Biochimica et Biophysica Acta 932 (1988) 71–83
Elsevier

BBA 42690

The quaternary structure of the plasma membrane *b*-type cytochrome of human granulocytes *

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(Received 25 August 1987)

Key words: Quaternary structure; Cytochrome *b*; (Granulocyte plasma membrane)

Hydrodynamic, crosslinking and immunoprecipitation studies were performed on detergent solubilized cytochrome *b* to demonstrate that the two copurifying polypeptides of molecular weight 91 000 (glycosylated) and 22 000 [1,2] formed a molecular complex. The hydrodynamic studies indicated that the cytochrome *b*/detergent complex had a sedimentation coefficient, partial specific volume and Stokes radius of 5.25 S, 0.82 cm^3/g and 6.2 nm in Triton X-100 and 6.05 S, 0.80 cm^3/g and 5.6 nm in octylglucoside, respectively. These studies also indicated that the detergent-protein complex has a molecular mass of 202 and 188 kDa in Triton X-100 and octylglucoside, respectively, is asymmetric in shape with a frictional coefficient of 1.3–1.4 and binds significant amounts of detergent. The molecular mass of the protein portion of the detergent-cytochrome complex was estimated to be between 100 and 127 kDa. Crosslinking studies with disuccinimidyl suberate and alkaline cleavable bis[2-(succinimidooxy-carbonyloxy)ethyl]sulfone revealed that the M_r = 91 000 and M_r = 22 000 components of purified cytochrome *b* are closely associated and can be covalently bound to form a polypeptide which, by SDS-polyacrylamide gel electrophoresis, has M_r values of 110 000–120 000 and 120 000–135,000 on 8% and 11% (w/v) SDS-polyacrylamide gels, respectively. Cleavage of the crosslinked species resulted in the reappearance of the M_r = 91 000 and M_r = 22 000 species. Sedimentation profiles of crosslinked cytochrome *b* in linear sucrose density gradients made up in H_2O were identical to those of non-crosslinked controls. A close association of the two protein species was further confirmed by the ability of antibody specific for the smaller subunit to immunoprecipitate the larger one also. Experiments aimed at identifying the heme-carrying subunit(s) were inconclusive, since dissociation of the complex resulted in loss of cytochrome *b* spectrum. These results, in combination with our SDS-polyacrylamide gel electrophoresis molecular-weight estimates, provide strong evidence for the cytochrome *b* being an α-β-type heterodimer composed of a glycosylated M_r = 91 000 and non-glycosylated M_r = 22 000 polypeptide.

* Publication No. 4593 IMM from the Department of Immunology.

Abbreviations: ABTS, 2,2-azino-di(3-ethylbenzthioline sulfonic acid); BSOCOES, bis[2-(succinimidooxy-carbonyloxy)ethyl]-sulfone; DSS, disuccinimidylsuberate; DMSO, dimethylsulfoxide; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PMSF, phenylmethylsulfonylfluoride; TEMED, *N,N,N′,N′*-tetramethylethylenediamine.

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Introduction

In recent years there has been considerable interest in a membrane-bound *b*-type cytochrome present in polymorphonuclear leukocytes. This heme protein has been implicated as a terminal oxidase in the production of superoxide anion by activated neutrophils [3–5]. It has a midpoint oxidation-reduction potential of −245 mV at pH

7.0 [6], binds CO in its reduced state [6,7] and becomes reduced when the O_2^--generating system in granulocytes in activated [4,5]. Its heme spectrum is reversibly altered by heterocyclic nitrogenous bases. This alteration is correlated with the reversible inhibition of O_2^--generating activity [8]. The heme spectrum is also absent in X-linked and some autosomal recessive forms of chronic granulomatous disease which is characterized by the inability of granulocytes to make O_2^- [3,9,10].

The molecular weight of the cytochrome *b* has been reported by several groups with poor agreement. These estimates ranged from 11 000 to 127 000 [11–16]. Our laboratory reported the existence of a smaller polypeptide of molecular weight of approx. 22 000, copurifying with a larger glycosylated polypeptide ($M_r \approx 91\,000$) and with the cytochrome spectral activity [1,2] which was confirmed by Segal [16]. The new method of purification provided an explanation for the wide range of molecular polyacrylamide weight estimates for this cytochrome [2]. This explanation was based on the effect of content on SDS-polyacrylamide gel electrophoresis mobilities and other technical factors. The resultant technical variation existing in the different laboratories could therefore explain the discrepancies in M_r reported for the heavy chain by Lutter [15] and Segal [16]. A species variant of the light chain may have been detected by the others [11,12] which appeared independent of the heavy chain probably because of the intrinsic difficulty in staining the latter glycopeptide. Also, the presence of the light chain most likely went undetected in earlier reports by Segal and coworkers because of lower-molecular-weight contaminants in their purified cytochrome *b* preparation.

In light of the confusion in the literature over the size and other properties of the cytochrome *b*, we have undertaken a characterization of the non-ionic detergent-solubilized protein using hydrodynamic, crosslinking and immunological techniques. The results of our hydrodynamic studies have provided estimates of the size, shape and surface area of the cytochrome *b* in contact with membrane lipids. In addition, analysis of the hydrodynamic data along with the results of crosslinking and immunoprecipitation studies has provided strong evidence for a heterodimeric structure of the human neutrophil *b*-type cytochrome.

Materials and Methods

Reagents. Reduced nicotinamide adenine dinucleotide (NADH), dihydrocytochalasin B, Na_2 adenosine triphosphate (ATP), pyruvate, bovine serum albumin, cytochrome *c*, chymostatin, hypoxanthine, human red cell carbonic anhydrase, bovine liver catalase, Type III peroxidase, dimethylsulfoxide, protein A-sepharose Cl-4B, *cis*-oxaloacetic acid, and 2,2-azino-di-(3-ethylbenzthioline sulfonic acid) (ABTS) were purchased from Sigma (St. Louis, MO). Superoxide dismutase, phenylmethylsulfonylfluoride (PMSF), octylglucoside, deuterium oxide (^2H_2O), dithiothreitol, rabbit muscle aldolase, rabbit muscle lactate dehydrogenase, and bovine xanthine oxidase were purchased from Calbiochem Behring Corp. (La Jolla, CA). Sodium dithionite was obtained from Fisher (Tustin, CA). Hepes was purchased from U.S. Biochemical Corp. (Cleveland, OH). EDTA was obtained from Fluka (Hauppage, New York). Ultrapure Triton X-100 was purchased from Boehringer Mannheim (F.R.G.). Ferritin and protein molecular weight standards were purchased from Pharmacia (Uppsala, Sweden). Sodium dodecyl sulfate (SDS), acrylamide, glycine, bis-acrylamide, sodium persulfate and TEMED were purchased from BioRad (Richmond, CA). Prestained high-molecular-weight protein standards for SDS gels were purchased from Bethesda Research Laboratories (BRL) (Gaitherberg, MD). Glycerol and 2-mercaptoethanol were from J.T. Baker (Phillipsburg, NJ). Sucrose and ultrapure urea were acquired from Schwartz-Mann (Spring Valley, NY). Fractogel TSK HW-55, disuccinimidyl suberate (DSS), bis[2-(succinimidooxy-carbonyloxy) ethyl]sulfone (BSOCOES), iodobeads and BCA-protein-staining reagents were purchased from Pierce (Rockford, IL) .

Buffer composition. DPBS(−) was a modified Dulbecco's phosphate-buffered saline containing 5 mM KCl, 147 mM NaCl, 1.9 mM KH_2PO_4, 1.1 mM K_2HPO_4, 1.5 mM $CaCl_2$, and 1.1 mM $MgCl_2$, pH 7.4. Membrane resuspension buffer (buffer 1) consisted of 100 mM KCl, 10 mM NaCl, 10 mM HEPES, 1 mM EDTA, 1 mM PMSF and 10 μg/ml chymostatin (pH 7.3). Column running buffer (buffer 2) consisted of 500

mM NaCl, 50 mM Hepes (pH 7.3), 5% (v/v) glycerol, 1 mM EDTA, 0.1 mM dithiothreitol and either octylglucoside or Triton X-100 in the amounts described in the text. Sucrose solutions consisted of the appropriate amount of sucrose dissolved in 100 mM KCl, 10 mM NaCl, 10 mM Hepes, 1 mM EDTA and either octylglucoside or Triton X-100 in the amounts described in the text (pH 7.3).

Preparation of neutrophil membranes and the purification of cytochrome b. Neutrophil membranes and cytochrome *b* were purified exactly as previously described [2].

Hydrodynamic characterization of cytochrome b. For Stokes radius determinations, purified cytochrome *b* and concentrated extracts of polymorphonuclear leukocytes membranes solubilized in 2% (w/v) octylglucoside or 1% (w/v) Triton X-100 were chromatographed on a column of Fractogel TSK HW-55 (2.6 × 60 cm) equilibrated with column running buffer at a flow rate of 50 ml/h. The elution volume of the cytochrome was then compared to those of characterized standard proteins with known Stokes radii. The void volume was measured using blue dextran and the included volume with tritiated sucrose. Standards included aldolase, catalase, xanthine oxidase and ferritin.

Since the cytochrome *b* is an integral membrane protein [17,11] and requires detergent for solubilization, classical techniques for determining hydrodynamic parameters could not be used. Instead, a technique first described by Edelstein [18] and Meunier [19] and later refined by Neer [20] and Clarke [21] was used. This procedure involves comparison of the sedimentation profiles of the protein of interest, in detergent-containing sucrose gradients made up in H_2O or ^2H_2O, relative to those of well-characterized standards. Using this technique, physicochemical information can be obtained even for proteins present in trace amounts or as impure mixtures.

The sedimentation coefficients and partial specific volumes of detergent solubilized cytochrome *b* were determined by analysis of sedimentation profiles in sucrose density gradients. Linear 5–20% (w/v) sucrose gradient were constructed which contained either 1% Triton X-100 or 2% octylglucoside in H_2O or ^2H_2O. Marker proteins with known sedimentation coefficients ($S_{20,w}$) and partial specific volumes ($\bar{V}$) used for standard curve constructions are listed in Table I.

Concentrated 2% octylglucoside membrane extracts were used for sedimentation studies in octyl glucoside-containing sucrose gradient. For sucrose gradients containing Triton X-100, iodinated purified cytochrome *b*-559, concentrated eluate from heparin ultrogel and the concentrate from the 100 000 × *g* supernatant of membranes solubilized in 1% Triton X-100 were used.

Velocity sedimentation experiments were performed in ^2H_2O and H_2O. The sucrose gradients were overlaid with 230 μl samples and centrifuged at 45 000 r.p.m. at 4°C in an SW 50.1 rotor (Beckman, Inc.) for 16 h (H_2O) and 30 h (^2H_2O). At least two internal standards were run in cytochrome *b*-containing gradients to allow comparison with standard gradients.

The gradients were analyzed by fractionation into 240–250 μl samples using a Buchler Densiflow pump (Saddler, NJ). Sedimentation coefficients and partial specific volumes were calculated using Eqns. 11–14 from Clarke [21]. The molecular mass of the protein detergent complex was estimated

TABLE I

PROTEIN STANDARDS USED FOR CALIBRATION OF SUCROSE DENSITY GRADIENTS

Globular protein standards and respective $S_{20,w}$ and $\bar{V}$ values used for determination of the $S_{20,w}$ and $\bar{V}$ of cytochrome *b* as described in Materials and Methods.

Protein	$S_{20,w}$ (10^{-13} s)	$\bar{V}$ (cm^3/g)	Reference
Carbonic anhydrase (human erythrocyte)	2.75	0.731	38
Horseradish peroxidase Type II	3.48	0.699	39
Malate dehydrogenase (bovine heart)	4.32	0.734	40
Bovine serum albumin	4.41	0.734	42,43
Lactoperoxidase	5.37	0.764	44
Lactate dehydrogenase (rabbit muscle)	6.95	0.730	45
Aldolase (rabbit muscle)	7.70	0.742	46,47
Glyceraldehyde-3-phosphate dehydrogenase (rabbit muscle)	7.71	0.725	48

using the equation (from Ref. 22)

$$M = \frac{S_{20,w} 6\pi\eta_{20,w} Na}{1 - \bar{V}\rho_{20,w}} \quad (1)$$

where $S_{20,w}$ is the sedimentation coefficient at 20 °C in water, $\eta_{20,w}$ is the viscosity of water at 20 °C, N is Avogadro's number, a is the Stokes radius, $\bar{V}$ is the partial specific volume of the complex and $\rho_{20,w}$ is the density of water at 20 °C.

To estimate the asymmetry of the detergent-protein complex, the frictional ratio was calculated by the equation (from Ref. 22)

$$\frac{f}{f_0} = a\left(\frac{4\pi N}{3M\left(\bar{V} + \frac{\delta}{\rho_{20,w}}\right)}\right)^{1/3} \quad (2)$$

where M is the molecular mass and δ is the solvation factor.

Biochemical assays. Cytochrome *b* was quantitated by reduced-minus-oxidized difference spectroscopy on a Cary 219 dual-beam spectrophotometer (Varian, Inc., San Jose, CA) assuming an extinction coefficient of $2.16 \cdot 10^4$ l/mol per cm [7]. Samples were reduced by the addition of 2 μl of a 1.0 M solution of sodium dithionite made up in H_2O immediately prior to use.

Carbonic anhydrase, aldolase, and bovine serum albumin were labelled with ^{125}I by the chloramine T method [23]. Peroxidase activity was quantitated by the change in optical absorbance at 415 nm of 0.5 mM ABTS and 30 mM H_2O_2 in 0.1 M citrate buffer (pH 4.2). Catalase and ferritin were quantitated by optical absorbance at 415 and 405 nm, respectively. Xanthine oxidase was quantitated by the increase in optical absorbance at 550 nm of a solution of 50 μM cytochrome *C* in column running buffer containing 0.5 mM hypoxanthine. Lactate dehydrogenase activity was measured by the decrease in absorbance at 340 nm after the addition of a solution of 0.15 mM NADH and 2.5 mM pyruvate in 0.1 M sodium phosphate buffer (pH 7.4), $T = 20$ °C. Malate dehydrogenase activity was measured by the decrease in absorbance at 340 nm after the addition of a solution of 3.75 mM NADH and 6 mM *cis*-oxaloacetic acid in 0.1 M sodium phosphate buffer (pH 7.4), $T = 20$ °C. Glyceraldehyde-3-phosphate dehydrogenase activity was quantitated using a coupled enzyme assay as described previously [24].

Electrophoresis. SDS-gel electrophoresis was carried out at room temperature in polyacrylamide slab gels containing 0.1% (w/v) SDS [25]. Samples and molecular-weight standards were mixed with an equal volume of sample buffer, boiled for 3 min and layered onto a 4% w/v stacking gel. Proteins were focused at 20 mA and electrophoresed at 40 mA ($T = 20$ °C). The electrophoretic mobility of samples reduced with 500 mM 2-mercaptoethanol was compared to the mobility of reduced molecular-weight standards. Proteins were visualized on slab gels by first staining for 30 min with 0.125% Coomassie blue G-250 in 50% methanol and 10% acetic acid. Gels were then destained in 25% isopropanol and 10% acetic acid until the background was clear and hydrated overnight in H_2O with several changes of water. Hydrated gels were then silver stained under basic conditions [26].

Crosslinking studies. Two-dimensional gel electrophoresis experiments were performed using the base cleavable homo-bifunctional crosslinking reagent BSOCOES as described by Zarling [27] with some modifications. To cytochrome *b* preparations, 100 mM BSOCOES (in DMSO) was added to a final concentration of 0.25 mM (0.25% DMSO, $T = 20$ °C). After 20 min of incubation, the reaction was terminated by the addition of glycine to a concentration of 20 mM. Samples were mixed with an equal volume of reduced sample buffer and subjected to SDS-polyacrylamide gel electrophoresis on 11% (w/v) polyacrylamide gels. After electrophoresis, individual lanes were excised from the slab gels and either frozen at −70 °C or fixed and stained as described in the previous section. For two-dimensional SDS-polyacrylamide gel electrophoresis, a frozen gel slice of the crosslinked material was thawed and incubated for 1 h at 37 °C in cleavage buffer consisting of 50 mM Tris base, 50 mM H_3PO_4, 0.1% SDS, and 100 mM 2-mercaptoethanol; the pH was adjusted to 11.0 with 5 M NaOH. The gel slice was then neutralized for 30 min ($T = 37$ °C) first in 100 mM Hepes (pH 7.0) and then for 30 min in 0.125 M Tris (pH 6.8). Both neutralization buffers contained 0.1% SDS and 100 mM 2-mercaptoethanol. The neu-

tralized gel slice was then subjected to a second dimension of SDS-polyacrylamide gel electrophoresis in an 11% (w/v) polyacrylamide gel.

The cytochrome *b* was also crosslinked with DSS in an analogous fashion. To samples containing purified cytochrome *b*, 100 mM DSS (in DMSO) was added to a final concentration of 0.25 mM ($T = 20\,°C$). After varying lengths of time, aliquots were removed, and the reaction terminated by the addition of glycine as described above. SDS-polyacrylamide gel banding patterns of the crosslinked material were then analysed by scanning densitometry.

For sedimentation studies, cytochrome *b* samples were treated with 0.25 mM DSS for 20 min ($T = 20\,°C$) and the reaction terminated with glycine as described above. Crosslinked samples and uncrosslinked controls were then sedimented in Triton X-100-containing sucrose gradients made up in H_2O, fractionated and analysed as described in the previous sections.

Immunoprecipitation studies. Immunoprecipitation studies were performed using antibody to the $M_r = 22\,000$ component of the cytochrome *b* which was affinity purified from a previously described polyclonal antibody [2]. To affinity purify the antibody, cytochrome *b* samples were subjected to SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose. The $M_r = 22\,000$ region of the nitrocellulose was excised and treated with buffer containing 10% goat serum, 1% bovine serum albumin in 500 mM NaCl, 20 mM Hepes (pH 7.4) to block nonspecific protein binding sites. The nitrocellulose was then incubated overnight ($T = 4\,°C$) with polyclonal antiserum containing 0.2% Tween 20. After extensive washing with buffer consisting of 250 mM NaCl, 0.2% Tween 20 and 10 mM Hepes (pH 7.4), the bound antibody was eluted by treatment for 7 min with 50 mM glycine/HCl (pH 2.8). The pH of the eluate was immediately adjusted to 8.0–8.6 with Tris-base and bovine serum albumin added to a final concentration of 5 mg/ml. After dialysis against DPBS(−), the affinity purified antibody was ready for use.

For immunoprecipitation studies with the affinity purified material, the antigen consisted of ^{125}I-labelled eluate from immobilized heparin prepared as described previously [2]. Antigen-antibody incubations were done as follows: to 0.25 ml of affinity purified antibody (eluted from approx. 0.7 μg of $M_r = 22{,}000$ subunit), was added 0.25 ml of 25% (w/v) sucrose prepared in 100 mM KCl, 10 mM Hepes (pH 7.4) and 0.2% Triton X-100. The pH of the solution was increased to 8.0–8.2 by the addition of 10 μl of 2.0 M Tris base (pH 8.3). To this solution was added 10 μl or $2.5 \cdot 10^6$ cpm of ^{125}I-labelled heparin eluate followed by incubation overnight at 4 °C. The next day, 25 μl of protein A conjugated to Sepharose 4B was added, and the suspension was incubated for 2 h at 4 °C. The immunoglobulin-bound protein A-Sepharose was then washed ten times with wash buffer consisting of 0.3 M NaCl, 25 mM Tris pH 8.2, 0.2% Triton X-100 and 1 mM EDTA.

After the final wash, the pellet of protein A-Sepharose 4B was resuspended in 50 μl of SDS-polyacrylamide gel electrophoresis sample buffer containing 500 mM 2-mercaptoethanol, heated to 100 °C for 3 min at the supernatant subjected to SDS-polyacrylamide gel electrophoresis on 11% (w/v) polyacrylamide gels. For control samples, the antibody solution was replaced by a solution of DPBS(−) + 5 mg/ml bovine serum albumin. After electrophoresis, the polyacrylamide gels were fixed overnight in a solution of 25% isopropanol, and 10% acetic acid, followed by hydration in water for 4 h. The slab gels were then dried on Whatman filter paper with the aid of a slab gel drier. Autoradiography was performed for 4–8 h at −70 °C, using a Dupont Cronex lightning-plus intensifying screen.

Dissociation of the components of b-cytochrome in sucrose density gradients. Experiments were performed to investigate the effect of dissociation of the $M_r = 91\,000$ and $M_r = 22\,000$ components of solubilized cytochrome *b* on the sedimentation profile of its heme spectrum. Concentrated eluate from immobilized heparin containing 0.8% w/v Triton X-100 was treated with various concentrations of SDS for 30 min in order to separate the components of the cytochrome *b*. The mixture was then sedimented in Triton X-100 containing sucrose gradients for 16 h and fractionated as described in the hydrodynamics section. The sucrose gradient fractions were then assayed for cytochrome *b* soret absorbance and subjected to reduced SDS-polyacrylamide gel electrophoresis as described in previous sections.

Results

Hydrodynamic properties of the b-cytochrome

Our previous studies suggest that the b-cytochrome consists of two polypeptides when analysed by SDS-polyacrylamide gel electrophoresis. Because the purification employed provided little information about the native conformation of this molecule, it was imperative to determine its molecular mass, size and shape in detergent solution as a first approximation of its structure in the membrane.

To gain information about the size and shape of detergent solubilized cytochrome *b*, experiments were performed to determine the hydrodynamic size, sedimentation coefficient, and partial specific volume in both octylglucoside and Triton X-100. From these physical parameters, the molecular size, Stokes radius, and frictional ratio for the protein-detergent complex could be calculated as described in the materials and Methods. The results of these experiments are summarized in Table II.

TABLE II

PHYSICOCHEMICAL PROPERTIES OF HUMAN NEUTROPHIL CYTOCHROME *b*

	Triton X-100	Octylglucoside
Stokes radius (nm)	6.2	5.6
$S_{20,w}$ (10^{-13} S)	5.2	6.0
$\bar{V}$ (cm^3/g)	0.82	0.80
f/f_0 [a]	1.43	1.33
Molecular mass [b] (kDa) (detergent-protein complex)	202	188
Detergent binding [c] (g/g protein)	0.6–1.0	
Molecular mass [d] (kDa) (detergent-free protein)	100, 127	
M_r (SDS-polyacrylamide gel electrophoresis)	91000, 22000	

[a] Frictional ratios were calculated assuming a solvation factor of 0.2 g solvent per g protein [20].

[b] Molecular mass was calculated assuming the protein binds the same amount of detergent in ^2H_2O as in H_2O [21].

[c] Detergent binding was estimated assuming a $\bar{V}$ of 0.7–0.75 for the protein portion of the detergent-protein complex.

[d] Calculated by substitution of the values for molecular mass and detergent binding into Eqn. 3 from Clarke [21].

The Stokes radius of the cytochrome *b* was determined by gel filtration in the presence of either Triton X-100 or octylglucoside. A column of Fractogel TSK HMW 55 was calibrated with proteins of known Stokes radii. Fig. 1 shows a comparison of the K_d and Stokes radii of the standard proteins and interpolation of the Stokes radius for the cytochrome *b* in both Triton X-100 and octylglucoside. The Stokes radius of the *b*-type cytochrome increased form 5.6 nm in octyl glucoside to 6.2 nm in Triton X-100, presumably due to the different properties of each detergent.

The sedimentation coefficients and partial specific volumes of the cytochrome-detergent complexes were determined by measuring the apparent sedimentation coefficients in both H_2O and ^2H_2O by velocity sedimentation in sucrose gradients containing detergent and comparison to those of known standard proteins. Fig. 2 shows the sedimentation profiles for the cytochrome *b* in sucrose gradients containing octylglucoside and Triton X-100. The shift in the sedimentation profile of cytochrome *b* relative to globular standards when comparing H_2O vs. ^2H_2O sucrose gradients indicates a higher partial specific volume than those of the globular protein standards. The partial specific volume of the cytochrome *b* increased from 0.80 ml/g in octyl glucoside to 0.82 ml/g in Triton X-100, while the sedimentation coefficient $S_{20,w}$ decreased from 6.0 to 5.2 when octylglucoside was replaced with Triton X-100. The significant deviation of the $\bar{V}$ of the cytochrome *b* from that of globular proteins indicates that a substantial amount of lipid or detergent is bound to the solubilized molecule.

The apparent molecular weights of the cytochrome *b*-detergent complexes in Table II were calculated assuming the protein binds the same amount of lipid or detergent in ^2H_2O as in H_2O. This approximation was shown by Clarke [21] to introduce only a small (3–10%) underestimation of the true molecular weight of several other well-characterized integral membrane proteins. The frictional ratio of the cytochrome, 1.3–1.4, was calculated assuming a solvation factor of 0.2 g solvent per g protein [20]. This range suggests asymmetry in the structure of the cytochrome *b*.

Although the source of cytochrome used for the sedimentation profiles shown in Fig. 2 was from detergent extracted membranes, the profiles of

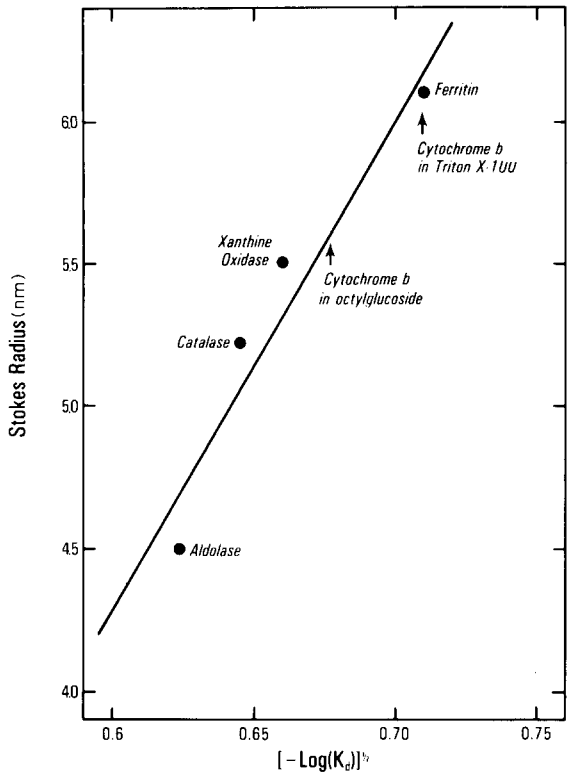

Fig. 1. Stokes radius determination of detergent solubilized cytochrome *b* by gel filtration. The Stokes radius of the cytochrome *b*-detergent complex was determined in both octylglucoside and Triton X-100. A column (2.6×60 cm) of Fractogel TSK HW-55 was equilibrated and eluted with column-running buffer (500 mM NaCl, 50 mM Hepes (pH 7.3), 5% glycerol, 1 mM EDTA, 0.1 mM dithiothreitol) containing either 0.8% octylglucoside or 0.1% Triton X-100. The column was calibrated by measuring the elution volume (V_e) of several proteins of known Stokes radii. Standard proteins and respective Stokes radii used were (1) Aldolase, 4.5 nm; (2) catalase, 5.22 nm; (3) xanthine oxidase, 5.5 nm; (4) ferritin, 6.1 nm. Void volume (V_0) was measured with blue dextran and included volume (V_i) was measured with tritiated sucrose. Cytochrome samples included iodinated, purified material and concentrated 100000×*g* supernatant of membranes solubilized in either 2% octylglucoside or 1% Triton X-100. K_d values were determined by the formula $K_d = (V_e - V_0)/(V_i - V_0)$. There was no difference in the gel filtration pattern of the standard proteins when either octylglucoside or Triton X-100 was used. Each data point is the average of at least four determinations. Enzyme activities were assayed as described in the methods.

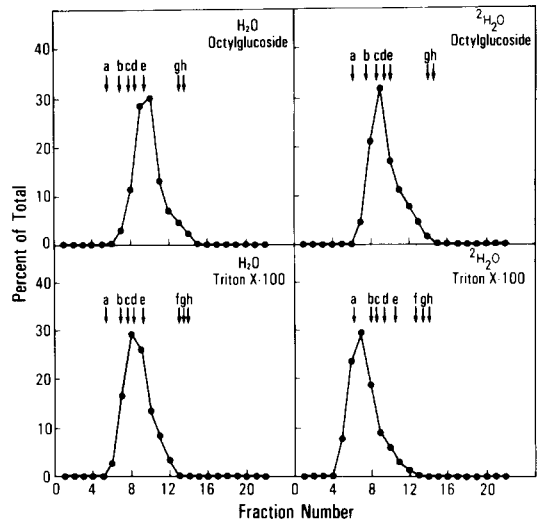

Fig. 2. Velocity sedimentation of cytochrome *b* in 5 ml 5–20% sucrose gradients to determine $S_{20,w}$ and $\bar{V}$. 180–300 pmol (230 µl) of cytochrome *b* from concentrated 2% octylglucoside (upper panels) or 1% Triton X-100 (lower panels) 100000×*g* supernatants from membranes were sedimented at 45000 r.p.m., $T = 4°C$ in an SW 50.1 (Beckman, Inc.) rotor for 16 h in sucrose gradients prepared in H_2O (left panels) or for 30 h in gradients prepared in ^2H_2O (right panels). Sucrose gradients in the upper panels contained 2% octylglucoside, whereas gradients in the lower panels contained 1% Triton X-100. Standard proteins mixed with the cytochrome *b* samples included: (a) 200000 cpm iodinated carbonic anhydrase; (b) 1 µg horseradish peroxidase Type II; (c) 10 µg malate dehydrogenase; (d) 200000 cpm iodinated bovine serum albumin; (e) 2 µg lactoperoxidase; (f) 10 µg lactate dehydrogenase; (g) 10 µg glyceraldehyde-3-phosphate dehydrogenase; (h) 200000 cpm iodinated aldolase. The midpoint of each enzyme activity is indicated with an arrow. Gradients were fractionated from the top into 22 fractions. The buffers used and enzyme assays were as described in Materials and Methods. Vertical axes refer to the percent of total cytochrome *b* recovered from gradients which was 60–80% of octylglucoside and 85–100% for the Triton X-100 containing gradients. ^{125}I-labeled purified cytochrome *b* (not shown) sedimentation profiles were indistinguishable from the profiles shown above. $S_{20,w}$ and $\bar{V}$ values were calculated as described in Materials and Methods.

cytochrome from the wheat germ agglutinin-Sepharose eluate, heparin eluate or purified ^{125}I-labelled cytochrome were unchanged, suggesting that there is no concentration-dependent association of the cytochrome *b* with other accessory proteins.

Crosslinking experiments

Our hydrodynamic studies suggest that the two polypeptides that copurify with the cytochrome spectrum are probably in a stable complex with one another, since they otherwise would have been easily resolved by the techniques employed. A

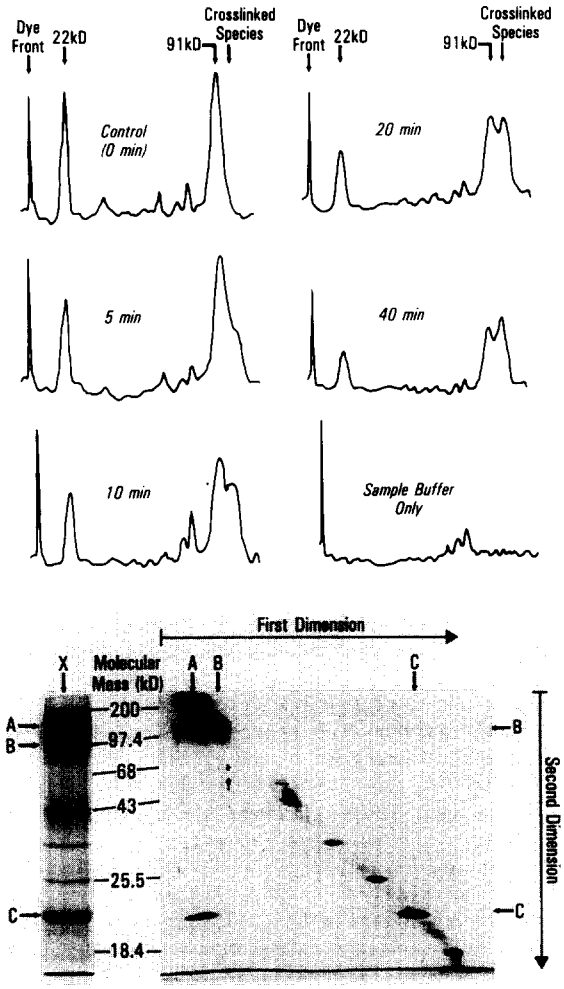

Fig. 3. Crosslinking and cleavage of crosslinked cytochrome *b*. Panel 1 represents the densitometric tracings of the SDS-polyacrylamide gel banding patterns of purified cytochrome *b* crosslinked for varying lengths of time with disuccinimidyl suberate DSS. To a 0.35 μM solution of purified cytochrome *b* in 10% sucrose, 100 mM KCl, 10 mM NaCl, 10 mM Hepes (pH 7.4) and 1% Triton X-100 was added DSS to a concentration of 0.25 mM. At the times indicated in Panel 1, 30 μl samples were removed and the reaction terminated by 20 mM glycine. Samples were then subjected to SDS-polyacrylamide gel electrophoresis on an 11% w/v polyacrylamide gel and silver stained as described in Materials and Methods. The individual sample lanes were scanned with a Zeineh soft laser scanning densitometer (Biomed Instruments, Inc., Chicago, IL). For the experiment depicted in panel 2, cytochrome *b* enriched eluate from immobilized heparin (3 μg protein) was crosslinked for 20 min with 0.25 mM BSOCOES as described in Materials and Methods. The lane marked X is the silver-staining pattern of the reduced, crosslinked material subjected to SDS-polyacrylamide gel electrophoresis on an 11% polyacrylamide gel. The arrows marked A, B and C denote the relative positions of crosslinked cytochrome *b*, uncrosslinked heavy chain and uncrosslinked light chain, respectively. An SDS-gel slice identical to the lane marked X was treated with pH 11.0 buffer to cleave the crosslinked protein and then subjected to a second dimension of SDS-polyacrylamide gel electrophoresis (11%) as shown to the right of lane X. The arrows marked B and C in the second dimension denote the position of cleaved $M_r = 91000$ and $M_r = 22000$ subunits, respectively. The molecular masses of standard proteins used are shown to the right of lane X and included myosin (200 kDa), phosphosylase B (97.4 kDa), bovine serum albumin (68 kDa), ovalbumin (43), α-chymotrypsinogen (25.5 kDa), β lactoglobulin (18.4 kDa).

close association of these two polypeptides is further confirmed by results of the crosslinking studies in this report. As shown in the densitometric tracings in panel 1 of Fig. 3, treatment of purified cytochrome *b* with DSS results in a time-dependent decrease in the staining density of the $M_r = 91000$ and $M_r = 22000$ polypeptides which is accompanied by the appearance and increase in staining density of a higher-molecular-weight species. The M_r of the crosslinked product ranges from 110000–120000 on 8% (w/v) polyacrylamide gels to 120000–135000 on 11% gels.

The composition of the crosslinked product was investigated using the base cleavable crosslinking reagent, BSOCOES. Treatment of purified cytochrome *b* with 0.25 mM BSOCOES results in the formation of a higher molecular-weight crosslinked species which was indistinguishable from that obtained with DSS. Panel 2 of Fig. 3 shows the results of an experiment performed to identify the constituents of the crosslinked material. Eluate from immobilized heparin was used as starting material so that protein contaminants would serve as an internal control for nonspecific crosslinking. SDS-polyacrylamide gel electrophoresis of the crosslinked material in lane "X" demonstrates the ability of BSOCOES to form a higher molecular weight species which has the same M_r value as that obtained with DSS. After cleavage of this higher molecular weight polypeptide by high pH electrophoresis in the vertical direction (second dimension), its protein constituents are identified as the $M_r = 91000$ and $M_r = 22000$ components of cytochrome *b*: the only two species that do not fall on the diagonal. The specificity of the crosslinking is confirmed by the other protein contami-

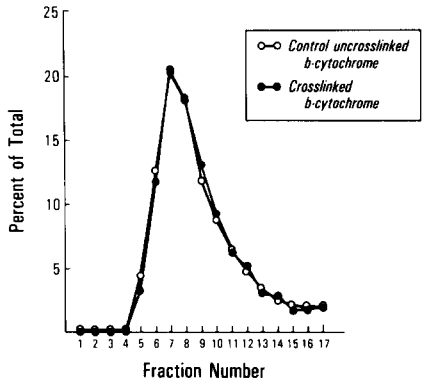

Fig. 4. Effects of crosslinking on the sucrose density gradient sedimentation profile of cytochrome *b*. A 350 µl sample of cytochrome *b* (2.5 µM) was crosslinked for 20 min with 0.25 mM disuccinimidyl suberate and sedimented for 16 h in a 5–20% sucrose density gradient containing detergent as described in Materials and Methods. The percentage of total recovered spectral activity (85–95%) is plotted against the fraction number. The sedimentation profile of crosslinked cytochrome *b* (filled circles) is compared to an uncrosslinked control (open circles).

nants which are present only on the diagonal of the gel.

If cytochrome *b* exists as a complex of proteins in detergent solution, then the effects of such crosslinking on its sedimentation characteristics should be minimal. Fig. 4 shows the results of such an experiment. Concentrated eluate from immobilized heparin was treated with 0.25 mM DSS for 20 min which resulted in crosslinking analogous to that shown in Fig. 3. The sedimentation profiles of the crosslinked cytochrome *b* in Triton X-100-containing sucrose density gradients made up in H_2O were indistinguishable from those of uncrosslinked controls. This result indicates that neither the hydrodynamic nor the spectral properties of detergent solubilized cytochrome *b* are altered by covalent coupling of the $M_r = 91\,000$ and $M_r = 22\,000$ components.

Immunoprecipitation studies

Experiments were performed with affinity-purified antibody to the $M_r = 22\,000$ component to confirm independently the close association between the $M_r = 91\,000$ and $M_r = 22\,000$ polypeptides and to investigate the stability of the complex. The specificity of the antibody for the $M_r = 22\,000$ polypeptide is demonstrated in the Western blotting profile shown in lane 1 of Fig. 5. There is a complete absence of labelling of the $M_r = 91\,000$ species even though at least 0.5 µg is present in this lane. The antibody was then incubated with a ^{125}I-labelled cytochrome *b* preparation depicted in lane 2 of Fig. 5 and immunoprecipitated using immobilized protein A. The autoradiograms of the immunoprecipitates are shown in Lanes 3 and 4 which demonstrate the ability of antibody specific for the $M_r = 22\,000$ component to immunoprecipitate the $M_r = 91\,000$ component also. This result confirms the strong association of the $M_r = 22\,000$ with the $M_r = 91\,000$ polypeptide.

The presence of aggregated material shown on the top of lanes 2, 3, and 4 of Fig. 5 has been a recurrent problem with SDS-polyacrylamide gel electrophoresis of ^{125}I-labelled cytochrome *b* preparations. Incubation of samples in the presence of sucrose was found to reduce but not eliminate

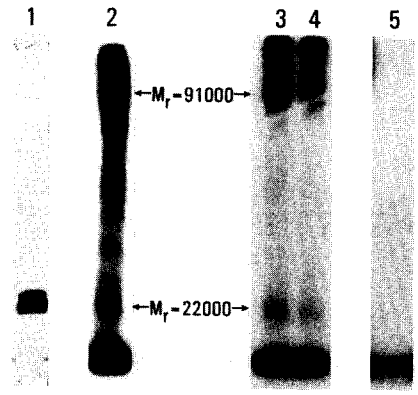

Fig. 5. Immunoprecipitation of ^{125}I-labelled cytochrome *b* with antibody immunopurified from the $M_r = 22\,000$ subunit. Lane 1 depicts the Western blotting profile of affinity purified anti-$M_r = 22\,000$ rabbit IgG to cytochrome *b*-enriched eluate from heparin-agarose done as previously described [2]. Lanes 2–5 represent autoradiograms of samples subjected to SDS-polyacrylamide gel electrophoresis on 11% (w/v) polyacrylamide gels. Lane 2: $2.5 \cdot 10^5$ cpm of ^{125}I-labelled heparin eluate. Lanes 3 and 4: duplicate experiments in which affinity purified antibody to the $M_r = 22\,000$ subunit was incubated with $2.5 \cdot 10^6$ cpm of ^{125}I-labelled heparin eluate, followed by incubation with protein A-Sepharose which was then washed and subjected to SDS-polyacrylamide gel electrophoresis as described in Materials and Methods. Lane 5: control incubation done identically as with lanes 3 and 4, except that DPBS(−) plus 0.5% bovine serum albumin was substituted for the antibody solution. See Materials and Methods for details.

aggregation. Although the $M_r = 91{,}000$ band in lanes 3 and 4 could have arisen from a nonspecific aggregate present in the initial ^{125}I-labelled mixture, the absence of the other labelled protein bands seen in lane 2 implies that the aggregated material consists only of the $M_r = 91\,000$ and $M_r = 22\,000$ components. Thus aggregation probably resulted from manipulation of the sample for electrophoresis. The absence of ^{125}I-labelled protein bands in lane 5 of Fig. 4 demonstrates that the immunoprecipitation is antibody specific.

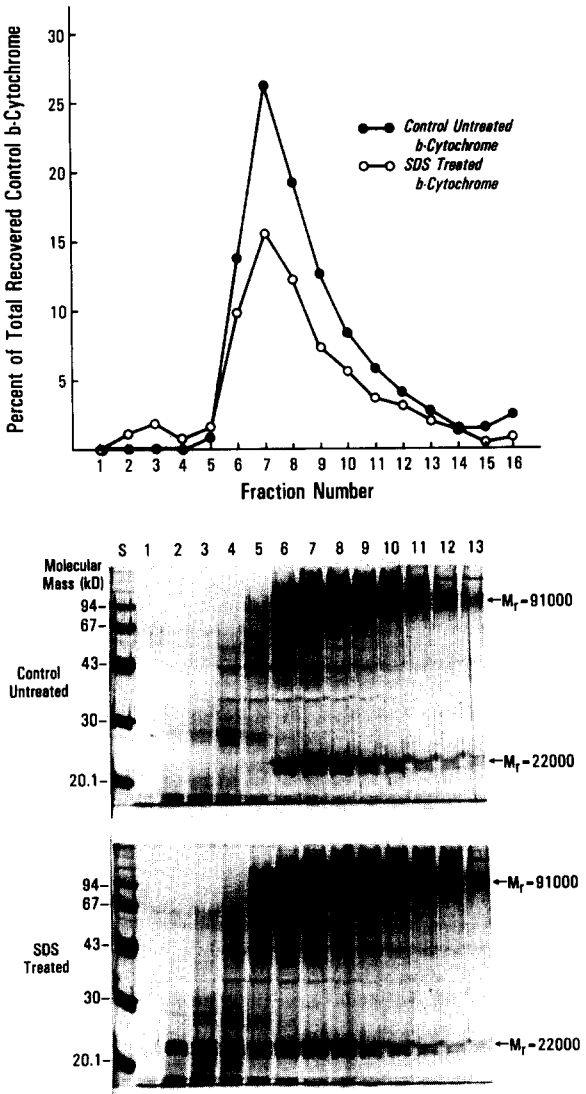

Fig. 6. Sedimentation of SDS treated cytochrome *b* in sucrose density gradients. To cytochrome *b* enriched eluate from immobilized heparin (2.0 μM cytochrome *b*) was added SDS at a molar ratio of SDS/Triton X-100 of 1:5. After 30 min of incubation at 20 °C, the sample was applied to the top of a linear 5–20% (w/v) sucrose density gradient containing 0.8% Triton X-100, sedimented for 16 h and fractionated as described in the Methods. The sedimentation profile of SDS-treated cytochrome *b* is compared to an untreated control. In panel A, percent of the total amount of cytochrome *b* spectral activity applied to the gradient is plotted against fraction number where 1 is the top of the gradient. Panel B represents the reduced SDS-polyacrylamide gel profiles of the sucrose gradients shown in panel A. 25 μl samples were mixed with an equal volume of sample buffer subjected to SDS-polyacrylamide gel electrophoresis on 11% (w/v) polyacrylamide gels and silver stained as described in Materials and Methods. Each numbered lane corresponds to the appropriated sucrose gradient fraction from panel A. Lane S represents standard proteins with their molecular masses listed to the left. The arrows to the right of panel B indicate the position of the $M_r = 91\,000$ and $M_r = 22\,000$ subunits of cytochrome *b*.

Dissociation of the components of solubilized cytochrome b

Since our experimental results indicated that cytochrome *b* exists as a complex of two proteins in detergent solution, experiments aimed at dissociation of the subunits were attempted in an effort to identify the heme carrying subunit(s). As can be seen in Figs. 3 and 4, SDS-polyacrylamide gel electrophoresis effectively separates the uncrosslinked components of cytochrome *b*. We investigated which aspect of sample preparation for SDS-polyacrylamide gel electrophoresis might be responsible for the dissociation of the $M_r = 91\,000$ and $M_r = 22\,000$ species and found that SDS treatment without boiling or reduction was adequate to separate the two proteins.

In analyzing the effects of SDS on the heme spectrum we found that treatment of octylglucoside or Triton X-100-solubilized cytochrome *b* with increasing amounts of SDS resulted in a time-dependent reduction in the α, β and Soret absorbancies. At a molar ratio of SDS/Triton X-100 of 1:5, the Soret absorbance of the cytochrome spectrum was diminished by 50% in 60 min and 100% after a few hours. Fig. 6 shows the effect of SDS on the sedimentation of cytochrome *b* in a sucrose gradient containing Triton X-100. Incubation of the cytochrome *b* in SDS at a molar ratio of SDS/Triton X-100 of 1:5 resulted in a 45%

decrease in recovered spectrum, but no shift in the sedimentation profile of the residual spectral activity when compared to untreated controls. SDS-polyacrylamide gel electrophoresis of the sucrose gradient fractions demonstrated effective resolution of approx. 30–40% of the total $M_r = 22\,000$ protein-staining density from fractions containing the $M_r = 91\,000$ component. As shown in Fig. 6, a small amount of recovered cytochrome *b* spectrum (2–3%) did co-isolate with fractions containing the dissociated $M_r = 22\,000$ polypeptide, but this amount is far less than the amount of spectrum which should have been in these fraction if no denaturation was taking place (i.e., 40%). This result implies that the conditions necessary to dissociate the two protein constituents may not be compatible with preservation of the heme spectrum. Sedimentation experiments done with lower concentrations of SDS failed to resolve these two proteins or perturb the spectra (data not shown).

Discussion

Recently, we have demonstrated by SDS-polyacrylamide gel electrophoresis that the purified human neutrophil cytochrome *b* consists of two polypeptides of $M_r = 91\,000$ and $M_r = 22\,000$ [1,2]. The purpose of this study was to analyze the structural relationship of these two polypeptides in order to gain further insight into their function and organization in the native cytochrome *b*. These investigations employed a combination of hydrodynamic, crosslinking, and immunological techniques which suggest that the cytochrome *b* is an integral membrane, α-β-type, heterodimeric protein.

The hydrodynamic studies in this report indicate that the molecular mass of the cytochrome *b* detergent complex is approx. 200 kDa. This size estimate is considerably larger than that obtained by SDS-polyacrylamide gel electrophoresis, suggesting that the protein may be comprised of several subunits. However, this is no doubt an over-estimate, since the high partial-specific volumes measured for the solubilized cytochrome suggest binding of large amounts of lipid or detergent. Assuming the bound lipid has been replaced by detergent and that the protein portion of the Triton X-100/cytochrome *b* complex has a $\bar{V}$ between 0.7 and 0.75 cm^3/g, then use of Eqn. 4 from Clarke [21] reveals that approx. 0.6–1.0 g Triton X-100 is bound per g protein. This amount of detergent binding falls within the range found for other integral membrane proteins such as rhodopsin [28], erythrocyte Band III and major sialoglycoprotein (PAS-1) [29]. It suggests, furthermore, that approx. 50% of the polypeptide surface area of the cytochrome is in contact with detergent (assuming it is a hard sphere [30]; and that the area occupied by a Triton X-100 molecule is 0.5 nm^2; Rohm and Haas data for an air-to-water interface) or, by inference, lipid [20,21] when in the membrane. Correcting for the estimated bound detergent, we calculate the molecular mass of detergent-free cytochrome *b* to be 100–127 kDa.

This estimate has an intrinsic uncertainty resulting from the high degree of glycosylation of the large subunit. Glycoproteins can have lower $\bar{V}$ values in the range of 0.68–0.70 cm^3/g and run anomalously on SDS-polyacrylamide gels [31]. Using the former value as a lower limit, the estimated molecular mass of the detergent-free complex is still 93 kDa. Given these uncertainties and those of SDS-polyacrylamide gel electrophoresis, our results are most consistent with cytochrome *b* being a 91 and 22 kDa α,β-type heterodimer but the possibility of it being an α,β,β-hetero-oligomer cannot be excluded. Both of these structures would be of sufficient size to span the plasma membrane lipid bilayer.

To verify this proposed structure of the cytochrome independently, immunoprecipitation and crosslinking experiments were performed on the detergent solubilized protein. A strong interaction between the two subunits was revealed by the ability of antibody specific for the light chain ($M_r = 22\,000$) to immunoprecipitate the heavy chain ($M_r = 91\,000$) also. The two components of the cytochrome could also be specifically crosslinked under conditions which did not crosslink other membrane proteins in solution, indicating that crosslinking was not secondary to random association of two proteins in a detergent micelle. Furthermore, the M_r of the crosslinked species agrees well with the predicted sum for the structure proposed. Lastly, the lack of an effect of crosslinking on the sedimentation profile of the heme spectrum of the cytochrome *b* indicates that

the molecular weight of 100–127 kDa predicted by our hydrodynamic studies must represent the sum of the molecular weights of the two components seen on SDS-polyacrylamide gels. This proposed stoichiometry is further supported by analysis of the relative protein staining densities of both subunits on SDS-polyacrylamide gels.

Our structural studies provided insufficient information to determine which subunit bears the noncovalently bound heme group responsible for electron transfer. SDS exposure was unable to dissociate the subunits without loss of spectral activity. However, a strong argument can be made for the light chain as the heme-bearing subunit of the cytochrome. First, the size of the $M_r = 22\,000$ component is in the same range as those of other non-mitochondrial *b*-type cytochromes such as the adrenal chromaffin granule cytochrome *b*-561 ($M_r = 30\,000$) [32] and microsomal cytochrome b_5 ($M_r = 16\,700$) [33]. Secondly, the presence of carbohydrate on the heavy chain would by atypical for a *b*-type cytochrome. Third, a recent confirmation of the primary defect responsible for X-linked chronic granulomatous disease being a deletion in the DNA encoding for the heavy chain of the cytochrome *b* [34–36] has provided the predicted amino acid sequence of that protein. This sequence has no significant homology to other cytochromes or proteins.

Although we have not shown that the cytochrome *b* exists as a heterodimer in the intact granulocyte membrane, recent immunological studies with neutrophils from patients with chronic granulomatous disease support a close association between the two in the membrane. Western blotting experiments have revealed a complete absence of both the heavy chain [35] and light chain [2] in X-linked forms of CGD. These results are supported by those recently reported by Segal [16]. Since the defect in X-CGD is a single deletion [34] in the gene coding for the heavy chain of the cytochrome *b*, the absence of the light chain in the same individuals suggests that the presence of intact heavy chain is required for cellular processing of the light chain.

The functional role of the non-heme containing subunit is a matter of speculation. Possible functions might include targeting and anchorage of the protein in the plasma membrane, facilitation of anion transport (O_2^-) through the membrane, or possibly vectorial redirection of electron flow to target surfaces which specifically bind the extracellular domain of the cytochrome. This type of surface redistribution has been shown to occur specifically for a number of cell-surface proteins including the Fc receptor of macrophages [37]. Further analysis of the structure and function of the subunits of cytochrome *b* using physicochemical, biochemical and molecular biological approaches will ultimately answer these important questions.

Acknowledgments

This work was supported by USPHS grants AI 17354, R0I AI 22735 and RR00833. C.A.P. is the recipient of National Institute of General Medicine Sciences National Research Award PHFGM07198 from the University of California School of Medicine, San Diego, CA. A.J.J. is the recipient of an American Heart Association Established Investigator Award, with funds contributed in part by the California Affiliate of the A.H.A. We wish to thank Velda Comstock for excellent secretarial and editorial work.

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