Human Neutrophil Cytochrome b Contains Multiple Hemes

EVIDENCE FOR HEME ASSOCIATED WITH BOTH SUBUNITS*

(Received for publication, October 9, 1991)

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Human phagocyte cytochrome b is the terminal component of the microbicidal superoxide generating system. Although the primary structure of this protein has been determined, little is known about the placement of the heme prosthetic groups in this heterodimeric integral membrane protein. Analysis of the cytochrome using lithium dodecyl sulfate-polyacrylamide gel electrophoresis at 0 °C followed by tetramethylbenzidine heme staining demonstrated the presence of heme in both the 91- and 22-kDa subunits identified by Western blot analysis using peptide specific antisera. Exposure of cytochrome b (purified or in isolated neutrophil plasma membranes) to Staphylococcal protease V8 or trypsin did not affect absorbance spectra. However, such treatment resulted in degradation of both subunits to smaller fragments, including characteristic immunoreactive 20-kDa fragments of both the large and small subunits of the cytochrome that retained one or both of the hemes. The spectral stability to proteolysis and size of the proteolytic heme-containing fragments generated explains previous reports which suggested that the heme resided in the small subunit. Our current results indicate that human neutrophil cytochrome b is a bi-heme or possibly tri-heme molecule with at least one heme residing in the large subunit and one shared between both subunits and that the heme-containing regions of the cytochrome probably lie within the membrane lipid bilayer. Such a multi-heme structure would be consistent with an electron transfer function for this cytochrome by providing an efficient mechanism for transferring electrons across the plasma membrane to the extracellular surface where oxygen could be reduced to create superoxide.

Plasma membrane electron transport and activatable oxidase function appears to be a widespread process observed in a variety of systems (1, 2). It is observed in fungi and plants such as Neurospora and Corn (Mais) as blue light (3, 4) or auxin-activated (5) NAD(P)H oxidase activity. In mammalian systems it is seen in the interplay between trans-plasma membrane redox systems and growth control (6), Ca2+ flux (7), or as activation of superoxide generation during the process of phagocytosis in phagocytes (8). In most cases an involvement of a unique type of plasma membrane cytochrome b is implicated (4, 9, 10). Our knowledge of these systems is most developed in the case of the phagocyte host defense process.

Neutrophils are the body's first line of defense against invading pathogens (11-13). Stimulation of these phagocytic cells with antibody-opsinized bacteria or chemotactants results in the activation of a latent reduced nicotinamide adenine dinucleotide diphosphate (NADPH) oxidase system to produce the so-called respiratory burst (14-17). The initial product of the respiratory burst is superoxide anion (O2-); however, subsequent reactions result in the formation of other toxic oxygen metabolites (H2O2, HOCI, etc.) that are important for microbicidal activity and inflammatory tissue injury (11-13).

Activation of the NADPH oxidase system appears to involve the translocation of cytosolic oxidase components to the plasma membrane (18) where they associate with a low-potential b-type cytochrome to form an electron transport chain that transfers electrons from cytosolic NADPH to molecular oxygen (19-21). The putative terminal component of this electron transport chain is the low-potential b-type cytochrome known as neutrophil cytochrome b (also known as cytochrome b559, b566, b245) (10, 20, 22).

Cytochrome b is a heterodimer (23, 24) composed of a 22-kDa (α) subunit (also known as p22-phox) (23, 25) and a glycosylated 91-kDa (β) subunit (also known as gp91-phox) (23, 26), both of which have been cloned and sequenced (25, 27). These two subunits are closely associated, as determined by cross-linking and immunoprecipitation studies (24), and can be dissociated only under extreme conditions (i.e. in SDS) that also destroy the cytochrome spectrum and result in loss of the non-covalently bound heme (23). Consequently, there has been uncertainty about which of the subunits actually carries the heme and also whether there is more than one heme.

In the studies presented here, we analyze cytochrome b to directly determine the location of the heme. Analysis of purified cytochrome b using lithium dodecyl sulfate polyacrylamide gel electrophoresis (LDS-PAGE) at 0 °C, followed by tetramethylbenzidine (TMBZ/H2O2) staining for heme demonstrated the presence of heme prosthetic groups in both the 91- and 22-kDa subunits. Proteolytic treatment of cytochrome b (either purified or in isolated neutrophil membranes) did not affect the cytochrome's absorbance spectrum although it degraded both subunits into smaller heme-containing frag-

* This work was supported by National Institutes of Health Grant 5R0126711. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ The abbreviations used are: phox, refers to phagocyte oxidase and is used to designate protein components of the respiratory burst of phagocytes; LDS, lithium dodecyl sulfate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TMBZ, 3,3',5,5'-tetramethylbenzidine; DFP, diisopropylfluorophosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TEMED, N,N,N',N'-tetramethylethylenediamine.
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EXPERIMENTAL PROCEDURES

Materials—Diisopropylfluorophosphate (DFP), 2,2'-azino-di-3-ethylbenzthiazoline sulfinic acid, disodium adenosine triphosphate (ATP), sucrose, aprotinin, chymostatin, bovine serum albumin, pepsin, diamidino-phenylindole, catalase, glycine, Tween 20, Triton X-100, cytochrome c (Type VII), formyl-methionyl-leucyl-phenylalanine, lithium dodecyl sulphate, trypsin (Type XI), and protease (Type XVII) from Staphylococcus aureus strain V8 were purchased from Sigma. Phenylmethylsulfonyl fluoride, octyl glucoside, and TMBZ were from Cal-biochem (La Jolla, CA). Gelatin, EDTA, sucrose, and dithiothreitol were purchased from Fischer (Tustin, CA). SDS, bis-acrylamide, ammonium persulfate, TEMED, Affi-Gel 10/50, and alkaline phosphatase-conjugated goat anti-rabbit were purchased from Bio-Rad. HEPES and ultrapure acrylamide was from U. S. Biochemical Corp. (Cleveland, OH). Normal goat serum was purchased from ICN Immunobiologicals (Lisle, IL). Prestained protein standards for SDS gels were purchased from Bethesda Research Laboratories (BRL).

Protease Treatment—Neutrophil Membranes and Cytochrome b—The methods of neutrophil isolation (from blood drawn from normal human donors) and membrane preparation were identical to those previously described (28). Neutrophil cytochrome b was purified to >90% purity from isolated neutrophil membranes as described previously (23) and was suspended in buffer composed of 10 mM HEPES, pH 7.4, 800 mM NaCl, 10 mM EDTA, 0.5 mM DTE, and 0.1% Triton X-100.

Electrophoresis—LDS-polyacrylamide gel electrophoresis (PAGE) was carried out at 0 °C using 7–18% gradient gels (60 × 300 × 1.5 mm) containing 0.1% (w/v) LDS (29–31). The temperature of the electrode buffer (0.1% LDS (w/v), 25 mM Tris base, 192 mM glycine) was maintained at 0 °C using a Hoyer refrigerated electrophoresis unit in a cold room. LDS-PAGE was carried out at room temperature (electrode buffer: 0.1% SDS (w/v), 25 mM Trizma base, 192 mM glycine) using 7–18% gradient gels (60 × 80 × 1.5 mm) containing 0.1% (w/v) SDS as described previously (28).

Samples analyzed by LDS-PAGE were mixed with an equal volume of LDS sample buffer (1 part 10% LDS (w/v) in H2O, 1 part 0.5 M Trizma base, pH 6.8, 1 part glycerol) at 4 °C and applied to the gel. The cytochrome samples gave identical migration patterns in the presence of 2-mercaptoethanol (not shown); however, since 2-mercaptoethanol can inhibit the heme-staining reaction, it was not used in the LDS-PAGE gels. Samples analyzed by SDS-PAGE were mixed with 2X sample buffer and boiled for 3 min in a boiling water bath prior to gel loading. SDS-PAGE was carried out at 37 °C with gentle shaking. At various time points, sample aliquots were taken, treated with 2 mM DFP, and boiled for 3 min in SDS-PAGE sample buffer (28). Samples of purified neutrophil plasma membranes were mixed with 2X sample buffer containing 500 mM 2-mercaptoethanol, 30% glycerol, 1% Triton X-100, and tetraborate stain (TMBZ/H2O2). Using a similar technique, we analyzed heme staining of an LDS-PAGE gel containing purified cytochrome b. Two major bands of staining were detected for heme (see below) or stained with 0.125% Coomassie Blue G in 50% methanol and 10% acetic acid. Gels were then destained in 25% isopropanol alcohol 10% acetic acid and hydrated in H2O for 2 days.

Heme Staining—Samples of purified cytochrome b were subjected to SDS-PAGE at 0 °C as described above and then stained for heme following the method of Thomas et al. (32) with slight modification. Briefly, the gels were incubated for 30 min at 25 °C in the dark in a solution containing 3 parts of 6.3 mM TMBZ dissolved in methanol and 7 parts of 0.25 M sodium acetate, pH 5.0. The gels were developed for 15–30 min by adding H2O2 to a final concentration of 60 mM and then placed in 0.01 M sodium acetate (pH 4.0) containing 10% isopropanol alcohol and photographed. The gels were then completely destained by incubating in 70 mM sodium sulfite (w/v) in H2O, washed extensively with 30% isopropanol alcohol, and stained with Coomassie Blue as described above.

Peroxidase Treatment—Samples of purified cytochrome b were incubated with 10 μg/ml or 50 μg/ml S aureus protease V8 at 37 °C with gentle shaking. At various time points, the oxidized spectrum was measured (see below) and sample aliquots were taken, treated with 2 mM DFP, and boiled for 3 min in SDS-PAGE sample buffer (28). Samples of purified neutrophil plasma membranes were mixed with 2X sample buffer containing 0.005% Triton X-100 at 37 °C with gentle shaking. At various time points, sample aliquots were taken, treated with DFP and centrifuged at 122,000 × g for 30 min in a Beckman TL-100 table top ultracentrifuge. The pellets and supernatants were then analyzed for cytochrome b using reduced-minus-oxidized difference spectrophotometry, SDS-PAGE, and Western blotting.

Measurement of Cytochrome b—Neutrophil cytochrome b was quantitated by reduced-minus-oxidized difference spectrophotometry (Shimadzu, Kyoto, Japan), assuming an oxidized Soret band (413 nm) extinction coefficient of 131 mM−1 cm−1 and a reduced-minus-oxidized Soret band (427–440 nm) extinction coefficient of 101 mM−1 cm−1 (33). Samples were reduced by the addition of 5 μl of a 1.0 M solution of sodium dithionite made up in H2O immediately before use.

Antibody Preparation—Rabbit antiserum to a peptide corresponding to residues 182–174 (NH2-EARKKPSSEEEAA-COOH) of the cytochrome b small subunit was prepared and affinity purified on a peptide affinity column as described previously (34). This antibody was specific for the cytochrome b small subunit and blotted identically to our previously characterized anti-cytochrome b holo-subunit antibody (23, 35).

Western Blotting—Electrophoretic transfer of proteins from LDS-PAGE or SDS-PAGE gels onto nitrocellulose and Western blotting was performed as previously described (23), except an alkaline phosphatase-conjugated goat anti-rabbit IgG secondary antibody was used in these studies. The blots were developed using a commercial kit (Kirkegaard & Perry Laboratories, Gaithersburg, MD), and the reaction was terminated by the transfer of the nitrocellulose membrane to distilled H2O. Western blots were quantitated using a Zeineth 1D/2D scanning laser densitometer (Biomed Instruments, Fullerton, CA). Laser Densitometry—Bands on Coomassie Blue-stained gels or Western blots were quantitated using a Zeineth 1D/2D soft laser scanning densitometer with software to determine the areas of the densitometric peaks.

RESULTS

We have postulated that human neutrophil cytochrome b is a multi-heme cytochrome. Indirect biochemical and spectral evidence (see “Discussion”) supports this hypothesis. To address this question directly, however, we attempted to identify the cytochrome b subunit or subunits in which the heme was localized. Widger et al. (31) reported success in identifying polypeptides to which non-covalently bound heme was localized in chloroplast cytochrome b59. Using a similar technique, we analyzed highly concentrated samples of purified human neutrophil cytochrome b. Fig. 1 (upper panel) shows the TMBZ/H2O heme staining of an LDS-PAGE gel containing purified cytochrome b. Two major bands of staining are quite evident: a broad band at ~91 kDa and a sharper band at ~22 kDa. Minor heme staining is also evident in the regions of 30, 44, and >200 kDa. Also evident is a hazy stained region at the bottom of the gel which is due to the presence of free heme that was released from the cytochrome during electrophoresis. Due to the release of a portion of the non-covalently bound heme (31, 32), the heme staining of the major bands decreased comparatively as the amount of cytochrome b applied to the lanes was decreased (see lanes 4–8).

Coomassie Blue staining of these lanes (Fig. 1, lower panel) after destaining to remove the heme stain indicated the presence of protein bands in the region of the heme stain, as well as a number of other protein bands that were not stained for heme. The major bands of heme staining corresponded exactly with the protein-staining pattern published for purified human neutrophil cytochrome b (23, 24), suggesting that both subunits of the cytochrome had associated heme.

To positively identify the heme-staining bands, these samples were fractionated by LDS-PAGE and immunoblotted using previously characterized anti-cytochrome b antibodies
phoresis and heme staining. Weight standards were used on all gels. In addition, immunoblots were subjected to LDS-PAGE and Western blotted with anti-cytochrome b small subunit peptide antibody (data not shown). In addition, immunoblotting also demonstrated that the heme-staining band at 43 kDa was immunoreactive with the anti-cytochrome b large subunit antibodies, suggesting it represented a dimer or multimer of the large subunit.

One of four experiments giving identical results. A previous report by Yamaguchi et al. (34) indicated that the 91-kDa heme-staining band is indeed the large subunit of cytochrome b (gp91-phox), and immunoblotting with an anti-cytochrome b small subunit peptide antibody indicated that the 22-kDa heme-staining band is the small subunit (gp22-phox). Immunoblotting with an antibody prepared against holo-cytochrome b small subunit peptide antibody indicated that the 22-kDa heme-staining band is the small subunit (gp22-phox). Immunoblotting with an antibody prepared against holo-cytochrome b small subunit peptide antibody indicated that the 22-kDa heme-staining band is the small subunit (gp22-phox).

Further analysis of the V8 protease-treated cytochrome b using LDS-PAGE followed by TMBZ/H2O2 heme staining was performed to identify heme-containing fragments of the proteolyzed cytochrome. In these experiments, a higher concentration of protease was utilized to ensure complete proteolysis of the cytochrome. Fig. 5 (left panel) shows a heme-stained gel containing control, untreated cytochrome b (lane 4), and cytochrome b treated with 50 μg/ml V8 protease for 30, 60, and 120 min (lanes 5–7, respectively). At 30 min of treatment, several heme-containing fragments were present, spectral properties was investigated. Fig. 3 (upper panel) shows that cytochrome b treated with Staphylococcal protease V8 retains a normal absorbance spectrum and that the apparent cytochrome content is no different than that of control samples, even after 4 h of protease treatment. In addition, the cytochrome b spectrum observed in the protease-treated samples was identical at all time points to the spectrum of control, untreated cytochrome. It should be noted, however, that there was a small decrease in Soret absorbance amplitude in both control and treated samples (~20%), possibly due to nonspecific aggregation and/or denaturation of the cytochrome at 37 °C.

To confirm that the cytochrome was proteolyzed, these samples were also fractionated by SDS-PAGE, Coomassie Blue stained, and the cytochrome b bands quantitated by scanning laser densitometry. Fig. 3 (lower panel) shows that indeed both subunits of the cytochrome were proteolyzed, with both large and small subunit bands being degraded to a similar extent. Immunoblots of the proteolyzed samples showed a time-dependent loss of immunoreactive large subunit with the appearance of smaller immunoreactive bands, including a major band at ~18–20 kDa (Fig. 4, left panel, double arrowheads). Immunoblotting with anti-cytochrome b small subunit antibody also showed a time-dependent loss of the small subunit with a corresponding appearance of an immunoreactive band also at ~18–20 kDa in the protease-treated samples (Fig. 4, right panel, double arrowheads). No decrease in the staining intensity of either subunit was evident in Western blots of control untreated samples, and, in addition, we did not observe any immunoreactive peptide fragments on the control blots (data not shown).

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Fig. 3. Effect of protease treatment on human neutrophil cytochrome b. Purified human neutrophil cytochrome b was treated with 1 µg/ml V8 protease for the indicated time periods, and samples were removed from the incubation and analyzed for oxidized absorbance spectrum (upper panel) as described under "Experimental Procedures." The results, expressed as mean ± S.D. (n = three experiments), represent percent of initial (time 0) cytochrome b absorbance spectrum, with 100% representing 50–60 pmol cytochrome b for control (O) and protease-treated (●) samples. Samples were also treated immediately with DFP, diluted in SDS-PAGE sample buffer, and subjected to SDS-PAGE as described under "Experimental Procedures." The cytochrome b bands (91 and 22 kDa) in the Coomassie Blue-stained gels were then quantitated densitometrically (lower panel) as described. The results, expressed as mean ± S.D. (n = three experiments), represent percent of initial (time 0) cytochrome b subunits intact, as determined by gel scanning of control (O) and protease-treated (●) samples. Since both subunits were proteolyzed with similar kinetics, only one plot is given to represent the data from both subunits.

including a heavily stained band at 18–20 kDa (the same location as the immunoreactive fragments from both subunits shown in Fig. 4). Several other heme-stained bands also appeared at ~50–60 kDa and at ~29–35 kDa, presumably as fragments of the large subunit of the cytochrome. It is interesting that light heme staining also was present in the ~29–35 kDa region in Fig. 1, suggesting that a small amount of proteolysis of the large subunit may occur during the cytochrome preparation. After longer treatment times with V8 protease (1 and 2 h), most of the larger (~29–35 kDa) heme-staining bands were lost (lanes 3 and 4) while the 18–20-kDa band became darker, suggesting that it contains the major stable heme-containing proteolytic fragments of the cytochrome. After destaining the gel with sodium sulfite, it was stained with Coomassie Blue to visualize the protein pattern. Fig. 5 (right panel), showing the Coomassie-stained gel, indicates that indeed the cytochrome b subunits (control shown in lane 4) were degraded by the protease (lanes 5–7) with the appearance of a major protein band at 18–20 kDa, which is identical to the heme-staining pattern.

To determine whether the heme containing regions of cytochrome b lie within the membrane bilayer, or whether they are on external loops, purified neutrophil plasma membranes were subjected to proteolysis. As was observed with purified cytochrome b, neither V8 protease nor trypsin treatment of membranes affected the cytochrome b spectrum. When the treated membranes were then pelleted by ultracentrifugation, the spectrum remained with the membrane pellet, suggesting that the heme containing regions were protected from proteolytic attack by the membrane structure (Fig. 6, upper panel). No cytochrome b was detected in the supernatants, as determined by absorbance difference spectroscopy and Western blotting (data not shown). SDS-PAGE analysis followed by Coomassie Blue staining confirmed that both of the cytochrome b subunits were indeed proteolyzed significantly by both of these proteases (Fig. 6, lower panel). Western blots of the supernatants and pellets from these samples confirmed that the cytochrome b and its immunoreactive fragments (for the proteolyzed samples) were present in the membrane pellets and that the cytochrome b in the pellets of the proteolyzed samples was indeed degraded into its characteristic fragments (data not shown). Some of the cytochrome b in these samples (~15–20%) appeared to be resistant to proteolysis, however, possibly because of sequestering inside closed vesicles, although 0.005% Triton X-100 was present to help permeabilize membrane vesicles to the proteases, or because of sequestering inside any possible membrane aggregates.

DISCUSSION

The theoretical heme content for a cytochrome b heterodimer of aggregate molecular mass of 113 kDa (91 + 22 kDa) containing only one heme/cytochrome b molecule is 8.9 nmol/mg of protein. However, the measured heme content of the purified cytochrome b is 20–30 nmol/mg of protein depending on the value used for the extinction coefficient (23, 38). This discrepancy allowed us to speculate that the cytochrome is a multi-heme protein (25). In subsequent studies, additional spectral evidence has now accumulated supporting this speculation. Iizuka et al. (39) have reported that the low temperature reduced-minus-oxidized absorbance spectrum of the
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FIG. 5. Lithium dodecyl sulfate-polyacrylamide gel electrophoresis and heme staining of proteolyzed neutrophil cytochrome b. Purified human neutrophil cytochrome b (≈6.5 nmol for each condition) was treated with buffer as a control (lane 4) or with 50 μg/ml V8 protease for 30 (lane 5), 60 (lane 6), and 120 (lane 7) min as described under "Experimental Procedures." After incubation, the samples were treated with DFP, analyzed by LDS-PAGE and TMBZ/H2O2, heme staining (left panel), destained, and stained with Coomassie Blue (right panel) as described under "Experimental Procedures." As a positive control for heme staining, cytochrome c was applied to lanes 1–3 and 8–10 (lanes 3 and 8 = 16 pmol; lanes 2 and 9 = 32 pmol; and lanes 1 and 10 = 64 pmol). Prestained molecular weight standards were used on all gels (Std) and molecular weights are as indicated. The locations of the large (91 kDa) and small (22 kDa) subunits of the cytochrome (large arrowheads) and the major proteolytic fragment(s) (double small arrowheads) are indicated.

FIG. 6. Effect of protease treatment on cytochrome b in neutrophil membranes. Purified human neutrophil membranes suspended in 10 mM HEPES, pH 7.4, 100 mM KCl, 10 mM NaCl, 0.005% Triton X-100 were treated with proteases as described under "Experimental Procedures," and aliquots were removed at the indicated time points and centrifuged to separate soluble and particulate fractions. The pellets and supernatants were assayed for cytochrome b content using reduced-minus-oxidized difference spectroscopy as described under "Experimental Procedures," and the results from the pellets are expressed as pmol of cytochrome b (upper panel). No cytochrome b was found in the supernatants. Pellet samples were also subjected to SDS-PAGE, stained with Coomassie Blue, and the cytochrome b bands scanned using a laser densitometer to determine the amount of the subunits remaining intact. The results represent percent of initial (time 0) cytochrome b subunits intact, as determined by gel scanning. Since both subunits were proteolyzed with similar kinetics, only one graph is given to represent the data from both subunits. One of two identical experiments.

cytochrome b α band is split, suggesting the presence of two heme species or that the cytochrome is present in two different states. Hurst et al. (40) using EPR and resonance Raman spectroscopy concluded that their spectra were compatible with a hibhistidinyl, multi-heme cytochrome with closely spaced hemes. These interpretations were recently confirmed by Ueno et al. (41). Our data substantiate the indirect spectral evidence by directly showing the locus of heme to be in both the large and small subunits.

Yamaguchi et al. (36) and Nugent et al. (37) recently reported evidence for a cytochrome b molecule in which the heme was bound by the small (22 kDa) subunit; however, several unexplained discrepancies are present in their data. First, Yamaguchi et al. (36) claimed to have purified the heme-containing 20–22 kDa subunit of cytochrome b, yet their reported amino acid composition was greatly different from the actual composition determined from the predicted amino acid sequence obtained from our molecular genetic studies (25). Second, the large (91 kDa) subunit disappeared during their cytochrome purification procedure, possibly due to proteolytic degradation, under conditions that normally do not disrupt the heterodimeric complex (i.e. octyl glucoside extraction) (23, 24). Third, Nugent et al. (37) reported the apparent dissociation of α and β subunits on sedimentation-equilibrium analysis at 15 °C for 36 h in the presence of octyl glucoside and provided an estimated size of 22–29 kDa for the heme-binding subunit. However, they were not able to reproduce these findings using gel filtration in the presence of octyl glucoside, irrespective of temperature (4–25 °C) or of NaCl concentrations (up to 1 M). Although their radiation inactivation analysis supported their hydrodynamic data in octyl glucoside, they were unable to inactivate to the recommended 100–1000-fold level (42–44) to assure linearity of the inactivation fit. In addition, they did not account for the presence of oligosaccharide on the cytochrome b large subunit, which can be ionized without leading to damage of the polypeptide chain (43). The result is an estimated target size that is substantially smaller than the actual glycoprotein molecule (43). Finally, analysis of the primary sequence of the cyto-
chrome b small subunit for heme-coordinating residues demonstrated the presence of only 2 histidines, 1 of which displays polymorphic substitutions that do not affect cytochrome spectrum (25, 45). This variability in primary structure of the small subunit suggests that it may not be capable of coordinating a heme by itself. It does not rule out, however, the possibility of a heme shared between two small subunits or between a small and large subunit.

Our current data clearly indicate that cytochrome b structure is more sensitive to proteolysis than is its absorbance spectrum. V8 protease treatment of purified cytochrome b as well as cytochrome b in isolated neutrophil membranes rapidly degraded both the large and small subunits of the cytochrome into significantly smaller heme-containing fragments although the spectrum remained essentially unchanged. The major heme-bearing fragments detected by heme staining, Western blot analysis, and gel staining with Coomassie Blue were close to 20 kDa in molecular mass, resembling more what was observed by Yamaguchi et al. (36) and Nugent et al. (37). In addition, we have observed that it is, in fact, very difficult to detect intact cytochrome b large subunit by Western blot analysis in the specific granule fractions of N. caviaeatis, DFP-treated neutrophils when they are separated on linear sucrose density gradients (31, 46). These fractions contain significant cross-contamination of elastase-rich azurin. It is due to the presence of a complex of proteolytic fragments of both subunits, as well as heme-bearing fragments of the cytochrome molecule.

This configuration is compatible with our proteolysis data in both the membrane and heme-coordinating residues are protected from proteolysis by the lipid bilayer.

These conclusions are also compatible with our view of the presumed function of cytochrome b in neutrophils, i.e., an electron transfer protein (53) for high energy electrons accessing extracellular oxygen to produce superoxide. Such a protein would require hydrophobic hemes in the membrane positioned so that electrons could be accepted from a flavoprotein donor and then transferred to a remote oxygen-binding pocket (41, 54) located near the extracellular aspect of the membrane, possibly by a quantum mechanical electron transfer process through structural bridges in the protein (55, 56).

Acknowledgment—We thank Dr. William R. Widerg for helpful discussions regarding the use of LDS-PAGE for the analysis of non-covalently bound hemes.

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