Purification of cytochrome *b*_{–245} from human neutrophils

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The low potential cytochrome *b* (*b*_{–245}) of the microbicidal oxidase of phagocytic cells has been purified from neutrophils from patients with chronic myeloid leukaemia. Cells were homogenized in the presence of proteinase inhibitors and centrifuged to remove the cytoplasm. The pellets containing membranes, granules and other organelles (15 mg/ml) were then washed with buffered sodium cholate (5 mg/ml). Residual pellets were subsequently solubilized with the non-ionic detergent Triton N101 (10 mg/ml) which extracted about 60% of the cytochrome *b*. About 10% of the cytochrome *b* was of mitochondrial origin which was removed on a column of *n*-amino-octyl-Sepharose that did not adsorb cytochrome *b*_{–245}. Cytochrome *b*_{–245} was chromatographed on a column of heparin-agarose and eluted with NaCl to give a peak specific content of 11–16 nmol of cytochrome *b*_{–245}/mg of protein, representing a 140–200-fold purification with a recovery of 15%. This technique results in the purification of approx. 100–150 nmol of highly purified cytochrome *b*_{–245} from (3–5) × 10¹¹ cells within 4 days. The most purified material gave a broad band with an apparent *M_r* of between 68 000 and 78 000 on sodium dodecyl sulphate/polyacrylamide gel electrophoresis, but gel filtration indicated an aggregated form of the protein in Triton N101. Purified protein (14 nmol of haem/mg of protein) did not contain FAD or FMN and had no NADPH-dependent O₂[–]-generating activity.

Neutrophil polymorphonuclear leucocytes, or neutrophils, are phagocytic cells of the blood and form part of the host defence system against bacterial infection. The mechanisms they possess to kill microbes include an oxidase system that generates H₂O₂ and oxygen radicals (see reviews by Badwey & Karnovsky, 1980; Root & Cohen, 1981). The nature of this oxidase system has been the subject of much study, but purification and characterization of its components have been hampered by the instability of the oxidase enzyme on cell fractionation (see, for example, Tauber & Goetzl, 1979; Light *et al.*, 1981; Wakeyama *et al.*, 1982). There is strong evidence, however, that a low potential cytochrome *b* (*E_m*_{7.0} = –245 mV) present in these (Segal & Jones, 1978) and other phagocytic cells (Segal *et al.*, 1981) is a component of the oxidase system (Cross *et al.*, 1981, 1982a; Roberts *et al.*, 1982; Segal *et al.*, 1983a) and is probably the terminal oxidase itself.

Abbreviations used: Dip-F, di-isopropyl phosphorfluoridate; PMSF, phenylmethanesulphonyl fluoride; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulphonate; SDS, sodium dodecyl sulphate.

These studies describe a 150–200-fold purification of this cytochrome *b* and we document some of its properties.

Experimental

Materials and buffers

Heparin (preservative free, 1000 i.u./ml) was purchased from Paines and Byrne (Greenford, Middx. U.K.), Trasylol (20 000 k.-i.u./ml) from Bayer U.K. (Haywards Heath, W. Sussex, U.K.). Detergents were purchased from Sigma and used without further purification. Molecular weight standards for SDS electrophoresis, carboxymethyl- and phenyl-Sepharoses, DEAE-Sephadex and Sephadex G-200 were from Pharmacia. Blue A-agarose was from Amicon (Woking, Surrey, U.K.) and heparin-agarose was obtained from Bethesda Research Laboratories (Cambridge, U.K.). *n*-Octylamino-Sepharose 4B, prepared as described by Guengerich & Martin (1980), was kindly provided by Dr. E. Shephard of the Department of Biochemistry, University College London. All other chemicals were purchased from Sigma.

Buffer A was a slightly modified buffer as used by van der Hoeven & Coon (1974) and contained Tris/acetate, pH 7.4 (100 mM), KCl (0.1 M), glycerol (20% v/v), dithiothreitol (1 mM), EDTA (1 mM) and butylated hydroxytoluene (30 μ M). Buffer B contained Tris/acetate, pH 7.4 (10 mM), glycerol (20% v/v), dithiothreitol (0.1 mM), EDTA (1 mM) and butylated hydroxytoluene (30 μ M).

Purification of cells and preparation of material for detergent extraction

Leucocytes were collected by leukapheresis from patients with chronic myeloid leukemia. Cells were pelleted and resuspended in 0.15 M-NH₄Cl (containing heparin, 5 i.u./ml) to lyse residual red cells. Homogenization, fractionation, and subsequent purification of cytochrome *b*₋₂₄₅ were carried out at 4°C and as a precaution against proteolysis PMSF (1 mM) was added throughout. In some experiments, as a further precaution, cells [(5–7) × 10⁸/ml] were treated with Dip-F (2 mM) in sucrose (8.6%, w/v) containing EDTA (1 mM, pH 7.4), heparin (5 i.u./ml), PMSF (1 mM) and Trasylol (100 k.i.u./ml) for several hours at 4°C and then washed and homogenized in the same mixture but without Dip-F. The proteinase inhibitors PMSF (1 mM) and Trasylol (100 k.i.u./ml) were then used throughout the extraction and purification of cytochrome *b*₋₂₄₅. Cells (3 × 10¹¹) were homogenized in sucrose (8.6%, w/v; 1 litre) containing EDTA (1 mM, pH 7.4) and heparin (5 i.u./ml) with a Polytron homogenizer (Northern Media Supply, Brough, North Humberside, U.K.) to give 90% breakage. The homogenate was centrifuged at 800g for 10 min to sediment nuclei and unbroken cells. The resulting post-nuclear supernatant was removed and the pellet again homogenized and centrifuged as before. The post-nuclear supernatants were combined and centrifuged (69 900g_{av.}, 45 min), and the supernatant was discarded. The pelleted particulate material was resuspended in detergent in buffer A with the Polytron homogenizer.

Activation of neutrophils and preparation of sub-cellular particles

Human neutrophils were isolated from buffy coat residues as described previously (Segal & Peters, 1977). Neutrophils (2.5 × 10⁹) were activated with phorbol myristic acid and their oxygen consumption measured as described by Cross *et al.* (1981) except that activation was for 5 min. The cell suspension and control unstimulated cells were then taken into ice-cold 0.9% NaCl and a post-nuclear supernatant was prepared as described by Segal & Peters (1977) except that solutions additionally contained PMSF (1 mM). This was centrifuged (69 900g_{av.}, 45 min) and the supernatant was discarded.

Chromatography of extracts

The Triton N101 (10 mg/ml) extracts of cytochrome *b*₋₂₄₅ (20 ml, approx. 1 μ M, approx. 600 pmol of cytochrome *b*₋₂₄₅/mg of protein) in buffer A or buffer B were run at 5 ml/h onto columns (1 cm diameter), of CM-Sepharose (10 ml), DEAE-Sephadex (10 ml), *n*-amino-octyl-Sepharose (5 ml), heparin-agarose (5 ml), Blue A-agarose (5 ml) or phenyl-Sepharose (5 ml) pre-equilibrated with the same buffer. All chromatography was carried out at 4°C. Columns were washed with the appropriate buffer until there was no protein in the eluate and the adsorbed material was eluted with NaCl in the same buffer, except for the column of *n*-amino-octyl-Sepharose which was eluted with 1% sodium deoxycholate in buffer A, containing Triton N101. Gel filtration was carried out on a column of Sephadex G-200 (1 cm diameter, 20 ml volume) equilibrated with buffer A containing Triton N101. Blue Dextran was used to determine the void volume.

Spectroscopy

The concentration of cytochrome *b*₋₂₄₅ was assayed by reduced-minus-oxidized spectroscopy using $\Delta\epsilon_{559-540} = 21.6 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ (Cross *et al.*, 1982a). Where contaminating cytochromes were present, potassium ferricyanide was added to the reference sample to ensure these were fully oxidized. Addition of ferricyanide to the reference cuvette had no effect on the spectrum produced by cytochrome *b*₋₂₄₅. NADH or NADPH were added to the sample cuvette to initiate reduction, after cuvettes were made anaerobic as described by Cross *et al.* (1982a). CO-difference spectra were obtained by reducing both samples with dithionite and then bubbling CO through one cuvette. Oxidation reduction potentials were measured as previously described (Cross *et al.*, 1981). We are very grateful to Dr. A. R. Cross, Department of Biochemistry, University of Bristol, for performing these titrations.

An estimate of myeloperoxidase in extracts was made from the reduced-minus-oxidized spectrum ($\Delta\epsilon_{473-501} = 74000 \text{ M}^{-1} \cdot \text{cm}^{-1}$) (Wever *et al.*, 1976).

Fluorimetry and assay of FAD and FMN

Excitation and emission spectra were recorded with a Perkin-Elmer 3000 fluorimeter. FAD and FMN were extracted and assayed as described by Faeder & Siegel (1973) except that samples were boiled for 5 min to remove protein from the flavin.

Assay of enzymic superoxide production

NADPH-dependent superoxide generation was measured by following superoxide dismutase inhibitable reduction of cytochrome *c* as described by

Tauber & Goetzl (1979), except that incubations were at 37°C and both reference and sample cuvette additionally contained FAD (50 μM) and NaN₃ (2mM). An absorption coefficient of 21100M⁻¹.cm⁻¹ was used for (reduced-minus-oxidized) cytochrome *c* (van Gelder & Slater, 1962).

Protein determination

Protein concentrations were estimated as described by Lowry *et al.* (1951) or Bensadoun & Weinstein (1976) where protein concentrations were low and there were interfering substances present. The latter assay was modified as follows: precipitation with trichloroacetic acid was overnight and the precipitates were redissolved and assayed as described by Markwell *et al.* (1978).

Electrophoresis

Analytical SDS/polyacrylamide-gel electrophoresis was carried out on slab gels as described previously (Segal *et al.*, 1983b) except that samples were prepared for electrophoresis in buffer B (10mg of Triton N101/ml) additionally containing 100mM-dithiothreitol, 2mM-EDTA and 2% SDS. SDS gels were stained for carbohydrate with Schiff's reagent following the method of Glossmann & Neville (1971). Where gels were to be stained for haem, samples were first dialysed to remove dithiothreitol and butylated hydroxytoluene, and dithiothreitol was omitted from the sample buffer. Samples were not boiled. Gels were stained for haem and subsequently for protein according to Thomas *et al.* (1976).

Results

Solubilization of cytochrome *b*₋₂₄₅

Triton N101 and Triton X-100 were the most effective solubilizers of cytochrome *b*₋₂₄₅ (Table 1a), whereas sodium cholate and Tween 20 were very poor at a detergent: protein ratio of 1:1.8. Increasing the ratio of detergent to protein increased solubilization by various detergents, but lowering the ionic strength of the buffer or omitting KCl from buffer A decreased solubilization (results not shown). A two-stage detergent extraction procedure was thus developed for solubilization of cytochrome *b*₋₂₄₅ to increase its specific content. An initial solubilization of particulate material with cholate or Tween 20 followed by solubilization of cytochrome *b*₋₂₄₅ with Triton N101 gave the best combination of detergents (Table 1b). The concentration ratio of protein to detergent in the prewash was critical in order to optimize yield and specific content of cytochrome *b*₋₂₄₅ in the Triton N101 extract. In the standardized extraction (Fig. 1) pelleted organelles (15mg of protein/ml) were extracted twice with sodium

Table 1. Solubilization of cytochrome *b*₋₂₄₅

Organelles were initially extracted with various detergents (a) and then with Triton N101 (b). Neutrophil organelles were suspended in buffer A at 18 mg of protein/ml (with a specific content of cytochrome *b*₋₂₄₅ = 80 pmol/mg of protein), and 10 mg of detergent/ml except for CHAPS, where the concentration was 6 mg/ml (10mM). Mixtures were centrifuged immediately (69900 g, 45 min.), the pellets resuspended in Triton N101 (10mg/ml) and centrifuged again.

	Cytochrome <i>b</i> ₋₂₄₅			
	(a) In initial detergent extract		(b) In subsequent Triton extract	
	% of total	Specific content (pmol/mg of protein)	% of total	Specific content (pmol/mg of protein)
Sodium deoxycholate	27	72	35	139
Sodium cholate	†	†	51	236
CHAPS	12	45	19	139
Cetyl trimethyl ammonium bromide	†	†	18	109
Triton N101	38	130	16	85
Triton X100	31	111	18	98
Emulgen 911	28	72	21	113
Brij 35	10	40	34	180
Tween 20	6	26	54	299
				Concentration of myeloperoxidase (μM)
				0.32
				0.05
				0.17
				0.27
				1.1
				1.1
				1.1
				1.1
				1.5

* Not determined but well in excess of 4.6 μM.
† Minimum solubilization, exact quantification obscured by myeloperoxidase.

Fig. 1. Flow scheme for the isolation of cytochrome *b*₋₂₄₅ from human neutrophils

cholate (5 mg/ml) and then twice with Triton N101 (10 mg/ml). The combined Triton N101 extracts contained 60–65% of the cytochrome *b*₋₂₄₅ with a specific content of 600–700 pmol of cytochrome *b*₋₂₄₅/mg of protein. These figures include a mitochondrial cytochrome *b* component (usually 10%) which was not distinguished at this stage. Cytochrome *b*₋₂₄₅ was spectroscopically stable in buffer A containing Triton N101 (10 mg/ml) at 4°C for at least 3 weeks. The effect of different detergents on cytochrome *b*₋₂₄₅ was not systematically studied, although addition of cetyltrimethylammonium bromide to the Triton N101 extract was observed to denature the protein.

Chromatography of cytochrome *b*₋₂₄₅

About 20% of the Triton N101-solubilized cytochrome *b*₋₂₄₅ (specific content 600–800 pmol/mg of protein) was adsorbed to a column of *n*-amino-octyl-Sephacrose. The adsorbed material was eluted with sodium deoxycholate and found to contain a large number of mitochondrial cytochromes, including mitochondrial cytochrome *b*. Redox titration of the Triton N101-solubilized cytochrome *b*₋₂₄₅ indicated that there was an approx. 10% contamination with higher potential mitochondrial cytochrome *b* and that this was

removed on the column of *n*-amino-octyl-Sephacrose. Occasionally there was a higher percentage contamination with mitochondrial cytochrome *b* when extracts were made from cells with a greater proportion of immature forms.

Triton N101-solubilized cytochrome *b*₋₂₄₅ failed to bind to CM-Sephacrose and DEAE-Sephadex in buffer A. Lowering the ionic strength of the extract by dialysis to 10 mM-Tris/acetate, pH 7.4, (buffer B) resulted in binding to CM-Sephacrose but not to DEAE-Sephadex. Cytochrome *b*₋₂₄₅ was quantitatively eluted (> 70%) from CM-Sephacrose but further studies were not made because dialysis prolonged the procedure and dilution gave rise to large volumes that were impractical to handle. Cytochrome *b*₋₂₄₅ did not bind to phenyl-Sephacrose pre-equilibrated with buffer A containing 1% Triton N101. The addition of (NH₄)₂SO₄ to the running buffer to promote the strength of the hydrophobic interactions was impractical as it salted out the protein-detergent micelles. If columns of phenyl-Sephacrose were equilibrated with buffer A without detergent, cytochrome *b*₋₂₄₅ was absorbed to the resin from which it could be eluted with buffer containing Triton N101, but this was unsatisfactory in terms of a large-scale purification technique.

The majority of the cytochrome *b*₋₂₄₅ was found to bind to heparin-agarose and Blue A-agarose in buffer A and could be eluted (>70%) from both resins with NaCl. If the cholate prewash was omitted, adsorption of cytochrome *b*₋₂₄₅ to heparin-agarose was greatly reduced and erratic. Attempts were made to exchange detergents by eluting cytochrome *b*₋₂₄₅ from heparin-agarose with buffers containing sodium deoxycholate or cholate and NaCl. Problems were encountered, however, with a poor recovery of cytochrome *b*₋₂₄₅ from heparin-agarose (<50%) and there were difficulties in maintaining the eluted cytochrome *b*₋₂₄₅ in solution.

Our routine chromatographic purification of cytochrome *b*₋₂₄₅ (Fig. 1) was developed as follows: after a cholate prewash, the Triton N101-solubilized extract in buffer A was passed through a column of *n*-amino-octyl-Sepharose to remove mitochondrial cytochrome *b* contaminants (approx. 10%). The eluate was passed through a column of heparin-agarose connected in tandem to

Fig. 2. Chromatography on heparin-agarose of a Triton N101 extract of cytochrome *b*₋₂₄₅

A Triton N101-solubilized extract (430ml, 1.1 μM-cytochrome *b*₋₂₄₅, 1.11mg of protein/ml) was applied to a column of heparin-agarose (20ml, 1cm diameter) at a flow rate of 20ml/h. After washing (see Fig. 1) the cytochrome *b*₋₂₄₅ was eluted with a gradient of NaCl (70ml of buffer A containing 10mg of Triton N101/ml and 0.1–0.25M-NaCl; 14ml/h) and collected in 2.2ml fractions.

Fig. 3. SDS/polyacrylamide-gel electrophoresis of the different stages of purification of cytochrome *b*₋₂₄₅. Purification was from cells treated first with Dip-F and then in the presence of PMSF and Trasylol. Tracks: 1, organelles (165 μg of protein, <0.02 nmol of cytochrome *b*₋₂₄₅); 2, cholate wash (300 μg, 0 nmol); 3, Triton N101 extract (39 μg, 0.03 nmol); 4, cytochrome *b*₋₂₄₅ after passage through *n*-amino-octyl-Sepharose (16 μg, 0.02 nmol); 5, non-adsorbed material from heparin-agarose (14 μg, <0.003 nmol); 6, *M_r* markers [values (× 10⁻³) are indicated at the right of the Figure]; 7, peak eluted fraction from heparin-agarose (24 μg, 0.48 nmol); 8, buffer B + proteinase inhibitors.

Table 2. Purification of cytochrome *b*₋₂₄₅

Values are means ± s.d. for three experiments. Material before *n*-amino-octyl-Sepharose chromatography contains cytochrome *b* other than *b*₋₂₄₅ and thus the final values underestimate the purification.

	Cytochrome <i>b</i> ₋₂₄₅		
	Specific content (nmol/mg of protein)	Purification (fold)	Recovery (%)
Cells	0.09 ± 0.01	(1)	(100)
Organelles	0.12 ± 0.02	1.34 ± 0.03	88.2 ± 2.37
Triton N101 extract	0.90 ± 0.13	10.0 ± 0.29	50.4 ± 6.64
Non-adsorbed after <i>n</i> -amino-octyl-Sepharose	1.72 ± 1.23	18.8 ± 12.1	42.1 ± 7.83
After heparin-agarose	14.80 ± 4.63	166.0 ± 49.8	15.3 ± 2.27

adsorb cytochrome b_{-245} (90%) which was eluted with a gradient of NaCl (Fig. 2). Usually 60–80% of the adsorbed cytochrome b_{-245} was eluted from the resin. Those fractions with a specific content greater than 10–11 nmol/mg of protein (a concentration $>6\mu\text{M}$ in Fig. 2) were pooled and contained about 30% of the adsorbed cytochrome b_{-245} (Table 2). There was variation from experiment to experiment in the specific content of the most pure fractions (13–18 nmol/mg of protein) and the pooled fractions (11–17 nmol/mg of protein) but in an average of three experiments (Table 2) cytochrome b_{-245} was purified 166-fold from whole cells with a recovery of about 15%. Approximately 100–150 nmol of cytochrome b_{-245} could be prepared to this degree of purity from $(3-5) \times 10^{11}$ cells in 3–4 days. Fig. 3 shows the SDS/polyacrylamide-gel electrophoresis profile of the fractions from the different stages of purification of cytochrome b_{-245} .

Properties of cytochrome b_{-245}

Spectra. The oxidized, reduced-minus-oxidized and CO-difference spectra of purified cytochrome b_{-245} are shown in Fig. 4. The Soret maximum at 412 nm in the oxidized form shifts to 428 nm on reduction with dithionite. The cytochrome b_{-245} was not reduced by added NADH or NADPH. Approx. 60% of cytochrome b_{-245} was found to bind CO.

The midpoint potential of cytochrome b_{-245} in the initial Triton N101 extract (1.17 nmol of cytochrome b_{-245} /mg of protein) was -215mV and that for purified material (14.85 nmol/mg of protein) was -202mV . The redox titrations indicated the presence of about 10% contaminating higher potential mitochondrial cytochrome b in the initial Triton N101 extract, but there was no high-potential cytochrome b in the purified material.

Molecular weight. Electrophoresis. Fig. 5 shows the results of analysis of fractions eluted from heparin-agarose either in the presence of only PMSF or after treatment of the cells with Dip-F and then purified in the presence of PMSF and Trasylol. In both preparations the staining intensity of the broad band with an apparent M_r of about 68000–78000 coincided with that of the elution of the cytochrome b_{-245} and this band stained positively for haem (results not shown). In the absence of proteinase inhibitors there were a large number of other protein-staining bands with $M_r < 20000$, some of which also stained positively for haem.

Attempts were made to determine the reason for the smudged appearance on SDS gel electrophoresis of the cytochrome b_{-245} . If the sample was treated with a proteinase such as papain (Cleveland *et al.*, 1977) the smudged area disappeared indicating that it was due to protein and was not an artefact. It did not react positively with

Fig. 4. Spectral characteristics of purified cytochrome b_{-245}

(a) Oxidized spectrum of cytochrome b_{-245} (13.8 nmol/mg of protein), $9.14\mu\text{M}$ in buffer B containing Triton N101 (10 mg/ml). (b) Dithionite reduced-minus-oxidized (—) and CO (---) difference spectra of cytochrome b_{-245} (13.8 nmol/mg of protein), $0.46\mu\text{M}$ in buffer B containing Triton N101 (10 mg/ml).

the HIO₄/Schiff stain for carbohydrate. High-*M_r* (>100000) material was evident in the tracks, particularly if samples were boiled for too long (> 3 min) or the ionic strength of the samples was too high (> 0.2).

Gel exclusion chromatography. Cytochrome

b₋₂₄₅ was eluted in the void volume of a column of Sephadex G-200 equilibrated with buffer A containing Triton N101 (10mg/ml), indicating an *M_r* in excess of 600000, probably due to aggregation of the purified cytochrome *b*₋₂₄₅.

Flavin content. The cholate prewash was fluores-

Fig. 5. SDS/polyacrylamide-gel electrophoresis of cytochrome *b*₋₂₄₅ eluted from heparin-agarose (a) and (b) Purification in the presence of PMSF alone; (c) and (d) purification in the presence of Dip-F, PMSF and Trasylol. Tracks 1–9 in (a) and 2–6 in (c) show cytochrome *b*₋₂₄₅ eluted with increasing concentration of NaCl from heparin-agarose; the amounts of cytochrome *b*₋₂₄₅ (●) and protein (○) loaded on each track are indicated on (b) and (d). Tracks 10 in (a) and 1 in (c) contain *M_r* markers whose values ($\times 10^{-3}$) are indicated on the Figure. Track 7 in (c) contains buffer B+proteinase inhibitors.

Table 3. NADPH-dependent O₂⁻ production by fractions following solubilization of cytochrome *b*₋₂₄₅

Organelles were resuspended (15mg of protein/ml) in buffer A containing cholate (5mg/ml) but lacking dithiothreitol and butylated hydroxytoluene and centrifuged (69900*g*_{av.}, 30min). The pellets were resuspended in Triton N101 (10mg/ml) followed by centrifugation.

	O ₂ ⁻ generation				Cytochrome <i>b</i> ₋₂₄₅ (nmol)
	Unstimulated		Stimulated		
	Specific activity (nmol/min per mg of protein)	Total activity (nmol/min)	Specific activity (nmol/min per mg of protein)	Total activity (nmol/min)	
Cells	3.11	137	19.40	1550	15.0
Organelles	0.71	45.6	8.00	936	12.9
Cholate extract	0.89	4.8	1.20	28.6	0.0
Triton N101 extract	2.50	12.3	1.39	20.7	8.6
Residual pellet	0.00	0.0	3.16	162	3.0

cent. This spectrum had an emission band of 520 nm with excitation maxima near 350 and 460 nm and was similar to the spectrum of FAD. Analysis of the flavin content indicated that over 80% was FAD and that the specific content of FAD was approx. 170 pmol/mg of protein. None of the Triton N101 extracts and subsequent fractions, including the final preparation of purified cytochrome b_{-245} , gave a similar fluorescence spectrum.

NADPH-dependent superoxide generation. An extraction was made on leucocytes from healthy donors after stimulation with phorbol myristic acid. To minimize the purification time the organelles were extracted only once with cholate and then with Triton N101. Over 75% of the NADPH-dependent O_2^- generating activity was lost on homogenization of cells and detergent solubilization (Table 3). The Triton N101 extract which contained over 50% of the cytochrome b_{-245} contained less than 2% of the original O_2^- generating activity with a specific activity of 1.39 nmol/min per mg of protein.

Discussion

This paper describes the purification of cytochrome b_{-245} from human neutrophils. We have referred to it as cytochrome b_{-245} for the sake of clarity, even though it had a slightly more positive midpoint potential when solubilized with Triton N101. Cytochrome b_{-245} shows a dual localization in the membranes and specific granules of human neutrophils (Segal & Jones, 1979). Thus the starting material for our extraction was an organelle pellet rather than purified membranes, even though the specific content of the cytochrome is twice as great in the membrane. The cholate prewash differentially extracted proteins including myeloperoxidase, resulting in the cytochrome b being the major chromophore in the subsequent Triton N101 extract. The cholate also removed a fluorescent flavoprotein as has also been observed by Gabig (1983). This protein is believed to be the electron donor to the cytochrome in the functional oxidase (Cross *et al.*, 1982b), which would account for the absence of NAD(P)H-dependent reduction or superoxide-generating activity in the purified material. About 60% of the purified cytochrome bound CO, in keeping with the results of Cross *et al.* (1982a) who found that this was due to the low affinity of the protein for CO rather than to heterogeneity of the cytochrome. Their results indicated that at high partial pressures, all the cytochrome would form a CO complex.

Apart from the tendency of the haem to dissociate from the cytochrome during electrophoresis, aggregation of the protein and proteolysis

were the major causes of difficulty in identifying the molecule after SDS/polyacrylamide-gel electrophoresis. Similar aggregation has been observed with other cytochromes b (Marres & Slater, 1977; Duong & Fleming, 1982) and hydrophobic proteins (Albert & Litman, 1978). Amrein & Stossel (1980) found that the only effective means of preventing proteolysis during homogenization of neutrophils was by using Dip-F, which can penetrate intact neutrophils, but that PMSF was as an effective inhibitor as Dip-F once cells were broken. We established that PMSF alone was insufficient to prevent proteolysis. Thus we first treated cells with Dip-F before homogenization and subsequently utilized the less toxic PMSF and Trasylol. Although these measures were effective in removing most of the low- M_r components seen on SDS gels, cytochrome b_{-245} ran as a broad band with an apparent M_r of between 68 000 and 78 000. The upper M_r of 78 000 would give a maximum specific content of cytochrome b_{-245} of 13 nmol/mg of protein, although our most purified preparation had a specific content of 18 nmol/mg of protein. This indicates either that the protein runs anomalously upon SDS/polyacrylamide-gel electrophoresis, that the assay underestimates the amount of protein, or that material other than protein is a constituent of the molecule. Some integral membrane proteins bind SDS differently from water-soluble proteins (Helenius & Simons, 1975). Glycoproteins may run anomalously and can appear as broad bands on SDS gels (Fairbanks *et al.*, 1971; Segrest & Jackson, 1972; Frank & Rodbard, 1975). Cytochrome b_{-245} did not react with the HIO_4 /Schiff stain for carbohydrate, but some carbohydrates are resistant to periodate oxidation (Eckhardt *et al.*, 1976).

The purification of cytochrome b_{-245} will facilitate further investigations of its properties and function. Various groups have solubilized and in some cases partially purified the oxidase system containing cytochrome b (Light *et al.*, 1981; Gabig *et al.*, 1982; Bellavite *et al.*, 1983) and they should now be able to identify the cytochrome constituent of their preparations.

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