Purification and some properties of the 45 kDa diphenylene iodonium-binding flavoprotein of neutrophil NADPH oxidase

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The 45 kDa diphenylene iodonium-binding flavoprotein of the human neutrophil superoxide-generating oxidase has been purified by affinity chromatography. The polypeptide was eluted from Blue Memsep or 2',5'-ADP-agarose columns with either NADP or low concentrations of the specific inhibitor diphenylene iodonium. The purified protein was shown to bind FAD at a ratio of 1.09 mol of FAD/mol of protein. The reconstituted flavoprotein had a fluorescence spectrum similar, but not identical, to that of free FAD. It had an isoelectric point of approx. 4.0. The reconstituted flavoprotein displayed no diaphorase activity towards a range of artificial electron acceptors. Polyclonal antibodies raised against the pure protein inhibited superoxide generation by solubilized oxidase in a dose-dependent manner, and inhibited superoxide generation when incubated with either cytosol or membrane fractions in a reconstituted system. These antibodies precipitated the 45 kDa polypeptide together with a haem-containing 23 kDa protein thought to be the small subunit of cytochrome *b*₋₂₄₅. Antibodies raised against cytochrome *P*-450 reductase also precipitated these two polypeptides. These results are consistent with the 45 kDa polypeptide being the flavoprotein of the neutrophil superoxide-generating oxidase.

INTRODUCTION

Polymorphonuclear leukocytes and other phagocytes contain a NADPH-dependent enzyme system which, when activated, produces superoxide [1]. The system is believed to consist of a FAD-containing flavoprotein and a low-potential *b*-type cytochrome (cytochrome *b*₋₂₄₅) arranged as in the scheme [2,3]:

The cytochrome has been purified and sequenced and shown to be a heterodimer of subunit molecular masses 23 kDa and 70–90 kDa [4,5]. The properties of the flavoprotein moiety of the complex have not been fully established [6]. Partial purifications of the flavoprotein have resulted in preparations where the major component polypeptide has had a molecular mass of 51 kDa (by gel filtration [7]), 87 kDa (by non-denaturing electrophoresis [8]) or 65–67 kDa (by SDS/polyacrylamide-gel electrophoresis (PAGE) [9–11]). Affinity labelling with reactive NADPH analogues has also identified proteins of 65–68 kDa [9] and 45, 55 and 66–70 kDa [10]. It has recently been shown, however, that the 66 kDa membrane component labelled with *o*-dialdehyde NADPH is probably not part of the oxidase [12]. We have used the potent specific NADPH oxidase inhibitor diphenylene iodonium (DPI) to label a single band of 45 kDa (by SDS/PAGE) in whole neutrophils or solubilized preparations of oxidase [13]. This compound has previously been shown to inhibit (at higher concentrations) mitochondrial NADH dehydrogenase [14]. A band of identical molecular mass is labelled in macrophages [15] and eosinophils [16], and is induced during interferon gamma treatment of a human monocytic cell line in synchrony with the ability of the cells to produce superoxide [17]. We have also shown the polypeptide to be present in Epstein-Barr-virus-transformed lymphocyte cell lines,

which have the ability to produce superoxide in a DPI-sensitive manner. These cells also contain the cytochrome *b*, which is absent from those cell lines which cannot produce superoxide [18]. A band of similar molecular mass has been shown to be phosphorylated during the activation of neutrophil superoxide production, and phosphorylation of this band is absent in the autosomal form of chronic granulomatous disease [19]. The reaction of a low concentration of DPI with NADPH oxidase is partially inhibited by NADPH, and DPI prevents the reduction of the flavoprotein by NADPH [13,20]; this has led us to suggest that the 45 kDa DPI-binding protein is the flavoprotein of the oxidase.

This report describes the purification of the polypeptide and shows that its properties are consistent with its role as the flavoprotein component of the neutrophil NADPH oxidase.

MATERIALS AND METHODS

Purification of the DPI-binding 45 kDa protein

Neutrophils were isolated from buffy coats obtained from the South West Regional Blood Transfusion Unit, Southmead Hospital, Bristol, U.K., as previously described [21]. PMA (phorbol 12-myristate 13-acetate)-activated neutrophil membranes were prepared by nitrogen cavitation and were solubilized in 0.25% (v/v) Lubrol PX and 0.25% (w/v) sodium deoxycholate as described previously [22].

A 45 kDa DPI-binding protein was purified using a membrane column with a blue dye attached via a spacer (Blue Memsep 1000; Domnick Hunter, Co. Durham, U.K.). A sample (0.5 ml) of the solubilized oxidase was applied to a column (pre-equilibrated with solubilization buffer) at a flow rate of 0.5 ml/min, placed on ice for 30 min and then washed with solubilization buffer at a

flow rate of 0.2 ml/min. Fractions (1 ml) were collected at 0 °C. The 45 kDa protein was released from the column in a volume of approx. 20 ml. Inclusion of 1 mM-NADP in the elution buffer increased the rate of release of 45 kDa protein but the product was less pure.

FAD binding of purified 45 kDa protein

Free FAD was added to a preparation of purified DPI-binding protein to a concentration of approx. 40 μ M and incubated on ice for 1 h. Separation of bound and free FAD was performed on a Sephadex G-25 column (360 mm $\times$ 15 mm) pre-equilibrated with 10 mM-Mops (pH 7.4)/2 mM-MgCl₂. The void volume of the column was determined by running a sample of cytochrome *c* (36 ml). A portion (0.5 ml) of the FAD-saturated protein preparation was loaded on to the column, which was eluted with the same buffer at 1 ml/min. Fractions (1 ml) were collected and each was assayed for FAD by fluorescence spectroscopy, by monitoring emission at 525 nm (excitation 450 nm) on a Perkin-Elmer LS5B scanning fluorescence spectrophotometer. Protein was assayed by the method of Bradford [23].

SDS/PAGE

SDS/PAGE was performed using the discontinuous buffer system of Laemmli [24]. Proteins were revealed by Coomassie Blue staining or by silver staining as described by Blum *et al.* [23a].

Isoelectric focusing

Isoelectric focusing was performed on either a Pharmacia Phastgel system using pH 3.5–9.5 acrylamide gels (Pharmacia) or on a LKB Multiphor system using 1% agarose gels (pH 3–10). Both techniques were performed using the manufacturers' protocols.

Determination of superoxide production

Superoxide production by activated membranes and solubilized oxidase was assayed as previously described using the superoxide dismutase-sensitive rate of ferri-cytochrome *c* reduction [25,26].

Reconstitution of activity in a cell-free system

Human neutrophil membranes and cytosol were isolated and reconstituted according to the method of Curnutte *et al.* [27].

Spectroscopy

Absorption spectra were recorded using a scanning split-beam spectrophotometer as described previously [26].

Diaphorase activity

NADPH-dependent diaphorase activity was assayed with various electron acceptors in a dual-wavelength spectrophotometer using wavelength pairs 550–540 nm (cytochrome *c*, 100 μ M), 600–522 nm (dichlorophenol indophenol, 50 μ M) and 500–600 nm (indonitrotetrazolium, 200 μ M) in 50 mM-triethanolamine, pH 8, containing 2 mM-MgCl₂.

Generation of polyclonal antibody

Polyclonal antibody was raised in New Zealand White rabbits by fortnightly subcutaneous immunization with 50 μ g of the Blue-Memsep-purified 45 kDa polypeptide. The first injection was administered with Freund's com-

plete adjuvant, and subsequent injections used Freund's incomplete adjuvant. Animals were bled 1 week after the fourth injection. The blood (10 ml) was allowed to clot overnight at 4 °C, and the serum was removed after centrifugation. IgG was purified from serum by the method of Hurn & Chantler [28].

Immunoprecipitation and autoradiography

Solubilized neutrophil membranes were radioiodinated using a solid phase oxidant method [29] modified by Mr. E. Davies of this department. Briefly, polypropylene tubes were precoated with Iodogen (Amersham) according to the supplier's protocol. Reaction tubes were washed with 0.25 M-phosphate buffer (pH 7.4). A sample (100 μ l) of the solubilized neutrophil oxidase was incubated in the tubes containing 5 μ l of the phosphate buffer and 0.5 mCi of ¹²⁵I (Amersham) for 5 min at 25 °C with occasional agitation. The reaction mixture was diluted with 100 μ l of 0.05 M-phosphate buffer, pH 7.4, removed from the tube by aspiration and allowed to stand for a further 15 min to permit unreacted iodide species to decay. Separation of free iodine and protein fractions was performed on a 5 ml Sephadex G-25 gel filtration column which had been pre-equilibrated with bovine serum albumin (100 mg/ml) in 0.05 M-phosphate buffer, pH 7.4. Samples (0.5 ml) of this preparation were incubated with 100 μ l of pre-swollen Protein A-agarose (Sigma) for 2 h at 4 °C with continuous stirring to remove non-specific binding components. The treated oxidase suspension was centrifuged and the supernatant was incubated with an equal volume of antiserum for 4 h at 4 °C with continuous stirring. A further portion (200 μ l) of Protein A-agarose was added, and stirring was continued for a further 1 h. The Protein A-agarose was pelleted and washed four times with 2 vol. of phosphate-buffered saline (140 mM-NaCl/10 mM-K₂HPO₄, pH 7.4), and the antibody-antigen complex was eluted with 0.5 M-acetic acid. Samples were neutralized with a small volume of NaOH before SDS/PAGE or spectroscopy.

After SDS/PAGE, the gel was dried and autoradiographed against Fuji RX film for 3 days.

RESULTS AND DISCUSSION

Purification

Purification of the 45 kDa polypeptide from detergent-extracted neutrophil membranes using a Blue Memsep column yielded a single polypeptide of molecular mass 45 kDa (Fig. 1). The most concentrated preparations of the 45 kDa polypeptide that we were able to obtain (250 μ g/ml) yielded only one observable band on silver-stained gels. We estimate we would be able to detect contaminating proteins present at less than 5% of the abundance of the 45 kDa protein. Attempts to purify the protein using Blue Agarose columns were not successful, possibly due to the proximity of the dye to the matrix. A similar degree of purification was achieved using a 2',5'-ADP-agarose column in place of the Blue Memsep column.

Elution from these columns was increased markedly by inclusion of NADP (1 mM) or DPI (50 nM) in the elution buffer. The ability to bind to such affinity columns and the increased elution caused by the inclusion of NADP in the running buffer are characteristic of proteins containing NAD⁺- or NADP⁺-binding domains. The

Fig. 1. SDS/gel electrophoretogram of the purified 45 kDa protein eluted from a Blue Memsep column

Solubilized neutrophil extracts were prepared and chromatographed as described in the Materials and methods section. Lane 1, eluted fraction from the Blue Memsep column (5 μ g of protein); lane 2, molecular mass markers (bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; glyceraldehyde-3-phosphate dehydrogenase, 36 kDa; carbonic anhydrase, 29 kDa; trypsinogen, 24 kDa; trypsin inhibitor, 20.1 kDa; lactalbumin 14.2 kDa).

observation that low concentrations of DPI also increase elution of the protein is consistent with our previous suggestion that DPI and NADPH compete for the same binding site [13]. Incubation of the purified polypeptide with [125 I]DPI resulted in its radiolabelling (results not shown).

Isoelectric focusing of the product of the Blue Memsep column as described in the Materials and methods section resulted in a single band of pI approx. 4.0.

Purified 45 kDa protein showed low but variable fluorescence similar to that typical of flavins. Since the polypeptide was purified in the absence of exogenous FAD, and since the FAD is known to be non-covalently bound, we attempted to assess the ability of the protein to rebinding FAD lost during chromatography. FAD binding to the purified 45 kDa protein was assayed as described in the Materials and methods section. The elution profile of the purified 45 kDa protein matches the first peak of fluorescence eluted in the void volume, shown in Fig. 2. The protein/FAD ratio was estimated from the fluorescence of free FAD standards and was 1.09 mol of FAD/mol of 45 kDa protein. The second peak of fluorescence corresponds to the elution of free, unbound FAD. The fluorescence spectrum of the peak fraction is shown in Fig. 3. This spectrum is very similar, but not identical, to that of free FAD, with the excitation and emission bands being slightly broader and the emission maximum shifted to a slightly shorter wavelength. The differences suggest specific interaction between the protein and the prosthetic group. The purified 45 kDa polypeptide, which contained little FAD, displayed no NADPH-dependent diaphorase activity towards cytochrome *c*, dichlorophenol indophenol or indonitrotetrazolium. Reconstitution of the flavoprotein with FAD (Fig. 3) did not alter these negative findings, consistent with the previous conclusions of ourselves and others [30,31] that the neutrophil oxidase exhibits no intrinsic diaphorase activity.

Immunological studies

Inhibition of oxidase activity. Polyclonal antibodies were raised to the purified preparation as described in the Materials and methods section. Incubation of polyclonal antiserum with either solubilized enzyme or activated membranes resulted in the inhibition of superoxide

Fig. 2. Elution of reconstituted flavoprotein from a Sephadex G-25 gel-filtration column

Reconstitution of the purified 45 kDa protein with FAD and column chromatography were performed as described in the Materials and methods section. ○, FAD relative fluorescence (excitation 450 nm, emission 525 nm); ●, protein concentration.

Fig. 3. Relative fluorescence spectra of the reconstituted flavoprotein

The flavoprotein was purified and reconstituted with FAD, and the spectrum was recorded as described in the Materials and methods section. (a) Excitation spectra (emission at 525 nm), (b) emission spectra (excitation at 450 nm). A, Reconstituted 45 kDa flavoprotein; B, free FAD (800 nM).

Fig. 4. Dose-dependent inhibition of superoxide generation by polyclonal IgG raised against the purified 45 kDa polypeptide

Solubilized human neutrophil oxidase was incubated with polyclonal IgG for 2 h at 0 °C and assayed as described in the Materials and methods section. Inhibition is expressed as a percentage of control incubations containing an equivalent amount of non-immune IgG.

production. Treatment of solubilized oxidase with purified anti-45 kDa IgG inhibited superoxide production in a dose-dependent manner (Fig. 4). In order to identify the localization of the 45 kDa antigen, reconstitution experiments were performed using SDS to activate oxidase activity in a cell-free system containing a mixture of resting cytosol or membranes [27]. Incubation of antibody with either cytosol or resting membranes before activation of oxidase activity in mixtures caused an inhibition of superoxide production of 21% or 34% respectively. Treatment of both cytosol and membranes

Table 1. Inhibition by anti-45 kDa antibody of superoxide production in a cell-free system

Superoxide-generating activity was reconstituted from cytosolic and membrane fractions as described in the Materials and methods section. Where stated, the cytosol or membrane fractions were incubated with 1 mg of polyclonal antiserum/ml for 90 min at 0 °C. Results are expressed as a percentage of activity of fractions which were incubated with an equivalent volume of non-immune serum.

Fractions	Relative activity (%)
Control cytosol + control membranes	100
Control cytosol + treated membranes	66
Treated cytosol + control membranes	79
Treated cytosol + treated membranes	37.5

with antiserum resulted in 62.5% inhibition of oxidase activity. These results suggest that the antigen has a dual localization in plasma membranes and cytosol (Table 1). This is consistent with labelling studies with [¹²⁵I]DPI in which the 45 kDa polypeptide is found in both fractions (Fig. 5).

Immunoprecipitation. Incubation of antibody with solubilized enzyme resulted in the immunoprecipitation of both the 45 kDa polypeptide and spectrally detectable cytochrome *b*₂₄₅, suggesting an interaction between the two components. The ability of the antibody to recognize the 45 kDa polypeptide was further investigated by incubation of purified IgG fractions with radioiodinated solubilized enzyme. The antibody-antigen complexes were immobilized by Protein A-agarose treatment, eluted, and subjected to SDS/PAGE and autoradiography. This treatment revealed the polypeptide antigen and avoided obscuring the gel with antibody and serum-derived proteins. The procedure resulted in the detection of a major radiolabelled band at 45 kDa and a

Fig. 5. [^{125}I]DPI labelling of human neutrophil membranes and cytosol

Human neutrophil membranes and cytosol were prepared as described in the Materials and methods section and incubated with [^{125}I]DPI as described previously [13]. A 50 μg sample of protein was loaded in each track. Lane 1, membranes; lane 2, cytosol.

Fig. 6. Densitometer scans of autoradiographs of immunoprecipitated radioiodinated solubilized NADPH oxidase

Solubilized human neutrophil membranes were radioiodinated, immunoprecipitated and autoradiographed as described in the Materials and methods section. The resulting film was scanned using a Joyce-Loebl densitometer. (a) Anti-45 kDa immunoprecipitate, (b) anti-(cytochrome *P*-450 reductase) immunoprecipitate.

Fig. 7. Absorption spectrum of Protein A-agarose eluate

Spectra were recorded of the fraction eluted by acetic acid from Protein A-agarose as described in the Materials and methods section. (a) Acetic-acid-eluted Protein A fraction, (b) acetic-acid-treated purified cytochrome b_{-245} .

minor band at 23 kDa (Fig. 6). The band at 23 kDa has a similar molecular mass to that reported for the haem-bearing subunit of cytochrome b_{-245} [4]. Treatment of radioiodinated oxidase as described above with antiserum raised against the small subunit of cytochrome b_{-245} (generously given by Professor A. W. Segal, University College Hospital, London, U.K.), which is not inhibitory to oxidase activity, revealed a band of identical molecular mass. To confirm that the 23 kDa band which co-precipitated with the 45 kDa protein was the cytochrome subunit, spectra were recorded of the Protein A-agarose eluate. These spectra indicated the presence of haem and had similar absorbance characteristics to those of pure cytochrome b_{-245} after incubation in 0.5 M-acetic acid, the solvent used to elute the 45 kDa protein from Protein A-agarose (Fig. 7).

Other workers have previously shown that polyclonal antibodies raised to guinea pig cytochrome *P*-450 reductase are inhibitory to the superoxide generating system of that species [32]. The cytochrome *P*-450 system is analogous to the superoxide-generating system in that it too consists of a low-potential cytochrome which is capable of binding oxygen and CO [33,34] and of a flavoprotein cytochrome reductase (utilizing NADPH). There are, however, differences between the systems. Cytochrome *P*-450 reductase is a protein of molecular mass 78 kDa [35] containing both FAD and FMN, unlike the neutrophil oxidase flavoprotein which contains only FAD (reviewed in [6]). We have also shown that cytochrome *P*-450 reductase is not inhibited by concentrations of DPI 100 times greater than those required to inhibit the oxidase.

We found that antibodies to rat *P*-450 reductase (generously given by Dr. E. Shephard, University College Hospital, London, U.K.) were inhibitory to rat (66% inhibition), pig (24%) and human (11%) solubilized neutrophil oxidases. Incubation of the anti-(*P*-450-reductase) antibodies with radioiodinated solubilized human oxidase yielded results similar to those obtained with the anti-45 kDa antibody, i.e. immunoprecipitation

of both 45 kDa and 23 kDa polypeptides, although the interaction appeared weaker (Fig. 6).

The results reported here lead us to propose that the 45 kDa DPI-binding flavoprotein is the flavoprotein component of the phagocyte superoxide-generating oxidase responsible for the donation of electrons from NADPH to the terminal component of the oxidase, cytochrome b_{-245} . In addition to these two electron transfer moieties, there appear to be several other proteins involved in either electron transfer or activation processes. There are reported to be at least four essential cytosolic components, two of which are missing in complementing types of chronic granulomatous disease [36], and may be the GTP-binding proteins of 47 kDa and 67 kDa recently described [37–39]. Other electron transfer components have been suggested by purification and affinity labelling studies as described in the Introduction, as well as two low molecular mass membrane proteins identified by the use of inhibitory antibodies [40,41].

We are grateful to the Arthritis and Rheumatism Council, the Medical Research Council and the Wellcome Trust for financial support.

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Received 12 April 1989/14 July 1989; accepted 10 August 1989