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# PURIFICATION OF CYTOCHROME $b_{558}$ FROM BOVINE POLYMORPHONUCLEAR NEUTROPHILS

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**Summary:** A 110 fold purification of cytochrome $b_{558}$ from resting bovine neutrophils has been achieved. The plasma membrane bound cytochrome **b** was extracted with aminoxide WS35, a non ionic detergent. The purification procedure included liquid column chromatography on CM-C50 Sephadex, chromatofocusing on the anion exchanger PBE94, and gel filtration on P30 Biogel. The purified preparation was characterized by an heme to protein (nmol/mg) ratio of 7.7. The isoelectric point of cytochrome **b** was at pH 6.5. Upon polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate three bands corresponding to apparent Mr 64,000, 56,000 and 20,000 were revealed by staining with Coomassie Blue. 

Most **b**-type cytochromes are intrinsic membrane proteins whose prosthetic group is the non covalently bound iron protoporphyrin IX . Polymorphonuclear neutrophils contain a **b**-type cytochrome which is characterized by a spectrum with an absorption maximum α band at 558 nm and a redox midpoint potential of -256 mV at pH 7 (**1**). Considerable effort has been made to determine the role of cytochrome $b_{558}$ in the respiratory burst of neutrophils, but our understanding of its function is still limited (for review see Rossi (**2**)). A clearer picture of the role of this cytochrome may be achieved through purification and determination of its mode of action in a reconstituted liposomal system. A few reports have appeared concerning purification of cytochrome $b_{558}$ ; however, the purity of these cytochrome preparations is questionable, since molecular weight values reported for the product have ranged between 11,000 and 127,000 (**3-8**). A procedure for the purification of cytochrome **b** from a membrane fraction of resting bovine neutrophils was

**Abbreviations**: **MOPS**, 3- N-morpholino propane sulfonic acid ; **EDTA**, ethylene diamine tetraacetate ; **EGTA**, ethyleneglycol-bis(β-amino-ethyl ether)tetraacetate ; **PMSF**, phenylmethyl sulfonyl fluoride ; **TPCK**, N-tosyl-L-phenylalanine chloromethyl ketone ; **TLCK**, N-p-tosyl-L-lysine chloromethyl ketone ; **TMBZ**, 3,3'-5,5' tetramethylbenzidine ; **DTT**, dithiothreitol ; **SDS**, sodium dodecyl sulfate ; **PAGE**, polyacrylamide gel electrophoresis.

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reported earlier in a preliminary communication from this laboratory (**9**). This purification procedure has been improved by including chromatofocusing, and is described in detail in the present report. As neutrophil cytochrome **b** is highly hydrophobic, its behaviour during the purification procedure and especially during chromatofocusing was found to be variable. Conditions affecting its resolution are discussed.

## MATERIALS AND METHODS

**Chemicals.** Percoll, CM-C50 Sephadex, anion exchanger PBE 94, and Polybuffer 96-acetate were purchased from Pharmacia. Ampholines and Ultrodex were from LKB. Sucrose and dithionite were from Merck. The non ionic detergent aminoxide WS35 which is a mixture of aminoxides of different chain lengths was purchased from Onyx. Hydroxylapatite was from Bio-Rad. Asolectin, 3,3'-5,5' tetramethyl benzidine (TMBZ), sodium dodecyl sulfate (SDS), N-tosyl-L-phenylalanine chloromethyl ketone (TPCK), N-p-tosyl-L-Lysine chloromethyl ketone (TLCK), phenylmethyl sulfonyl fluoride (PMSF), soybean trypsin inhibitor, leupeptin and 3-N-morpholinopropane sulfonic acid (MOPS) were from Sigma Chemical Company.

**Preparation of plasma membrane.** Resting bovine neutrophils were prepared as described (**10, 11**). For extraction and purification of cytochrome **b**, the suspension of purified neutrophils was diluted to reach a final concentration ranging between $5 \times 10^8$ to $1 \times 10^9$ cells per ml in 10 mM MOPS buffer (pH 7.4) also containing 0.25 M sucrose, 5 mM EDTA, and the protease inhibitors, TPCK (0.1 mM), TLCK (0.1 mM), PMSF (1 mM), leupeptin (10 µg/ml) and trypsin inhibitor (1 µg/ml) (standard medium). In one experiment (Figure 4) the suspending solution also contained EGTA and mercaptoethanol. Neutrophils were disrupted at 0°C using a Branson sonifier operating at 40 W. Cells were subjected to four successive ice cold sonications of 15 s each time separated by 30 s intervals.

The high speed pellet enriched with plasma membrane was recovered by differential centrifugation of the neutrophil homogenate (**10, 11**). It consisted of two layers : a dense green bottom layer overlayered by a loose white material. The top layer (TL membrane fraction) contained most of the cytochrome **b** and a trace amount of myeloperoxidase. This fraction was recovered by gentle agitation in 0.1 M NaK phosphate, (pH 8.0), 20% (v/v) glycerol, 2 mM EDTA and 0.2 mM DTT. The membrane suspension was adjusted to a final concentration of 20 mg protein/ml, and cytochrome **b** was extracted with aminoxide WS 35. The dense bottom fraction of the membrane pellet contained most of the neutrophil myeloperoxidase activity and was discarded.

**Protein assay.** Protein was estimated as described by Lowry (**12**) with bovine serum albumin as a standard. Protein samples were precipitated using 10% (w/v) trichloracetic acid, collected by centrifugation, and then solubilized in 10% SDS for protein determination. In some experiments, protein was estimated spectrophotometrically at 280 nm.

**Measurement of cytochrome b.** Reduced vs oxidized difference spectra of cytochrome **b** were recorded at 77 K with a Perkin Elmer 557 spectrophotometer operating in the split beam mode. Sodium dithionite was used to reduce cytochrome **b** (**13**). Cytochrome **b** content was

calculated from extinction coefficients of 21.6 $mM^{-1}$ and 106 $mM^{-1}$ for reduced minus oxidized peaks at 558 nm and 425 nm respectively.

**Heme catalysed peroxidase activity.** During cytochrome **b** purification, the heme content in liquid column chromatography fractions was determined with the TMBZ reagent (**14**). A 500 µl sample of 6.3 mM TMBZ in methanol plus 0.25 M Na acetate buffer (pH 5.0), (3/7, v/v) was added to an equal volume of elution sample. The reaction was initiated by addition of 30 mM $H_2O_2$ and the intensity of blue colour formed was measured spectrophotometrically at 660 nm.

**Incorporation of cytochrome b into asolectin vesicles.** Asolectin was suspended in distilled water to a concentration of 100 mg/ml, and stirred at 4°C under nitrogen. After 2 h, the material was dispersed by sonication with a Branson sonifier operating at maximal output,until the solution was clear. Protein samples enriched in cytochrome **b** were incubated with the dispersed asolectin suspension for 15 min at 4°C. To improve protein incorporation into liposomes, the mixture was subjected to four successive bursts of ultrasonic irradiation, each one for 10 s.

**Isoelectric focusing** : Isoelectric focusing of cytochrome **b** was carried out in sucrose medium, or in Dextran gel. In the former system case, a linear 0-30 % sucrose gradient was prepared at 4°C in 0.1% (w/v) aminoxide WS35 and 0.4 % (w/v) LKB ampholines pH 3-9. The final volume of the gradient was 110 ml. Crude soluble membrane protein extract was applied to the middle of the ampholine-sucrose column. The reservoir at the cathode (bottom of the column) was filled with 5 mM NaOH in 50% sucrose (w/v) and the reservoir at the anode with 5 mM sulfuric acid. Electrofocusing was carried out at 4°C for 15 h at 400 V, and then for 30 h at 1200 V. Afterwards, 3-ml fractions were collected and the pH value, and the protein and cytochrome **b** content were determined.

For isoelectric focusing in Dextran gel, Ultrodex gel was suspended at the concentration of 4% (w/v) in a solution consisting of 20% (w/v) glycerol, 2% (w/v) ampholines pH 3-10, and 0.2% (w/v) aminoxide WS35. The slurry gel was poured onto a plate and subsequently dried to a limit corresponding to about 35% water evaporation. Protein extract in 0.5% (w/v) aminoxide WS35 was dialysed in 0.01 M NaK phosphate buffer (pH 8), 0.2 mM DTT, 2 mM EDTA, 0.2% (w/v) aminoxide WS35 and 20% (v/v) glycerol. Dialysed sample was then incorporated into asolectin liposomes, as described above, using a 1:1 (w:w) protein to asolectin ratio. Incorporation of cytochrome **b** into liposomes appeared to prevent heme dissociation from the apoprotein. Isoelectric gel focusing was carried out at 4°C first at 600 V. After 10 h the voltage was increased to 1000 V. Separated proteins were recovered from the gel by elution into the same buffer used for dialysis. Collected fractions were assayed for pH, and protein. The hemoprotein content was assayed by the TMBZ peroxidase test. Peroxidase positive fractions were pooled and cytochrome **b** content was determined spectrophotometrically.

**Chromatofocusing** : The anion exchanger PBE94 was used and elution was carried out with polybuffer 96-acetate, pH.6, as described in results.

**SDS-polyacrylamide gel electrophoresis (SDS-PAGE).**
Prior to denaturing SDS-PAGE slab gel electrophoresis, protein in aminoxide WS35 was precipitated with 0.1 N perchloric acid. The precipitate was washed with cold acetone and redissolved in 0.06 M Tris-HCl buffer (pH 6.8) containing 15% (v/v) glycerol, 5% β-mercapto-ethanol, 2.3% SDS, and 0.001% bromophenol blue. Solubilized samples were incubated at room temperature for 12 h with gentle stirring. SDS-PAGE was performed as described by Laemmli and Favre (**15**) with a 5% stacking

gel and a 15% resolving gel. Protein molecular weight standards used were phosphorylase b (Mr 94,000), bovine serum albumin (Mr 67,000), ovalbumin (Mr 43,000), carbonic anhydrase (Mr 30,000), soybean trypsin inhibitor (Mr 20,100) and α-lactalbumin (Mr 14,400).

## RESULTS AND DISCUSSION

### Solubilization and purification of cytochrome b from bovine neutrophil cytochrome b

Cytochrome **b** present in the TL membrane fraction obtained from the neutrophil homogenate (cf. Materials and Methods) was incubated with aminoxide WS35 for 1 h at 4°C. The solubilized material was recovered by centrifugation at 140,000 g for 1 h at 4°C. The data in Fig.1 A and B

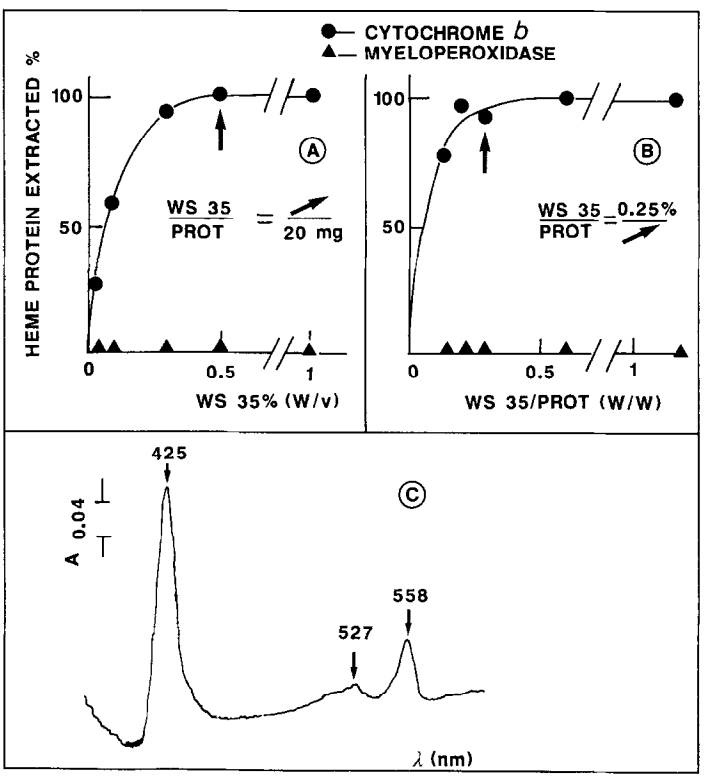



**Figure 1.** Extraction of cytochrome **b** from the plasma membrane of bovine neutrophils by aminoxide WS 35.
The plasma membrane fraction of resting neutrophils (cf. Materials and Methods) in 0.1 M phosphate buffer (pH 8), 20% (v/v) glycerol, 2 mM EDTA and 0.2 mM DTT, was mixed with aminoxide WS35 in a Dounce homogeneizer and incubated for 1h at 4°C. After centrifugation at 140,000 g for 1h at 4°C, the supernatant was analyzed for protein, cytochrome **b** and myeloperoxidase content. **A** and **B** . Effect of the concentration of aminoxide WS35 (w/v), and the aminoxide/protein ratio (w/w) on the efficiency of cytochrome **b** extraction. **C** . Dithionite-reduced vs oxidized absorption spectrum of membrane extract.

**TABLE I** Three-step purification of cytochrome $b_{558}$ from an extract of bovine neutrophil plasma membrane in aminoxide WS35

| Fraction | Total protein (mg) | cyt **b** (nmol/mg protein) | Total cyt **b** (nmol) | Recovery % | Purification factor |
|---|---|---|---|---|---|
| Aminoxide extract | 140 | 0.7 | 98 | 100 | 1 |
| CM-C50 Sephadex chromatography | 7.7 | 2.3 | 17.7 | 18 | 3.3 |
| Chromatofocusing | n.d | — | 10.0 | 10 | — |
| Biogel P30 filtration | 1.5 | 7.7 | 11.6 | 12 | 11.0 |

Details are given in the Text.

illustrate the effect of the detergent concentration and the detergent to protein ratio on the efficiency of cytochrome **b** extraction. Optimal conditions for cytochrome extraction were 0.5% (w/v) aminoxide concentration with an aminoxide to protein ratio of 0.3 (w/w). Solubilized cytochrome **b** prepared in this manner was then stored at - 80°C for future use, or purified further. It should be noted that the TL membrane fraction may also contain small amounts of myeloperoxidase localized in azurophile granules. However, this peroxidase activity was not released by the low-salt, detergent conditions used. A similar observation was reported in studies on rabbit neutrophils (**16**).

Cytochrome **b** purification consisted of three steps (Table I). A solubilized cytochrome **b** sample containing 100 to 150 mg of protein was loaded onto a CM C50 Sephadex column (50 cm x 2.5 cm) which had been equilibrated with 10 mM phosphate, 20% (v/v) glycerol, 2 mM EDTA, 0.2 mM DTT and 0.2% (w/v) aminoxide WS 35. A continuous phosphate gradient was applied to the column. Cytochrome **b** was eluted with 220-230 mM phosphate. The fractions containing cytochrome **b** were pooled and dialysed against 0.25 M imidazole-acetate buffer (pH 7.4), supplemented with 0.2 mM DTT, 2 mM EDTA, 20% glycerol (v/v) and 0.2% (w/v) aminoxide WS35 (imidazole-glycerol buffer). Dialysed cytochrome **b** enriched solution was then incubated together with asolectin liposomes and sonicated (see Material and Methods). The asolectin to protein ratio was 10 (w/w). The proteoliposome mixture was finally subjected to chromatofocusing on a column (15 x 2 cm) prepared with PBE 94, an anion exchanger, which had been equilibrated with imidazole-glycerol buffer

(see above). Elution from PBE94 was carried out with polybuffer 96-acetate (pH 6.0) diluted 13 fold in 20% glycerol, 0.2 mM DTT, 2mM EDTA and 0.2% (w/v) aminoxide WS35. Protein was monitored at 280 nm. Fractions enriched in cytochrome **b**, which focused between pH values of 5.8 and 6.5 were freed from polybuffer 96 by gel filtration on a column (120 x 1,5 cm) of Biogel P30. Biogel P30 was equilibrated in 50 mM phosphate buffer (pH 8), containing 0.2 mM DTT, 2 mM EDTA, 20% (v/v) glycerol and 0.2% (w/v) aminoxide WS35 (phosphate-glycerol buffer). Cytochrome **b** was eluted with the same buffer. The peak of eluted cytochrome **b** was very broad, with a large fraction of cytochrome **b** being retarded with respect to its expected behaviour based on the molecular weight of its components (see thereafter).

The percent recovery and the purification factor of cytochrome **b** at the different steps of the purification procedure are shown in Table I. Cytochrome **b** was purified 11 fold with a yield of 12%. As cytochrome **b** was already purified about 10 fold during preparation of the TL neutrophil plasma membrane fraction from the whole neutrophil cells, an overall purification of approximately 110-fold was achieved.

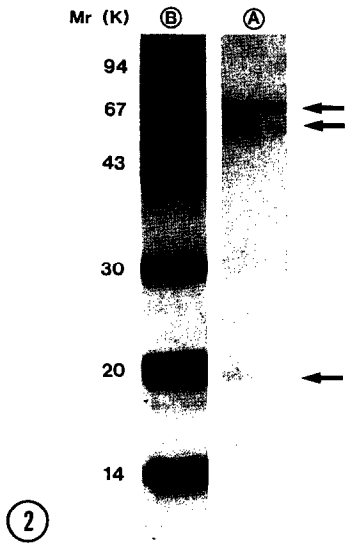
**Properties of the purified bovine neutrophil cytochrome b preparation.**

The reduced minus oxidized difference spectrum of isolated cytochrome **b** at 77 K was identical with that of the plasma membrane fraction of bovine neutrophils with peaks at 425 nm, 527 nm and 558 nm (see Fig. 1 and reference 10). From the spectral data, it can be calculated that the heme to protein ratio in our purified preparation of cytochrome **b** is 7.7 (Table I), which compares favorably with values given in recent reports (**3**, **4**, **7**).

Upon denaturing SDS-PAGE followed by Coomassie Blue staining, the purified cytochrome **b** preparation showed three peptide bands with apparent Mr ranging from 66,000 to 64,000, 58,000 to 56,000, and 20,000 to 18,000 (Fig.2). Components of similar molecular weight were reported in a preliminary communication (**9**), which also reported purification of bovine neutrophil cytochrome **b** using a different procedure based upon Sephacryl chromatography instead of chromatofocusing.

A pI of 6.5 for the purified cytochrome **b** was found by isoelectric focusing in a sucrose gradient (Fig.3). A similar value was found when isoelectric focusing was carried out in a Dextran gel. In contrast, chromatofocusing performed on cytochrome **b** incorporated into liposomes with a 10 fold excess of lipid with respect to protein resulted in a broad band of cytochrome **b** located between pH 5.8 and pH 6.5.

Subsequently to our preliminary communication (**9**), Parkos et al (**7**) and Segal (**8**) have reported that cytochrome **b** purified from human



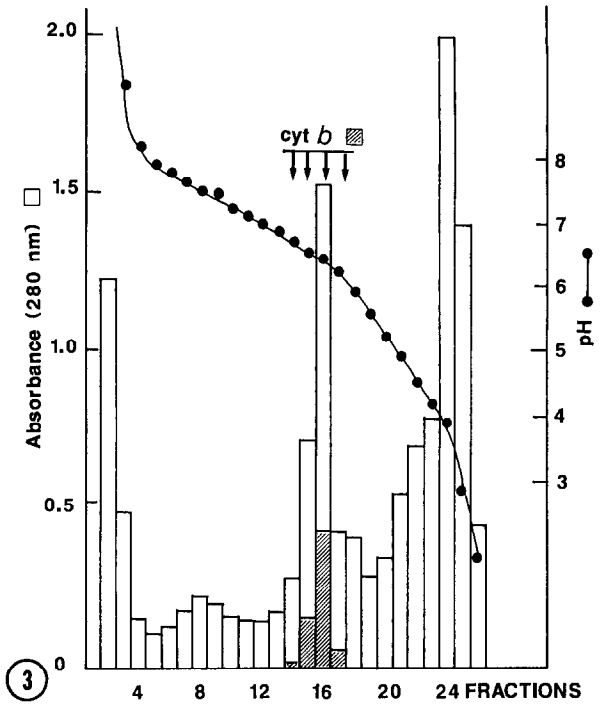




**Figure 2.** Identification of components in purified cytochrome **b** by SDS-PAGE followed by Coomassie Blue staining. Track A - Molecular weight markers ; Track B : purified cytochrome **b** preparation. Arrows designate the three components of Mr ≃ 64,000, 56,000 and 20,000.

**Figure 3.** Isoelectric focusing in sucrose gradient. Experimental conditions are described in Materials and Methods.

neutrophils is an heterodimer consisting of two subunits of Mr 22-23,000 and 76-92,000. The high molecular weight subunit of this dimer was found to be a glycoprotein that upon deglycosylation yielded a 50,000 Mr species. On the other hand, complete absence of the low molecular weight component was observed in neutrophils from patients with X-linked granulomatous disease (7). The similarity of the gel electrophoresis data for human and bovine neutrophil cytochrome **b** is noteworthy. However, whether the high and low molecular weight components of bovine neutrophil cytochrome **b** are subunits of an heterodimer, as proposed for human neutrophil cytochrome **b** (8), or whether they derive from each other by aggregation or cleavage cannot yet be decided.

In the course of our experiments, cytochrome **b** exhibited different patterns during isoelectric chromatofocusing. This depended upon the method of membrane preparation used. Routinely, membrane associated cytochrome **b** was prepared in 10 mM MOPS buffer (pH 7.4) with sucrose, EDTA and protein inhibitors (see Materials and Methods). Under this condition, cytochrome **b** focused as a single broad band between pH 5.8 and 6.5. In contrast, when membrane was treated in a similar buffer

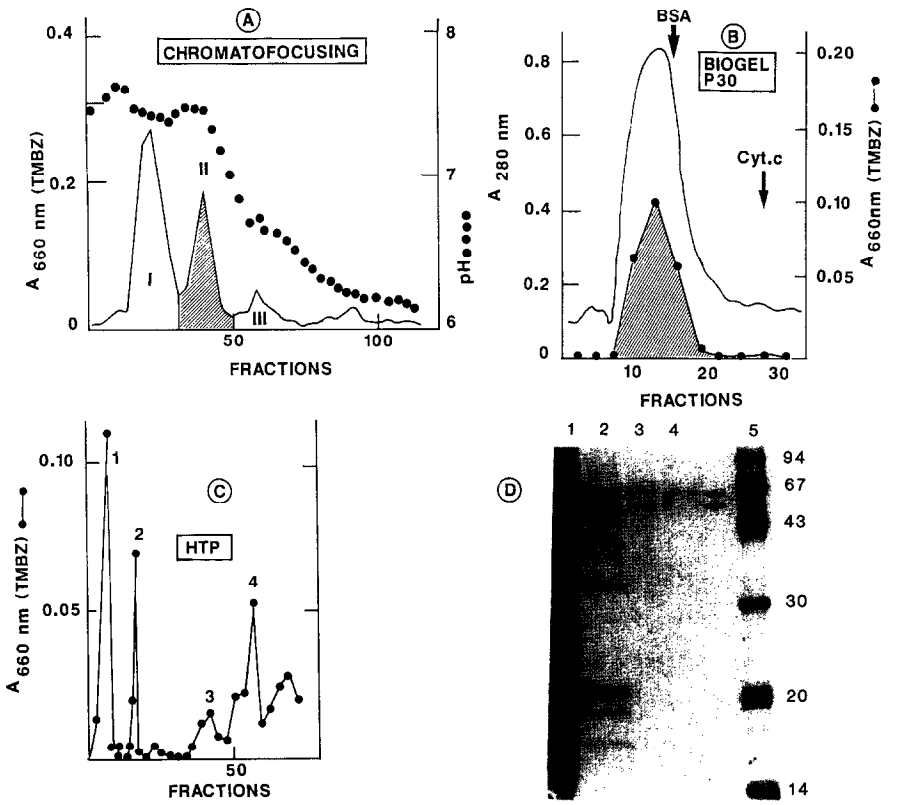



**Figure 4.** Resolution of several cytochrome **b** species by chromatofocusing and analysis of these species by Biogel P30 chromatography and hydroxylapatite chromatography.
In the present experiment, the standard sonication medium used for the purification of cytochrome **b** was supplemented with 5 mM EGTA and 20 mM mercaptoethanol. **A** Chromatofocusing was carried out with the CM-C50 Sephadex fraction as described in Results and Discussion. The concentration of aminoxide WS35 in the elution medium was increased to 0.5%. The TMBZ reactive material of peak II (spectrally characterized as cytochrome **b**) was subjected to Biogel P30 chromatography **B** The Biogel P30 column was equilibrated with 0.05 M phosphate pH 8.0, 20% (v/v) glycerol, 0.25 M KCl, 0.2 mM DTT, 0.2 mM EDTA and 0.5% aminoxide WS35. Elution was performed with the equilibration buffer. The protein content was monitored at 280 nm and cytochrome **b** was revealed by reaction with TMBZ. Bovine serum albumin (BSA) and cytochrome **c** were used as molecular weight markers (indicated by arrows). **C** The TMBZ reactive material from Biogel P30 was placed out to hydroxylapatite equilibrated with the same medium as that used for Biogel P30 chromatography. Elution was performed with 0.3 M phosphate pH 8.0, 20% glycerol, 0.2 mM DTT, 2 mM EDTA, 0.1 M KCl and 0.5% aminoxide WS35. **D** The TMBZ reactive fractions 1, 2, 3 and 4 were analyzed by SDS-PAGE followed by Coomassie Blue staining (tracks 1, 2, 3 and 4). The arrows correspond to the components of Mr 64,000 and 56,000 of purified cytochrome **b**. Track 5 corresponds to standard molecular weight markers (cf. Materials and Methods).

solution supplemented with 5 mM EGTA and 20 mM mercaptoethanol, cytochrome **b** formed three bands at pH 7.5, 7.1 and 6.6 (Fig. 4,A). The material of peak II (pI 7.1) and peak III (pI 6.6) was applied to Biogel P30, and the eluted fractions containing cytochrome **b** were subjected to SDS-PAGE. The material of peak III gave a broad band of cytochrome **b** by

filtration on Biogel P30 ; the material of this band was comprised of two major protein components which exhibited apparent molecular weight values of 64,000 and 56,000 respectively, and a minor one of Mr 20,000 (not shown). These components are probably identical to those of the purified cytochrome **b** preparation in Fig.2. The protein material of peak II (pI 7.1) migrated on Biogel P30 as a single band of cytochrome **b** activity whose rate of migration was significantly faster than that of bovine serum albumine (Fig. 4B). This material, however, resolved into four separate bands of cytochrome **b** activity on hydroxylapatite (Fig. 4C). The content of each of the four bands was subjected to SDS-PAGE and then stained with Coomassie Blue (Fig. 1, 4 D). Both bands 1 and 2 (lanes 1 and 2) represented a mixture of membrane proteins with cytochrome **b** material. Fraction 3 and 4 (lanes 3 and 4) were, however, comprised of two major proteins with apparent Mr of 64,000 and 56,000 respectively. From SDS-PAGE, both bands 3 and 4 seemed identical ; yet they migrated differently on hydroxylapatite as noted before. Whether this apparent heterogeneity of cytochrome **b** on chromatofocusing and hydroxylapatite is due to the presence of covalently linked residues, for example carbohydrate residues as in the case of human neutrophil cytochrome **b** (**7, 8**) or to different aggregation states of cytochrome **b** cannot be decided at present.

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