preparation was placed on the column. The column was then washed with 7–15 ml of the sucrose/equilibration buffer mixture. Finally, the column was eluted with 7–9 ml of an elution buffer made up of equal volumes of 0.34 M sucrose and detergent-free equilibration buffer. One-milliliter fractions were collected from the column during the washing and elution procedures. Each fraction was assayed for O_2^--forming activity (Fig. 1). Active fractions were pooled, divided into aliquots, and stored at −70°C. The purification procedure is summarized in Table 1.

Characterization of the Oxidase by Gel Electrophoresis. Purified preparations of active oxidase and of its counterpart from resting neutrophils were subjected to electrophoresis on a nondenaturing gel (Fig. 2). The active preparation showed two major protein bands, well-defined and closely spaced (R_f = 0.51 and 0.56 with respect to the dye front). These bands were not seen in the preparation from resting neutrophils, which showed instead several more diffuse bands that migrated only a small distance into the gel (R_f = 0.05–0.20). A few minor contaminants were also seen in both preparations.

By $NaDodSO_4$/PAGE, the active preparation showed only one major band, which migrated with an apparent molecular mass of ≈65 kDa (Fig. 3).† This band was missing from the resting preparation. When the two major bands in a nondenaturing gel prepared from the active oxidase were separately excised and subjected to reelectrophoresis in the presence of $NaDodSO_4$, identical patterns were observed in the two tracks, each showing a major band at ≈65 kDa together with some minor contaminants (Fig. 4).

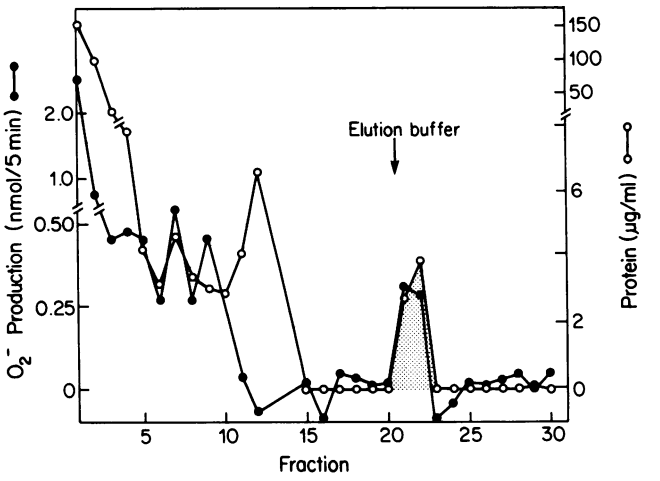

FIG. 1. Chromatography of NADPH oxidase over a red agarose column. At the time the column fractions were assayed, the solubilized enzyme preparation used for this purification step contained enough activity in 1.0 ml to generate O_2^- at a rate of 695 nmol/5 min. Calculations based on that figure and on the use in the O_2^- production assays of 50-μl portions of each column fraction showed that 82% of the enzyme in the applied sample was taken up by the column and that 2.0% of the adsorbed activity was released by the eluting buffer. The yield of enzyme in this particular preparation was lower than average. The *stippled area* represents the fractions containing the purified enzyme.

The following conclusions are consistent with the foregoing results: (*i*) the major band(s) observed in the nondenaturing and $NaDodSO_4$ gels probably correspond to the respiratory burst oxidase, since these bands appear only in preparations from activated neutrophils; (*ii*) the subunit molecular mass of the respiratory burst oxidase is ≈65 kDa; (*iii*) despite their different mobilities, both major bands in the nondenaturing gel probably represent oxidase, since they give identi-

†From time to time, the ≈65-kDa band on the $NaDodSO_4$ gel would be of rather low intensity, and an additional prominent band would appear in the vicinity of 32–33 kDa. We speculate that this pattern resulted from the proteolytic cleavage of some of the enzyme during the course of purification, despite the precautions taken to prevent this from occurring in the protease-rich neutrophil homogenate.

Table 1. Summary of the purification procedure

Step	Specific activity, units*/mg of protein	Purification, fold	Yield, %
Particles	0.106	1.0	(100)
Membranes	0.56	5.3	29.6
Solubilized preparation	1.7	16.0	21.8
Red agarose eluate	5.1	48.1	1.6

Results of a representative purification are shown. The total amount of activity in the particles used as starting material was 4.33 units. Yields for steps in which only a portion of the preparation was used were calculated so as to give yields for the total preparation. The yield of the red agarose eluate was corrected for the loss of 55% of the oxidase activity during the time required for chromatography, as measured in a sample of solubilized preparation that was stored at 4°C during dye-affinity chromatography and then assayed along with the red agarose eluate.

*μmol of O_2^- per min.

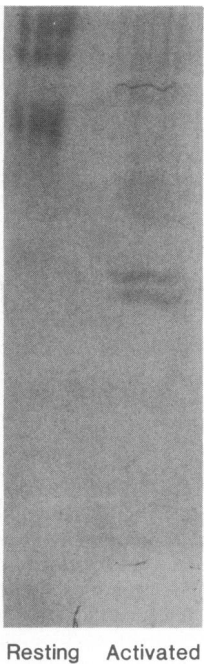

FIG. 2. Electrophoresis of purified NADPH oxidase and a comparable preparation from resting neutrophils on a nondenaturing gel. For each track, three oxidase preparations, each from neutrophils isolated from 300 ml of blood, were combined (the pooled preparations from activated cells contained 4.5 units of oxidase activity in 9 ml total volume) and concentrated to 0.4 ml. After removal of aggregates by centrifugation, the protein concentrations in the pooled, concentrated preparations from resting and activated cells were 0.9 and 1.2 $\mu g/\mu l$, respectively. For electrophoresis, 95 μg of protein was placed in each well. Proteins were visualized with Coomassie blue. The striations at the top of the left-hand track are an artifact of staining.

cal patterns when separately reelectrophoresed in the presence of $NaDodSO_4$.

Other Properties of the Oxidase. Several attempts to identify the major bands in the nondenaturing gel by direct staining for oxidase activity or by elution of oxidase activity from gel slices were unsuccessful, probably because the highly labile oxidase lost all its activity during the course of the electrophoresis. An experiment in which the gel was examined for flavin content, however, provided strong additional evidence that the major bands were in fact the oxidase. For this experiment, pooled oxidase preparations were concentrated and subjected to electrophoresis on adjacent tracks of a nondenaturing gel. One of the two tracks was stained for protein, while the other was sliced and assayed for flavin as described in *Materials and Methods*. The results (Fig. 5) showed a single peak of flavin [identified by excitation maxima at 385 and 465 nm and an emission maximum at 525 nm (25)] that comigrated with the more rapidly moving of the two major protein bands in the gel. The flavin appeared to be physically associated with the protein, since free FAD migrated with the tracking dye under these conditions. No flavin was detected in a similar experiment carried out with resting material. These findings, together with the results discussed in the previous section, establish with a reasonable degree of assurance that the two major bands in the nondenaturing gel do in fact represent the respiratory burst oxidase. The presence of flavin in the faster band is consistent with the notion that this band corresponds to the oxidase, since the oxidase is known to be a flavoprotein. Though flavin is absent from the slower band, this band also appears to correspond to the oxidase, because its pattern on $NaDodSO_4$ gel electrophoresis was identical to that of the faster band.

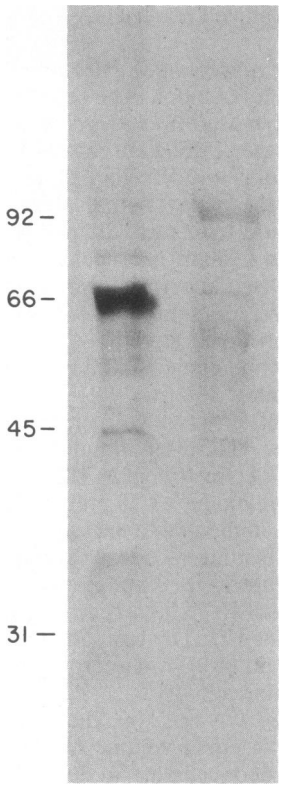

FIG. 3. $NaDodSO_4$ gel electrophoresis of purified NADPH oxidase and a comparable preparation from resting neutrophils. The experimental conditions were as described in the legend to Fig. 2. The pooled preparations from activated cells contained 1.2 units of oxidase activity in 9 ml total volume. The pooled, concentrated preparations from resting and activated cells contained 1.1 and 1.0 μg of protein per μl, respectively. For electrophoresis, 0.1 μg of protein was placed in each well. Proteins were visualized with Coomassie blue. (*Left*) The preparation from activated neutrophils. (*Right*) The preparation from resting cells. Molecular masses are given in kDa.

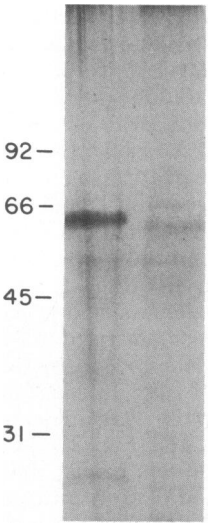

FIG. 4. $NaDodSO_4$ gel electrophoresis of individual bands from a native gel electrophoretogram of purified active oxidase. The two major bands in the gel shown in Fig. 2 were excised, minced, and placed along with some sample buffer into the wells of a $NaDodSO_4$ slab gel. Proteins were visualized with silver stain. The upper and lower bands were run in the left and right tracks, respectively. Molecular masses are given in kDa.

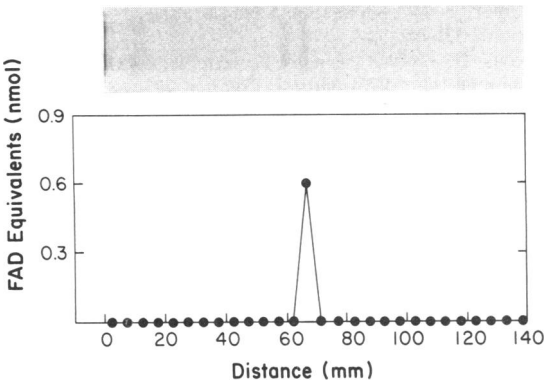

FIG. 5. Association of flavin with the faster-moving band in a nondenaturing gel electrophoretogram of the purified NADPH oxidase. Seven preparations of purified NADPH oxidase, each from neutrophils isolated from 150 ml of blood, were combined and concentrated to 0.1 ml. For electrophoresis, 50 μl of the concentrate was placed into each of two adjacent wells of a nondenaturing slab gel. After electrophoresis, one track was stained with Coomassie blue (above), while the other was sliced and assayed for flavin.

Its lower mobility is probably due at least in part to its lack of flavin, which would lead to a reduction in electrophoretic mobility under nondenaturing conditions because of loss of charge.

Neutrophils are known to contain a low-potential cytochrome (cytochrome b_{559}) that appears to play an important, though as yet incompletely defined, role in O_2^- production by these cells (15, 26–32). Fig. 6 indicates that substantial quantities of cytochrome b_{559} were present in the solubilized enzyme preparation, because a spectrum of this preparation both before and after treatment with dithionite showed peaks close to those characteristic of this cytochrome (413 nm for the oxidized form and 429, 529, and 559 nm for the reduced form). In spectra of the purified oxidase, however, no peaks could be detected either before or after treatment with dithionite. The failure to detect spectral peaks was most likely due to the concentration of enzyme in the purified preparations [enzyme concentration in a typical preparation (specific activity = 10.6 μmol of O_2^- per min/mg of protein) was 34 nM, assuming 100% purity and a molecular mass of 65 kDa], which was so low that any associated chromophores would have been too dilute to be detected by spectrophotometry.

DISCUSSION

Dye-affinity chromatography of deoxycholate-solubilized respiratory burst oxidase from human neutrophils yielded an enzyme preparation with a specific activity of 8.8 ± 3.5 μmol of O_2^- per min/mg of protein. Analysis of this preparation by $NaDodSO_4$/PAGE showed a single major protein band migrating with an apparent molecular mass of 66 kDa. On a nondenaturing gel, the same preparation gave rise to two prominent closely spaced bands that when rerun separately on $NaDodSO_4$ gels showed identical patterns, each characterized by a single major ≈65-kDa protein band. On the basis of the finding that these bands do not appear on gels from resting neutrophil preparations carried through the same purification procedure, and the observation that the faster-moving of the two major bands seen on the nondenaturing gel is associated with flavin, a known cofactor for the oxidase, we believe that the protein represented by these gel electrophoretic bands is the respiratory burst oxidase.

Purification of this enzyme has been difficult because of its instability. Its rapid loss of activity at 4°C under the most favorable storage conditions ($t_{1/2}$ ≈ 1–2 days) is aggravated by its very rapid inactivation by even modest concentrations of

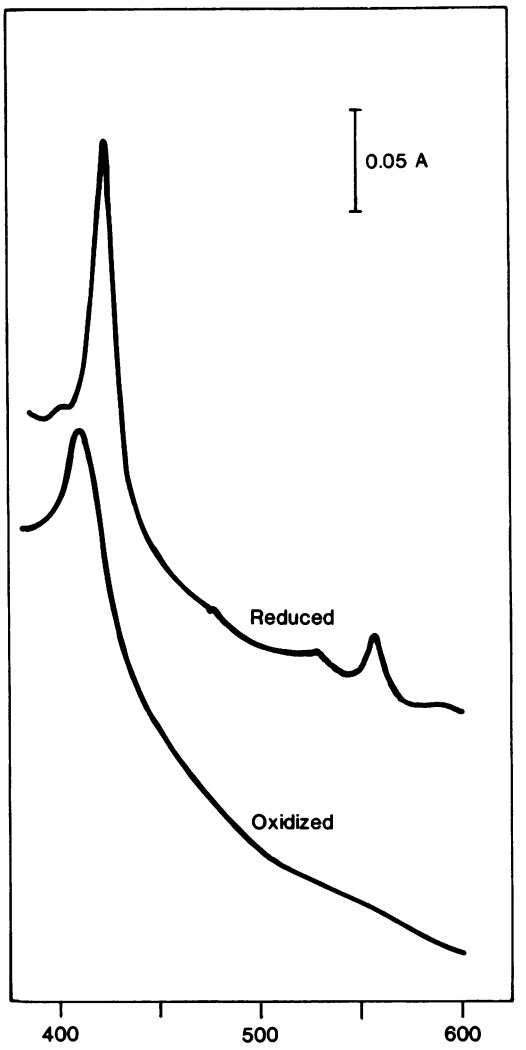

FIG. 6. The spectrum of solubilized oxidase preparation. The protein concentration in the extract used for spectroscopy was 0.25 mg/ml; the specific activity of the oxidase in the extract was 2.5 μmol of O_2^- per min/mg of protein.

salts, a property that precludes the use of any form of ion-exchange chromatography in its purification. A number of affinity and dye-affinity columns were tried in order to achieve a rapid purification, including 2′,5′-ADP-agarose, oxidized and dithionite-reduced NAD- and NADP-agarose, glucose-6-phosphate dehydrogenase-reduced NADP-agarose and blue Sepharose (Pharmacia). The enzyme was not retained by any of these columns. Of all the columns tested, only red Sepharose was able to retain the oxidase and then only when it was equilibrated with Lubrol-containing buffer before applying the enzyme. Furthermore, the oxidase once bound could not be released by any of its ligands nor could a salt gradient be employed because of the sensitivity of the enzyme to ionic strength. It was fortunately possible to release a portion of the enzyme by a sharp detergent-free buffer front, permitting the purification to be accomplished.

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