NADPH–cytochrome *c* reductase from human neutrophil membranes: purification, characterization and localization

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Neutrophil-membrane-associated NADPH–cytochrome *c* reductase and cytochrome b_{558} were separately eluted and highly purified by a combination of ion-exchange Sepharose, *N*-amino-octyl-agarose, 2′,5′-ADP–Sepharose and heparin–Sepharose column chromatographies. The purified cytochrome *c* reductase with an apparent molecular mass of 68 kDa contained FMN and FAD (FMN/FAD approx. 1). Cytochrome b_{558} prepared in the presence of phospholipids and FAD showed marked $O_2^{-\cdot}$-producing activity ($V_{max.}$, 8.53 μmol of $O_2^{-\cdot}$/min per mg of cytochrome; K_m for NADPH 58.8 μM) in a cell-free assay system consisting of cytosol, arachidonate and GTP[S]. However, when it was obtained without FAD added to the purification process, it had negligible FAD and little or no $O_2^{-\cdot}$-forming activity in the reconstituted system. The NADPH oxidase activity was not markedly stimulated on incubation of the purified reductase with either flavinated or flavin-depleted cytochrome b_{558} in the cell-free system, suggesting that the reductase is not likely to be involved in neutrophil $O_2^{-\cdot}$ generation. The purified reductase cross-reacted with polyclonal antibodies against both hepatic NADPH–cytochrome *P*-450 reductase and a synthetic peptide, ILVGPGTGIAPFRSF, which indicates residues 529–543 located in the glycine-rich NADPH-binding domain of the *P*-450 reductase, but cytochrome b_{558} did not produce any immunoreactive bands to these antibodies. These antibodies also produced a positive reaction with a 76 kDa protein from dimethyl sulphoxide-induced HL-60-cell microsomes. After solubilization of the microsomal membranes, the 76 kDa protein was readily converted into a partially proteolysed form (68 kDa) even in the presence of antiproteases. In addition, the microsomal fraction shows a CO difference spectrum with a peak at about 454 nm and a trough at 476 nm in the presence of dithionite, indicating the presence of a cytochrome *P*-450-like haemoprotein.

INTRODUCTION

The respiratory-burst oxidase is a complex enzyme characteristic of phagocytes which catalyses the reduction of oxygen to $O_2^{-\cdot}$ at the expense of NADPH [1]. Among the components of the oxidase is cytochrome b_{558}, a membrane-bound cytochrome with a haem group that has been proposed as the terminal electron acceptor of NADPH oxidase, accepting electrons from another catalytic centre probably containing flavin and transferring them to molecular oxygen [2,3]. Cytochrome b_{558} has been shown to be a heterodimer composed of a 92 kDa glycoprotein (large subunit) and 22 kDa polypeptide (small subunit) [4–6], and the large subunit is thought to have a domain(s) that interacts with cytosolic components. Two cytosolic factors, p47phox and p67phox, have been considered to be essential components of the NADPH oxidase system, and Rac-related GTP-binding proteins have been found to be necessary for activation of $O_2^{-\cdot}$ generation [7–10]. However, other groups [11–14] support the idea that, as well as cytochrome b_{558}, a membrane-bound flavoprotein is required to accept electrons from NADPH and to donate them to the haemoprotein which reduces molecular oxygen to $O_2^{-\cdot}$. These workers have produced a diaphorase oxidase transition model in which a membrane-bound NADPH–cytochrome *c* reductase is converted into an $O_2^{-\cdot}$-generating oxidase during stimulation [13–15]. In fact, it has been demonstrated that partially purified Nitroblue Tetrazolium (NBT) reductase from neutrophil membranes greatly activates the $O_2^{-\cdot}$-forming activity in a reconstituted cell-free system containing cytochrome b_{558}, cytosol, myristic acid and guanosine 5′-[γ-thio]triphosphate GTP[S] [16]. Isogai et al. [17,18] also reported that $O_2^{-\cdot}$ is produced on incubation of the purified flavin-free cytochrome b_{558} with hepatic NADPH–cytochrome *P*-450 reductase in the presence of sucrose monolaurate.

We have already purified and characterized an NADPH-dependent cytochrome *c* reductase from differentiated HL-60-cell cytosol [19]. In the present study the cytochrome *c* reductase (FMN/FAD approx. 1) with a molecular mass of approx. 68 kDa was highly purified from human neutrophil membranes, and the effect of this flavoprotein on $O_2^{-\cdot}$ production was examined in a reconstituted cell-free system consisting of cytochrome b_{558} and cytosol.

Shak and Goldstein [20] reported that the leukotriene B_4 ω-hydroxylation reaction in human neutrophils requires molecular oxygen and NADPH and that it is inhibited by CO suggesting the role of electron-transfer system consisting of cytochrome *P*-450 and flavoprotein. Sumimoto et al. [21] have provided direct evidence that the oxygen-activating component of the leukotriene B_4 ω-hydroxylase in human neutrophils is a microsomal cytochrome *P*-450, and that NADPH–cytochrome *P*-450 reductase is also involved in the hydroxylation reaction. We have also observed a cytochrome *P*-450-like haemoprotein in the microsomal fraction of differentiated HL-60 cells. Therefore the biological function of our purified NADPH–cytochrome *c* reductase is discussed in the present paper.

MATERIALS AND METHODS

Materials

The following materials were purchased from the indicated suppliers: cytochrome *c* (type VI), NBT, PAGE standards,

Abbreviations used: Dip-F, di-isopropyl fluorophosphate; PMSF, phenylmethanesulphonyl fluoride; NBT, Nitroblue Tetrazolium; OG, n-octyl glucoside; SOD, superoxide dismutase; *P*-450 reductase, microsomal NADPH–cytochrome *P*-450 reductase from rabbit liver; DTT, dithiothreitol; GTP[S], guanosine 5′-[γ-thio]triphosphate; KLH, keyhole limpet haemocyanin.

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NADPH, NADH, n-octyl glucoside (OG), di-isopropyl fluorophosphate (Dip-F), phenylmethanesulphonyl fluoride (PMSF), Tos-Lys-CH_2Cl ('TLCK') and *N*-amino-octyl-agarose were from Sigma (St. Louis, MO, U.S.A.). GTP[S] was purchased from Boehringer-Mannheim. HESPAN (6% hetastarch in 0.9% NaCl) and lymphocyte separation medium (6.2% Ficoll plus 9.4% sodium diatrizoate) were obtained from American Critical Care Division of American Hospital Supply (McGaw Park, IL, U.S.A.) and Biovetics (Kensington, MD, U.S.A.) respectively. Superoxide dismutase (SOD) and dithiothreitol (DTT) were from Wako Pure Chemical Co., and prestained molecular-mass standards for SDS/PAGE were obtained from Bio-Rad. Heparin–Sepharose CL-6B, DEAE-Sepharose CL-6B, CM-Sepharose CL-6B and 2′,5′-ADP–Sepharose were purchased from Pharmacia LKB. All other reagents were of the best grade commercially available.

Isolation of human neutrophils

Normal human neutrophils were obtained from peripheral blood obtained by phlebotomy. Erythrocytes were sedimented with HESPAN, and the mononuclear cells were removed from the resulting supernatant by centrifugation through lymphocyte separation medium [22]. The resulting cells were greater than 95% neutrophilic granulocytes. Neutrophils were resuspended in break buffer (6 mM Pipes, pH 7.35, containing 60 mM KCl, 18 mM NaCl, 2.3 mM $MgCl_2$, 1 μM Dip-F, 1 mM PMSF, 1 μg/ml Tos-Lys-CH_2Cl and 6%, w/w, sucrose).

Preparation of soluble membrane fraction from neutrophils

Membrane preparation was as described previously [23]. Cells (6×10^9) in 10 ml of ice-cold break buffer were disrupted by sonication at 20 W for 10 s ($\times 3$) with constant cooling at 3 °C. The sonicate was centrifuged (800 *g*, 5 min) to remove nuclei and unbroken cells. The supernatant was loaded on to a 30–50% discontinuous sucrose gradient in the break buffer and centrifuged at 150000 *g* for 1 h. Plasma-membrane and specific granule fractions were collected, and 5 vol. of relaxation buffer (10 mM Pipes, pH 7.35, containing 100 mM KCl, 3 mM NaCl and 3.5 mM $MgCl_2$) was added. The suspension was centrifuged at 250000 *g* for 1.5 h and then precipitates were resuspended in 5 ml of buffer A (0.1 M Tris/acetate buffer, pH 7.4, containing 0.1 M KCl, 20% glycerol, 1 mM DTT, 1 mM EDTA, 1 mM PMSF, 1 μg/ml Tos-Lys-CH_2Cl, 0.34 mM dimyristoyl L-α-phosphatidylethanolamine, 0.34 mM dimyristoyl L-α-phosphatidylcholine, 40 mM OG, 0.5% sodium cholate and 10 μM FAD). The solubilized membrane was centrifuged at 150000 *g* for 1 h, and the supernatant was used for purification of NADPH–cytochrome *c* reductase and cytochrome b_{558} by affinity chromatography.

Separation of microsomes from differentiated HL-60 cells

Differentiation was induced by addition of 1.25% dimethyl sulphoxide for a week at 37 °C in a humidified incubation chamber with 7% CO_2. Cells were grown and harvested as previously described [19]. Cells (6.5×10^{10}) in 100 ml of ice-cold relaxation buffer (PBS, pH 7.35, containing 2 mM $CaCl_2$, 0.5 mM PMSF, 50 μM Dip-F, 1 μg/ml Tos-Lys-CH_2Cl and 10 μg/ml each antipain, chymostatin and pepstatin) were gently disrupted by a Dounce homogenizer with constant cooling at 3 °C. The homogenate was centrifuged (800 *g*, 5 min) to remove nuclei and unbroken cells. As described by Koizumi et al. [24], the supernatant was loaded on to a 20, 42 and 70% discontinuous sucrose gradient and centrifuged at 52000 *g* for 1 h. The fraction that precipitated at the interface between 20 and 42% sucrose was collected and suspended in 4 vol. of PBS containing 1 mM EDTA, 1 mM DTT, 4 mM $MgCl_2$, 1 mM PMSF and 1 μg/ml Tos-Lys-CH_2Cl. The suspension was centrifuged at 13000 *g* for 15 min, and the resulting precipitate was removed and the supernatant further centrifuged at 150000 *g* for 1 h. The high-speed pellet was suspended in 50 mM Hepes, pH 7.25, containing 20% glycerol, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 4 mM $MgCl_2$ and 1 μg/ml Tos-Lys-CH_2Cl, and was used as the microsomal fraction.

Purification of cytochrome b_{558} from solubilized membranes

Detergent-solubilized cytochrome b_{558} was purified by the method of Segal et al. [2] with some modifications. The solubilized membrane fraction was passed through a mixed bed of DEAE-Sepharose, CM-Sepharose and *N*-amino-octyl-agarose (1.57 ml each bed volume), followed by chromatography on heparin–Sepharose (2.36 ml bed volume). The two columns were equilibrated and washed well with buffer A, and cytochrome b_{558} was adsorbed to the column of heparin–Sepharose. The affinity column was further washed stepwise with buffer A containing 0.1 M NaCl and buffer A containing 0.5 M NaCl. The cytochrome *b* bound to the column was eluted with buffer A containing 0.5 M NaCl. This cytochrome *b*-rich fraction was pooled, concentrated and applied to a desalting column (excellulose GF-5) equilibrated with buffer B (50 mM Tris/acetate buffer, pH 7.4, containing 20% glycerol, 2 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA, 1 mM PMSF and 1 μg/ml Tos-Lys-CH_2Cl) to remove free flavin and NaCl. The sample with high haem content was used as a finally purified preparation of cytochrome b_{558}. The concentration of cytochrome b_{558} was determined by reduced-minus-oxidized absorbance difference spectroscopy or by directly measuring the oxidized absorbance spectrum (350–600 nm) on a Hitachi 156 dual-beam scanning spectrophotometer, assuming a reduced-minus-oxidized Soret band (427–440 nm) absorption coefficient of 161 $mM^{-1} \cdot cm^{-1}$ or an oxidized Soret band (414 nm) absorption coefficient of 131 $mM^{-1} \cdot cm^{-1}$ [25]. Protein was quantified by the method of Lowry et al. [26] with BSA as standard.

Purification of NADPH–cytochrome *c* reductase from solubilized membranes of human neutrophils

After elution of protein fractions containing cytochrome b_{558} from a mixed-bed column of DEAE-Sepharose, CM-Sepharose and *N*-amino-octyl-agarose, the column was fully washed with equilibration buffer C (25 mM Hepes, pH 7.6, containing 10% glycerol, 1 mM DTT, 0.2 mM EDTA, 40 mM OG, 1 mM PMSF and 1 μg/ml Tos-Lys-CH_2Cl). The column was eluted with an NaCl linear gradient (0–0.5 M) at 8 ml/h, the absorbance at 280 nm was recorded, and fractions of volume 2 ml were collected. Fractions showing high NADPH–cytochrome *c* reductase activity were pooled and dialysed overnight against 2 litres of buffer C. The dialysed sample was loaded on to a 2′,5′-ADP–Sepharose column (10 mm × 20 mm) equilibrated with buffer C. The column was washed with 20 ml each of buffer C, buffer C plus 5 mM 2′-AMP and buffer C plus 0.5 M NaCl. Eluted fractions with high cytochrome *c* reductase activity were pooled, concentrated to a final volume of about 1 ml using a Centricon-10 microconcentrator (Amicon) and employed in subsequent studies.

Purification of NADPH–cytochrome *P*-450 reductase from rabbit liver

Detergent-solubilized *P*-450 reductase was isolated by ion-exchange chromatography on DEAE-Sepharose CL-6B followed by affinity chromatography on 2′,5′-ADP–Sepharose and hydroxyapatite (Bio-Rad) column chromatography [27]. Limited trypsinolysis of purified *P*-450 reductase was carried out by the method of Black and Coon [28].

Assay of flavin content

Prosthetic groups associated with the enzyme preparations were analysed by a method using reversed-phase h.p.l.c. with a fluorescent detector. The sample was boiled at 100 °C for 5 min and centrifuged at 26000 *g* for 20 min. A portion (50 μl) of the supernatant was subjected to h.p.l.c. analysis using a column of Cosmosil $5C_{18}$-AR (Nacalai Tesque). Flavins eluted from the column were determined on-line with a Hitachi 650-60 fluorimetric detector in which the light emission was measured at 530 nm with an excitation wavelength at 450 nm. FMN and FAD obtained from Sigma were further purified by h.p.l.c. and used as authentic standards.

Antibodies to synthetic peptides

Peptides were synthesized for immunization, and antibodies were used for identification and characterization of purified enzymes. Synthetic peptide was separated on a C18-reversed-phase column by elution with a linear gradient of acetonitrile in 0.1 % trifluoroacetic acid and water. The glycine-rich NADPH-binding domain of hepatic microsomal *P*-450 reductase, IMVGPGTGIAPFMGFC, which indicates residues 529–543 (15 underlined residues) common to brain, endothelial nitric oxide synthase (residues 1245–1259) and a large subunit of cytochrome b_{558} (residues 405–419) were synthesized [4,5]. The two synthetic C-terminal peptides, CSNPRGVHFIFNKENF and CAGGPPGGPQVNPIPVTDEVV, were also prepared which correspond to residues 558–570 of the large subunit (underlined) and residues 175–194 of the small subunit (underlined) of human cytochrome b_{558} [29–31]. These three peptides ranged from 70 to 80 % pure as assessed by h.p.l.c. and yielded the correct amino acid sequences from the major peaks purified by h.p.l.c. As described by Kennedy et al. [32], the synthetic peptide was conjugated through the -SH group on the cysteine to amino groups on keyhole limpet haemocyanin (KLH, for immunization of rabbits) and BSA (for assaying antibody activity) by means of the heterobifunctional cross-linkers, *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester and *N*-succinimidyl 3-(2-pyridylthio)propionate respectively. Three rabbits were immunized with each peptide–KLH emulsified in Freund's complete adjuvant (approx. 0.1 mg per dose). The rabbits received one subcutaneous injection every 2 weeks for a total of three injections, and serum was obtained after each injection. The three rabbits produced each detectable antibody response (as measured by peptide–BSA) after a single injection of each peptide–KLH. Antibodies (IgG fraction) were isolated from serum proteins by chromatography on a column (10 mm × 35 mm) of Protein A–agarose (Pierce, Rockford, IL, U.S.A.) and dialysed against 20 mM phosphate buffer, pH 7.4, containing 0.9 % NaCl for 24 h at 3 °C. This preparation was used for Western-blot analysis. The IgG fraction from non-immune serum was also prepared by the same procedure.

PAGE

SDS/PAGE was carried out by the method of Rudolph and Krueger [33]. After electrophoresis for 2 h, the gels were cut into two fragments, one of which was used for protein staining with 2D-silver stain II reagent (Daiichi Pure Chemical Co. Ltd.) and the other was subjected to Western-blot analysis.

Western-blot analysis

Proteins separated by SDS/PAGE (10 % gel) were transferred to an Immobilon-P membrane (Millipore Co., Bedford, MA, U.S.A.) by the method of Towbin et al. [34]. The membrane was incubated at 25 °C for 2 h in 4 % skimmed milk in 20 mM phosphate buffer, pH 7.3, containing 0.14 M NaCl and 2.7 mM KCl. After a washing procedure, the membrane was allowed to react first with antibodies raised against *P*-450 reductase, cytochrome b_{558} (two C-terminal peptides of large and small subunits) or glycine-rich NADPH-binding peptide of *P*-450 reductase and then with a horseradish peroxidase-linked secondary antibody (IgG, 1:5000 dilution) developed in goat. Antibodies with diluted with 20 mM PBS, pH 7.3, containing 0.1 % Tween 20, and each incubation was carried out for 1 h at 25 °C. The Immobilon-P membrane was washed extensively three times with 20 mM PBS, pH 7.3, containing 0.1 % Tween 20 (20 min each). The Western blots were stained for 15 min with 3,3′-diaminobenzidine in 20 mM PBS, pH 7.3, containing 0.03 % H_2O_2.

Determination of enzyme activities

The cell-free NADPH oxidase activity was assayed by measuring the arachidonic acid-elicited $O_2^{-\cdot}$ generation, which was determined by measuring the SOD-inhibitable ferricytochrome *c* reduction. The reaction mixture contained appropriate amounts of cytochrome b_{558}, cytosol, 10 μM GTP[S] and 250 μM arachidonate in a total volume of 0.2 ml. Two 95 μl portions of the reaction mixture were transferred to reference and sample cuvettes and then incubated for 10 min at 25 °C. At the end of the preincubation period, NADPH (0.2 mM), cytochrome *c* (0.08 mM) and 0.86 ml of buffer (10 mM Pipes, pH 7.0, containing 0.1 M KCl, 3 mM NaCl, 4 mM $MgCl_2$ and 0.5 mM PMSF) were added to initiate $O_2^{-\cdot}$ generation. SOD (80 μg) was added to the reference cuvette but not to the sample cuvette. When purified NADPH–cytochrome *c* reductase was added as a component for assay of $O_2^{-\cdot}$-generating activity, the reaction mixture containing purified cytochrome b_{558} and cytosol was first incubated with an appropriate amount of the reductase. Thereafter, a portion (95 μl) of the reconstituted mixture was assayed for $O_2^{-\cdot}$-forming activity as described above. Exogenous FAD was not added to the assay medium unless specified. An absorption coefficient of 18.5 $mM^{-1} \cdot cm^{-1}$ at 550 nm was used to calculate the quantity of cytochrome *c* reduced [35]. The rate of SOD-insensitive cytochrome *c* reduction was also measured in 1 ml of reaction medium consisting of 0.2 nM NADPH, 0.08 mM cytochrome *c*, purified enzyme and 80 μg of SOD. The reaction was started by addition of 0.2 mM NADPH.

Spectrophotometric measurements

The microsomal fraction from differentiated HL-60 cells was used for spectral studies. Difference spectra between the presence and absence of CO were recorded with a Hitachi U-3200 spectrophotometer. After the addition of a trace amount of dithionite to the microsomal suspension, the sample was transferred to two cuvettes and a baseline was recorded. One of the cuvettes was gently bubbled with CO at 3 °C. The difference spectrum between 400 and 600 nm was repeatedly scanned until no further change in absorbance was observed. Cytochrome *P*-450 was determined using a difference absorption coefficient (450 minus 490 nm) of 91 $mM^{-1} \cdot cm^{-1}$ [36].

RESULTS

Purification of cytochrome b_{558} and NADPH–cytochrome *c* reductase from neutrophil membranes

After solubilization of neutrophil membranes with 35 mM OG and 0.5% cholate in the presence of 10 μM FAD, the fractions containing NADPH–cytochrome *c* reductase activity and cytochrome b_{558} were separated, using a combination of column chromatography as shown in Figures 1–3. Cytochrome b_{558} was not adsorbed on the mixed-bed column (DEAE-Sepharose, CM-Sepharose and *N*-amino-octyl-agarose) equilibrated with buffer A, but it showed a strong affinity for the heparin–Sepharose column and was eluted with buffer A containing 0.5 M NaCl. The finally purified cytochrome b_{558} gave clearly distinguishable bands of the large and small subunits at 92 and 22 kDa regions by immunoblot analysis (Figure 3, inset). The dithionite reduced-minus-oxidized difference absorption spectrum (Figure 4, inset) of the purified preparation had absorption maxima at 427, 530 and 558 nm, which are characteristic of cytochrome b_{558} [37]. The

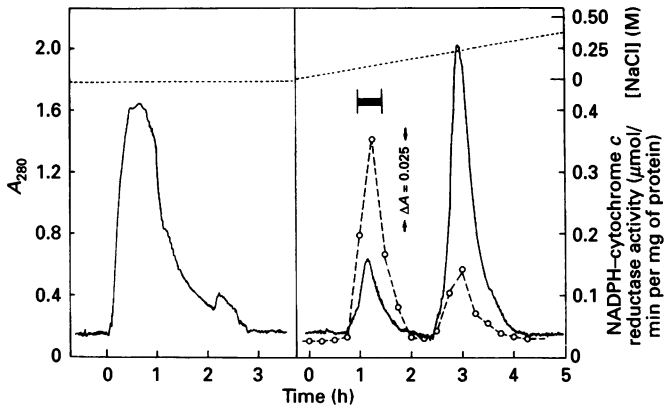


Figure 1 Mixed-bed (DEAE-Sepharose, CM-Sepharose and *N*-amino-octyl-agarose) column chromatography of solubilized neutrophil membranes

Freshly prepared membrane fraction (16.2 mg) from dormant cells was applied to the column, and membrane-associated proteins were separately eluted as described in the Materials and methods section. Absorbance at 280 nm (——) was monitored and 2 ml fractions were collected. A linear gradient (– – – –) was run from buffer C alone to buffer C containing 0.5 M NaCl. The activity of NADPH-dependent cytochrome *c* reductase (○) was measured at 550 nm. The fractions indicated by the horizontal bar were pooled and used for further purification.

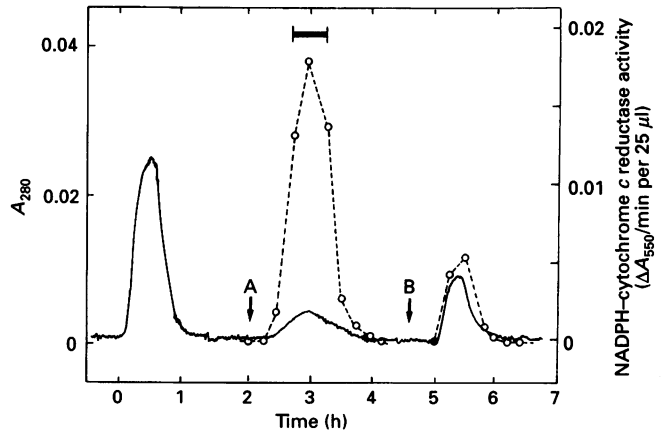


Figure 2 Affinity chromatography of cytochrome *c* reductase on 2′,5′-ADP–Sepharose

Pooled fractions from the mixed-bed column chromatography were dialysed and loaded on to the ADP–Sepharose column equilibrated with buffer C. Elution was changed at arrow A (buffer C plus 5 mM 2′-AMP) and arrow B (buffer C plus 0.5 M NaCl) respectively. The fractions were monitored for protein (——) at 280 nm and assayed for NADPH–cytochrome *c* reductase activity (○). The peak fraction indicated by the horizontal bar was pooled and represents the final preparation.

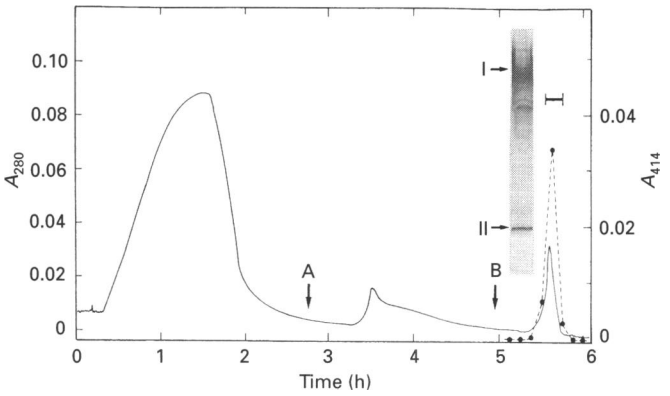


Figure 3 Affinity chromatography of cytochrome b_{558} on heparin–Sepharose

Cholate- and OG-solubilized membrane passed through a mixed bed of DEAE-Sepharose, CM-Sepharose and *N*-amino-octyl-agarose was applied to the column on heparin–Sepharose equilibrated with buffer A. After the column had been fully washed with equilibration buffer A, proteins were further eluted by a stepwise NaCl gradient (buffer A containing 0.1 M NaCl at arrow A and buffer A containing 0.5 M NaCl at arrow B). The flow rate was 12 ml/h and 1.5 ml fractions were collected. The fractions were monitored for protein (——) at 280 nm and cytochrome b_{558} was detected (– – – –) at 414 nm. The peak fraction indicated by the horizontal bar was loaded on to a desalting column equilibrated with buffer B, concentrated and applied to SDS/PAGE (3.4 μg) to examine the purity and to detect large (arrow I) and small (arrow II) subunits of cytochrome b_{558} by immunoblot analysis (inset). After transfer to an Immobilon-P membrane, the separated protein bands were treated with rabbit IgG that was immune against both the small and large subunits of human cytochrome b_{558}.

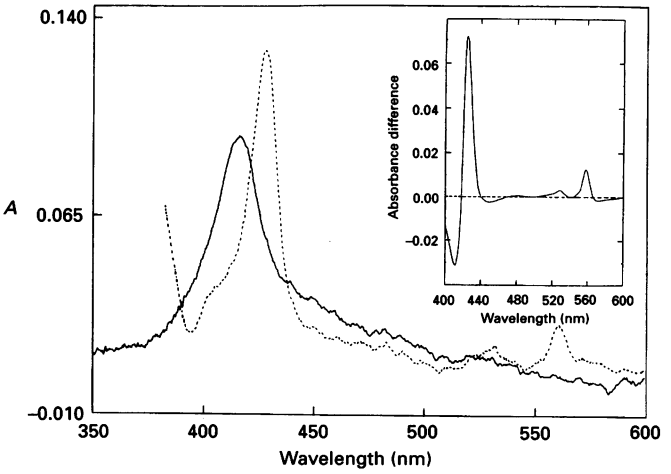


Figure 4 Absorption spectrum of the purified preparation of cytochrome b_{558}

The absorption spectra of both the oxidized form (——) and dithionite-reduced form (– – – –) of the cytochrome were measured in 1 ml of buffer B. The protein concentration of flavinated cytochrome b_{558} was 26.8 μg/ml. The reduced-minus-oxidized difference spectrum of the cytochrome is given in the inset.

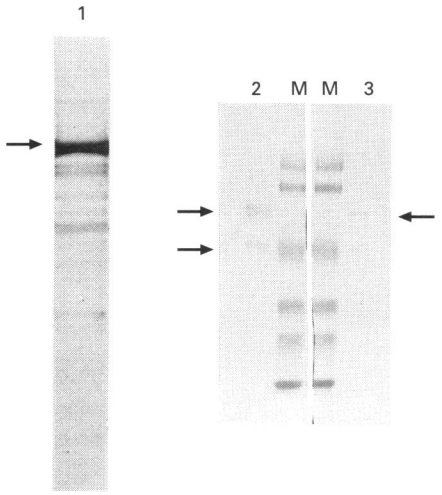


Figure 5 SDS/PAGE and immunoblot of cytochrome *c* reductase

The purified cytochrome *c* reductase (1.8 μg) was subjected to SDS/PAGE, followed by silver staining (lane 1). After transfer to an Immobilon-P membrane, the enzyme was treated with polyclonal antibody raised against either liver *P*-450 reductase (lane 2) or a synthetic peptide corresponding to the NADPH-binding domain of *P*-450 reductase (lane 3) as described in the Materials and methods section. Lane M shows prestained SDS/PAGE standards (phosphorylase *b*, 106 kDa; BSA, 80 kDa; ovalbumin, 50 kDa; carbonic anhydrase, 32.5 kDa; soyabean trypsin inhibitor, 27.5 kDa; lysozyme, 14.5 kDa). Visualized cross-reacting proteins and a purified major protein are indicated by arrows.

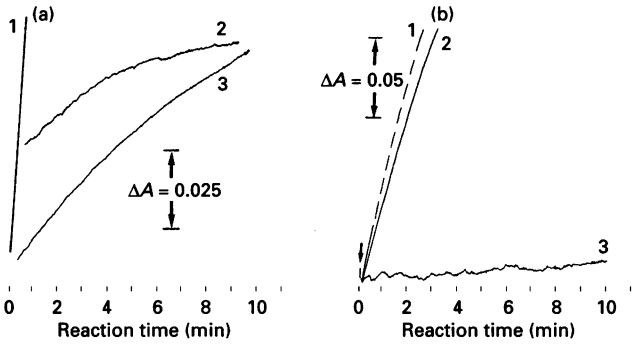


Figure 6 Time courses of SOD-insensitive and -sensitive NADPH-dependent cytochrome *c* reduction with purified cytochrome *c* reductase or flavinated cytochrome b_{558}

(**a**) SOD-insensitive cytochrome *c* reductase activity was measured with 0.36 μg of reductase (trace 1), 0.42 μg of cytochrome b_{558} (trace 2) or 8.6 μg of cytosol (trace 3) in the presence of 80 μg of SOD and 0.08 mM cytochrome *c*. The reaction was started by addition of 0.2 mM NADPH at 36 °C. (**b**) The cell-free system consisted of 0.84 μg of cytochrome b_{558}, 17.2 μg of cytosol, 10 μM GTP[S] and 250 μM arachidonate in 0.2 ml of medium A (10 mM Pipes, pH 7.0, containing 4 mM $MgCl_2$, 3 mM NaCl. 0.1 M KCl, 1 mM EDTA and 0.5 mM PMSF). This mixture was preincubated with (trace 1) or without (trace 2) purified reductase (0.72 μg) for 10 min at 25 °C. At the end of the preincubation period, 95 μl each of the preincubated mixture was added to reference and sample cuvettes containing 0.08 mM cytochrome *c* and medium A to measure $O_2^{-\cdot}$ generation. SOD (80 μg) was added to the reference cuvette only. The reaction was initiated by the addition of 0.2 mM NADPH to the assay medium (arrow). Cytosol was omitted from the incubation and flavinated cytochrome b_{558} alone was assayed for $O_2^{-\cdot}$-generating activity (trace 3).

final preparation passed through a desalting column (excellulose GF-5) contained 16.7 nmol of protohaem/mg of protein and 24.8 nmol of FAD/mg of protein, which appears to bind both the haem and flavin in a molar ratio of approx. 1 (flavinated cytochrome b_{558}). The yield of cytochrome was about 12.5 % on the basis of the haem content of solubilized membranes. After solubilization of the membrane fraction, cytochrome b_{558} was also prepared in the absence of FAD and phospholipids by using the same purification procedures as those for flavinated cytochrome b_{558} described in the Materials and methods section. The cytochrome purified without added FAD contained 16.5 nmol of haem/mg of protein, but a negligibly small amount of FAD (0.01 nmol of FAD/mg of protein).

After elution of cytochrome b_{558} from the mixed-bed column mentioned above, it was further washed with buffer C with or without a linear gradient of NaCl (0–0.5 M). Fractions from 2′,5′-ADP–Sepharose chromatography with the highest NADPH-dependent cytochrome *c* reductase activity were concentrated and employed in subsequent studies. The purity and minimum molecular mass of the purified cytochrome *c* reductase were determined by SDS/PAGE (Figure 5, lane 1). Silver staining of the polyacrylamide gel after electrophoresis revealed a major protein band migrating with a molecular mass of 68 kDa and several minor bands of lower molecular mass. Fluorophotometric analysis of flavins showed that the purified reductase contained nearly equimolar amounts of FAD (0.67 mol) and FMN (0.60 mol) per molecule of polypeptide chain. The low yields of the two flavins in the preparation appear to be due to their partial dissociation from the apoprotein during purification of the reductase.

Catalytic properties

Flavinated cytochrome b_{558} showed NADPH-dependent SOD-insensitive reduction of cytochrome *c*. The catalytic activity was 0.75 μmol of cytochrome *c* reduced/min per mg of cytochrome b_{558}, which was only 3.5 % of the activity of purified cytochrome *c* reductase (21.5 μmol of cytochrome *c* reduced/min per mg of protein) (Figure 6a). The cytosol also had very low NADPH-dependent cytochrome *c* reductase activity. A cytosolic flavoprotein with NADPH–cytochrome *c* reductase activity was highly purified from differentiated HL-60 cytosol, and its catalytic properties had similarities to those of liver microsomal *P*-450 reductase [19]. In contrast with liver *P*-450 reductase [17,18], our purified cytosolic cytochrome *c* reductase with a molecular mass of 68 kDa could hardly reduce NBT and cytochrome b_{558} [19]. These results suggest that the cytochrome *c* reductase is not likely to be related to the neutrophil respiratory-burst oxidase system [19].

As NADPH-dependent cytochrome *c* (or NBT) reductase activity is much higher in the neutrophil membrane than in the cytosol, and redox components for $O_2^{-\cdot}$ generation appear to be located in the membrane, we investigated the possible participation of the purified reductase in the reconstituted NADPH oxidase activity. The $O_2^{-\cdot}$-generating activity of the purified cytochrome b_{558} was measured in a cell-free assay system consisting of cytosol, arachidonate and GTP[S], with or without the presence of purified cytochrome *c* reductase. The flavinated cytochrome b_{558} alone generated an appreciable amount of $O_2^{-\cdot}$ (Figure 6b), but cytosol or cytochrome *c* reductase alone showed little $O_2^{-\cdot}$ production in the presence of arachidonate and FAD (Table 1). A reconstituted assay system composed of flavinated cytochrome b_{558} and cytosol showed markedly increased $O_2^{-\cdot}$-generating activity (Figure 6b). These results confirm previous reports [2,3] that cytosol and reflavination of purified cytochrome b_{558} are essential requirements for $O_2^{-\cdot}$ generation. In the presence of a constant amount of cytosol in the assay mixture, the purified cytochrome b_{558} indicated dose-dependent $O_2^{-\cdot}$-forming activity (Figure 7). The addition of neither FAD nor the purified

Table 1 Reconstitution of $O_2^{-\cdot}$-generating activity with purified cytochrome b_{558} and NADPH–cytochrome *c* reductase

The experimental conditions for reconstitution and assay systems were as described in Figure 6. In 1 ml of an assay medium, 0.36 μg of purified reductase, 0.42 μg of flavinated cytochrome b_{558} (or 0.42 μg of flavin-free cytochrome b_{558}*) and 8.6 μg of cytosol were added as indicated. Flavin-free cytochrome b_{558}* was prepared by the method described in the Materials and methods section except that FAD and phospholipids were not used in its purification process. For the assay system containing cytochrome b_{558}, the catalytic activities were expressed as μmol of cytochrome *c* reduced/min per mg of cytochrome.

Components	Haem content (nmol of haem/mg)	Flavin content (nmol/mg)	NADPH–cytochrome *c* reductase activity (μmol/min per mg of protein)	
			SOD-sensitive	SOD-insensitive
Cytochrome b_{558}	16.7	24.8 (FAD)	0.25	0.75
Cytosol	Negligible	0.02 (FAD)	Negligible	0.08
Cytochrome b_{558} + cytosol	–	–	8.53	–
Reductase	0.0	9.85 (FAD)/8.83 (FMN)	Negligible	21.5
Cytochrome b_{558} + reductase	–	–	0.30	–
Cytochrome b_{558} + reductase + cytosol	–	–	8.90	–
Cytochrome b_{558}*	16.2	0.01 (FAD)	Negligible	Negligible
Cytochrome b_{558}* + reductase	–	–	0.05	–
Cytochrome b_{558}* + reductase + 10 μM FAD	–	–	1.30	–
Reductase + cytosol	–	–	0.02	–

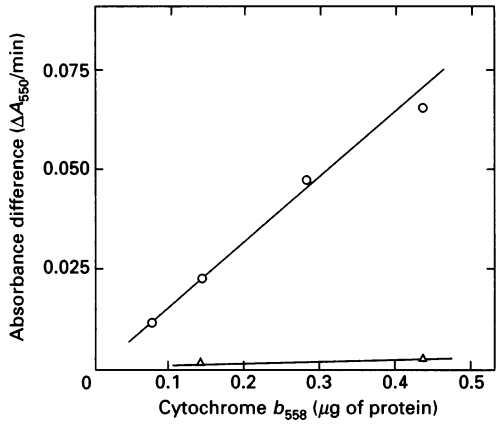


Figure 7 Effect of increasing concentrations of flavinated cytochrome b_{558} on $O_2^{-\cdot}$ generation

$O_2^{-\cdot}$ production was measured at different concentrations of cytochrome b_{558} (0.07–0.42 μg) without purified reductase (○). △, Cytosol was omitted from the incubation and assay media. The other conditions were as described in Figure 6.

cytochrome *c* reductase had any marked stimulatory effect on the reconstituted NADPH oxidase activity. In fact, the reconstituted system consisting of cytochrome *c* reductase, flavinated cytochrome b_{558} and cytosol had the same $O_2^{-\cdot}$-producing activity (8.90 μmol of $O_2^{-\cdot}$/min per mg of cytochrome b_{558}) as that seen in the system with flavinated cytochrome b_{558} and cytosol fraction (8.53 μmol of $O_2^{-\cdot}$/min per mg of cytochrome b_{558}) as shown in Figure 6(b) and Table 1. In agreement with previous results [19], it seems unlikely that electrons are catalytically transferred from NADPH to O_2 via the reductase and cytochrome b_{558} in the reconstituted system. On the other hand, purified flavin-free cytochrome b_{558} shows little or no NADPH-dependent diaphorase activity (Table 1). This cytochrome b_{558} did not exhibit $O_2^{-\cdot}$-forming capability in the presence of purified cytochrome *c* reductase and/or cytosol in the reconstituted system. The NADPH oxidase system containing flavin-free cytochrome b_{558} and cytosol was significantly stimulated by addition of 10 μM FAD to the assay mixture. However, the NADPH oxidase activity was approx. 15.3% of that observed in the assay system containing flavinated cytochrome b_{558} and cytosol. Purified cytochrome *c* reductase has little stimulatory effect on the oxidase system containing flavin-free cytochrome b_{558}.

When cytochrome *c* was used at fixed saturating concentration of 0.2 mM and NADPH was the variable substrate, the purified cytochrome *c* reductase exhibited typical hyperbolic kinetics with respect to NADPH concentrations. The K_m value for NADPH of the enzyme was determined from Lineweaver–Burk plots to be 2.20 μM, which was very close to the values obtained for the cytochrome *c* reductase of HL-60 cytosol (1.50 μM) and liver *P*-450 reductase (2.53 μM) [19]. With respect to NADPH, half-maximal activity in the reconstituted cell-free system consisting of flavinated cytochrome b_{558} and cytosol was observed at a concentration of about 58.8 μM which is a little higher than the value (40–50 μM) previously reported [14]. These considerably different K_m values between the purified cytochrome *c* reductase and reconstituted NADPH oxidase did not support the idea that cytochrome *c* reductase acts as an NADPH-binding and flavin-containing component to reduce cytochrome b_{558} in the respiratory-burst oxidase system.

Western blot

We examined whether the cytochrome *c* reductase purified from neutrophil membranes shares antigenic determinants with the microsomal *P*-450 reductase from rabbit liver. After SDS/PAGE of the purified reductase, an Immobilon-P membrane was incubated with either IgG fractions immune to *P*-450 reductase or the glycine-rich synthetic peptide (residues 529–543 of *P*-450 reductase) or non-immune fractions, and colour was developed using the peroxidase-linked second-antibody method. As shown in Figure 5, a band corresponding to an apparent molecular mass of 68 kDa was consistently detected in the purified flavoprotein. This molecular mass is a little lower than that (76 kDa) seen in freshly prepared neutrophil membrane (Figure 8, lane 1) or in

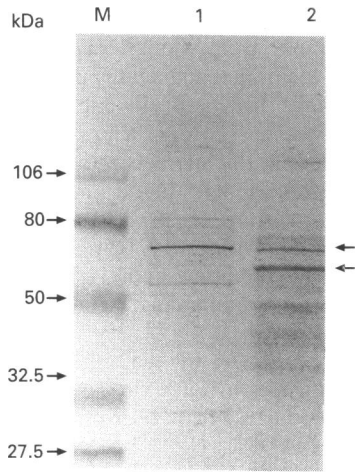


Figure 8 Immunoblot analysis of solubilized neutrophil membranes by antibodies against liver *P*-450 reductase

The solubilized membranes (each 25 μg of protein) were subjected to SDS/PAGE before (lane 1) and after (lane 2) their incubation for a week at 3 °C. After transfer to an Immobilon-P membrane, the separated protein bands were treated with either polyclonal antibodies (IgG fraction) to hepatic *P*-450 reductase developed in guinea pig or non-immune guinea-pig IgG. Lane M shows prestained SDS/PAGE standards which are the same as those described in Figure 5. The arrows on the right side indicate positively immunoreacted protein bands.

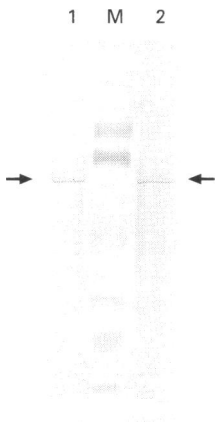


Figure 9 Binding of IgG against either liver *P*-450 reductase or a glycine-rich synthetic peptide by Western-blot analysis

Proteins of the microsomal fractions (12.5 μg) from differentiated HL-60 cells were subjected to SDS/PAGE and then transferred to an Immobilon-P membrane at 180 mA for 3 h using a Bio-Rad transblot cell. Microsomal proteins separated on the gel were allowed to react with antibodies raised against the synthetic peptide (lane 1) or the *P*-450 reductase (lane 2). Cross-reacting proteins indicated by arrows were visualized as described in the Materials and methods section. Lane M contained marked proteins with known molecular masses as described in Figure 5.

detergent-solubilized intact *P*-450 reductase from rabbit liver (results not shown). When the solubilized membranes were incubated for a week at 3 °C, one newly immunoreacted band of approximate molecular mass of 68 kDa was visualized and the 76 kDa band became weaker (lane 2). Hepatic *P*-450 reductase that had undergone a limited trypsinolysis also exhibited positive cross-reactivity at 68 kDa on immunoblot analysis (results not shown). However, non-immune IgG produced no clear positive bands to these proteins. These results possibly indicate that the purified cytochrome *c* reductase possesses regions that are closely homologous to liver microsomal *P*-450 reductase. In addition, immunoblot analysis using antibodies against the whole molecule of *P*-450 reductase (Figure 5, lane 2) indicated the weak presence of a smaller polypeptide with an approximate molecular mass of 50 kDa as well as the 68 kDa enzyme in the purified preparation. However, the immune IgG to the synthetic peptide showed only the 68 kDa protein. These results suggest that the 50 kDa band is a proteolysed form of the reductase which lacks an NADPH-binding domain. Together with the data demonstrated in Figure 8, the immunoblots indicate that the purified reductase with antigenic similarities to liver *P*-450 reductase is susceptible to undefined endogenous proteases and is rapidly degraded, resulting in loss of all catalytic activity except for cytochrome *c* reduction. In the present purification we were unable to isolate the cytochrome *c* reductase of molecular mass approx. 76 kDa, which corresponds to intact microsomal *P*-450 reductase, probably because of its rapid partial cleavage after disruption of neutrophils and solubilization of the membranes.

NADPH–cytochrome *c* reductase activity and CO difference spectrum of microsomal fraction from differentiated HL-60 cells

NADPH-dependent cytochrome *c* reductase activity was concentrated in the microsomal fraction prepared from HL-60 cells. The microsomal reductase showed a $V_{max.}$ of 0.12 μmol of cytochrome *c* reduced/min per mg of solubilized membrane and an apparent K_m for NADPH of 1.91 μM, which was similar to

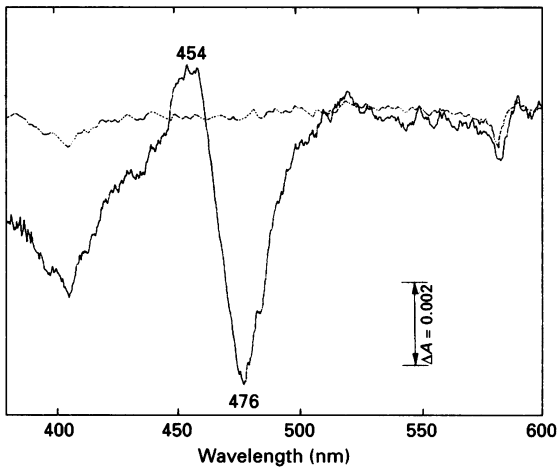


Figure 10 CO difference spectrum of differentiated HL-60 microsomal fraction

After addition of a trace amount of dithionite, the microsomes (1.25 mg of protein/ml) were added to reference and sample cuvettes and a baseline was recorded (····). The sample cuvette was then saturated with CO and the difference spectrum was measured (——).

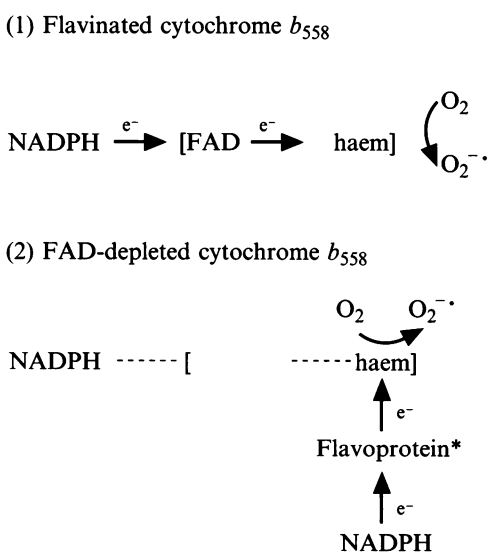
that obtained for the purified cytochrome *c* reductase from neutrophil membrane. In addition, the solubilized microsomes passed through a 2′,5′-ADP–Sepharose column significantly lost NADPH-dependent cytochrome *c* reductase activity (approx. 50% loss). Antibodies raised against either liver *P*-450 reductase or the synthetic peptide also demonstrated that the 76 kDa protein was clearly present in the solubilized microsomal fraction (Figure 9). The 76 kDa band was mainly lost on passing freshly prepared solubilized microsomes through an 2′,5′-ADP–Sepharose column (results not shown). In agreement with the results obtained for neutrophil membranes (Figure 8), incubation

of the solubilized microsomes caused a considerable conversion of the 76 kDa protein into the 68 kDa form.

When CO was bubbled into a suspension of the solubilized microsomal fraction after its reduction with dithionite, the difference spectrum showed a peak at about 450 nm (Figure 10), suggesting the presence of a cytochrome *P*-450-like haemoprotein. The specific content of the cytochrome *P*-450-like haemoprotein in the separated microsomal proteins was approx. 32 pmol/mg of protein. This value is much less than that in phenobarbital-treated liver microsomes (3.1 nmol/mg of protein) [38]. These spectral and immunological assays suggest that the purified cytochrome *c* reductase from human neutrophils is located in microsomes and transfers electrons from NADPH to cytochrome *P*-450 in a mixed-function oxidase system.

DISCUSSION

In the membrane fraction of human neutrophils we have demonstrated the presence of 76 and 68 kDa proteins cross-reacting with antibodies raised against liver *P*-450 reductase. However, the membrane-associated 76 kDa flavoprotein readily undergoes proteolysis, even in the presence of antiproteases, being converted into the 68 kDa form during prolonged incubation of the membrane (Figure 8) and purification of the NADPH–cytochrome *c* reductase. This limited cleavage appears to be very fast, and Western-blot analysis showed that the final purified preparation contained the 68 kDa protein and did not include the 76 kDa form (Figure 5). In addition, we tried to separate microsomes to the highest possible purity from differentiated HL-60 cells at the expense of yield. NADPH–cytochrome *c* reductase activity and the 76 kDa band were clearly found in the microsomal fraction of HL-60 cells. The 76 kDa enzyme was readily converted into a water-soluble 68 kDa form on incubation of solubilized microsomes at 3 °C, which consistently occurred in solubilized membranes from human neutrophils. Thus it is assumed that the cytochrome *c* reductase purified from neutrophil membranes is mainly distributed in microsomes and is in a partially proteolysed state. The 68 kDa flavoprotein (FMN/FAD approx. 1), which specifically interacts with antibodies to liver *P*-450 reductase, was occasionally found in the cytosolic fraction from human neutrophils and HL-60 cells; it was highly purified and characterized in a previous study [19]. These results highlight the fact that the native membrane-bound cytochrome *c* reductase is readily cleaved and contaminated in cytosol fractions after disruption of granulocytes.



Scheme 1 Pathways of electron transport in $O_2^{-\cdot}$ generation by cytochrome b_{558}

(1) Proposed physiological pathway; (2) non-physiological pathway operational when cytochrome b_{558} is depleted of FAD. In (2) the NADPH–cytochrome *P*-450 reductase acts as a flavoprotein for reduction of the cytochrome b_{558} haem.

Some enzymic properties of the purified reductase from neutrophil membrane are obviously similar to those of NADPH–cytochrome *P*-450 reductase from liver microsomes: almost the same K_m value for NADPH; NADH is a non-effective electron donor; the same coenzyme (FAD + FMN) specificities; the same susceptibility to limited proteolysis (76 kDa into 68 kDa conversion); higher affinity for cytochrome *c* than for NBT as an electron acceptor; and positive cross-reaction with antibodies raised against liver *P*-450 reductase. These results suggest that the two flavoproteins have identical and/or closely homologous regions in their protein structures.

The NADPH–cytochrome *c* reductase did not catalyse $O_2^{-\cdot}$ production in the presence or absence of cytosol. Furthermore, the purified reductase showed no stimulatory effect on $O_2^{-\cdot}$ production in the reconstituted system consisting of either flavinated or flavin-free cytochrome b_{558} and cytosol (Figure 6, Table 1). In contrast with our present data, Isogai et al. [17,18] reported that cytochrome b_{558}, which was depleted of FAD in the purification process, was found to be reduced by NADPH in the presence of native hepatic NADPH–cytochrome *P*-450 reductase and to generate $O_2^{-\cdot}$ in the reconstituted system without cytosolic components and amphiphiles (0.73 μmol of $O_2^{-\cdot}$/min per mg of cytochrome b_{558}) (Scheme 1). Miki et al. [16] also demonstrated that the $O_2^{-\cdot}$-producing activity was greatly stimulated by addition of a partially purified NADPH–NBT reductase to a reconstituted cell-free system composed of cytochrome b_{558}, and it was about seven times higher than that (2.26 μmol of $O_2^{-\cdot}$/min per mg of cytochrome b_{558}) observed in the system containing 5 μM FAD instead of the NBT reductase. On the basis of this evidence, they suggested that the flavin-dependent NADPH dehydrogenase and cytochrome b_{558} are essential membrane-bound components for $O_2^{-\cdot}$-forming NADPH oxidase activity. This inconsistency between their results and ours may be due to the fact that our highly purified flavoenzyme may have lost a small segment(s) during its purification, which is essential for protein–protein and protein–membrane interactions, but their flavoprotein, being in the native form, retained this membranous hydrophobic peptide. Thus it is assumed that our purified reductase, having lost a critical peptide(s), is incapable of interacting with cytochrome b_{558}, resulting in no stimulatory effect on $O_2^{-\cdot}$ generation in the reconstituted system. As shown previously [39], intact hepatic NADPH–cytochrome *P*-450 reductase is amphiphilic, the hydrophilic catalytic domain being C-terminal and the hydrophobic membranous segment being the N-terminal peptide, which readily undergoes tryptic attack. The detergent-solubilized intact *P*-450 reductase has activity toward cytochrome *P*-450 [40,41] and cytochrome b_5 [42] in a reconstituted system, whereas the protease-solubilized reductase still possesses catalytic activity for cytochrome *c* reduction but it is not functional in the reduction of either of these two membrane-bound cytochromes. Clearly, the hydrophobic peptide of the *P*-450 reductase is essential for physiological function, and it is suggested that specific interactions occur between the hydro-

phobic tail of the *P*-450 reductase and the hydrophobic domain of either cytochrome *P*-450 or cytochrome b_5.

In the present study we demonstrate that the purified cytochrome *c* reductase and cytochrome *P*-450-like protein are predominantly found in the microsomal fraction of HL-60 cells, and marked $O_2^{-\cdot}$-forming NADPH oxidase activity is observed in a cell-free system containing flavinated cytochrome b_{558} alone as redox component (Scheme 1). On the basis of the subcellular localization and enzymic characterization of NADPH–cytochrome *c* reductase purified from neutrophil membrane, it may be concluded that the flavoprotein is not likely to be related to the neutrophil respiratory-burst oxidase, but is involved in cytochrome *P*-450-dependent mixed-function oxidase in granulocyte microsomes. Thus it is assumed that electron transport in $O_2^{-\cdot}$ generation by cytochrome b_{558} proposed in Scheme 1 (pathway 1) is a physiological pathway, and pathway 2 in which NADPH–cytochrome *P*-450 reductase acts as a flavoprotein for reduction of the cytochrome b_{558} haem is non-physiological. In fact, a previous paper [21] demonstrated that NADPH–cytochrome *c* reductase is located in the microsomal membrane of human neutrophils and participates in the cytochrome *P*-450-dependent mixed-function oxidase because antibodies raised against liver *P*-450 reductase inhibited NADPH–cytochrome *c* reductase activity as well as leukotriene B_4 ω-hydroxylase activity of microsomes. In addition, isolation of cDNA clones for leukotriene B_4 ω-hydroxylase of human neutrophils showed that the hydroxylase is a novel form of cytochrome *P*-450 in microsomes and comprises a new subfamily of the cytochrome [43]. Although it is very difficult to isolate native 76 kDa flavoprotein and cytochrome *P*-450 from the microsomal fraction because of their low contents and rapid partial cleavage after solubilization, further studies are now in progress to investigate the biological function of the NADPH-linked electron-linked electron-transfer system in neutrophil microsomes.

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