Stoichiometry of p22-*phox* and gp91-*phox* in Phagocyte Cytochrome b_{558}[†]

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ABSTRACT: The phagocyte NADPH oxidase complex is an unusual electron transfer system. Its principal component, cytochrome b_{558}, is a heme-containing integral membrane protein consisting of two subunits, gp91-*phox* and p22-*phox*. We used a novel method to measure precisely the gp91-*phox*:p22-*phox* stoichiometry. Cytochrome b_{558} was isolated in high purity from human neutrophil membrane preparations using a novel affinity purification method. We performed direct peptide sequencing of purified cytochrome b_{558} and detected two amino acid sequences which matched predicted sequences for gp91-*phox* and p22-*phox*. We quantitated amounts of both amino acids released from p22-*phox* and gp91-*phox* in each sequencing cycle. Averaging over 25 cycles, the mean p22-*phox*:gp91-*phox* ratio of released amino acids was 0.93 ± 0.01. To correct for recovery differences between individual amino acids, we measured individual p22-*phox*:gp91-*phox* ratios for the eight different amino acids common to both p22-*phox* and gp91-*phox* in the first 25 positions. The mean of individual p22-*phox*:gp91-*phox* ratios for the eight common amino acids was 0.96 ± 0.05. The p22-*phox*:gp91-*phox* ratios for each of the eight common amino acids varied from 0.81 to 1.20. Taken together, measured ratios for total and individual amino acids are consistent with a predicted ratio of 1.0 for 1:1 p22-*phox*:gp91-*phox* stoichiometry in cytochrome b_{558}.

Myeloid-derived phagocytes, such as neutrophils, eosinophils, macrophages, and monocytes, are essential in host defense against pathogenic microorganisms. Stimulation of these cells leads to production of large quantities of superoxide, the parent compound to a family of reactive oxygen species (Umeki, 1994). The importance of superoxide to host defense is exemplified by chronic granulomatous disease, a genetically heterogeneous disease in which patients with defects in the ability to generate superoxide suffer frequent and life-threatening infections beginning in early childhood. Superoxide is produced by a membrane NADPH oxidase complex. Stimulation of neutrophils leads to assembly of the NADPH oxidase from an integral membrane cytochrome b_{558} and at least three cytosol proteins, p47-*phox*, p67-*phox*, and *rac*1 or *rac*2, that translocate to membrane and interact with cytochrome b_{558} (Segal & Abo, 1993). Cytochrome b_{558} is composed of two subunits, gp91-*phox* and p22-*phox*, which form a non-covalent but tightly associated complex (Parkos et al., 1987). gp91-*phox* is heavily glycosylated and its amino acid sequence predicts binding sites for FAD and NADPH (Kleinberg et al., 1989, Rotrosen et al., 1992, Segal et al., 1992). Cytochrome b_{558} binds two heme groups which are coordinated by histidines in both heme axial positions (Hurst et al.; 1991, Ueno et al.; 1991, Miki et al., 1992). These observations suggest that cytochrome b_{558} is the electron transfer entity of the NADPH oxidase. Consistent with this view, Koshkin and Pick (1994) showed that pure cytochrome b_{558} alone can transfer electrons from NADPH to molecular oxygen in the absence of p47-*phox*, p67-*phox*, and *rac* under certain *in vitro* experimental conditions.

Little is known about how gp91-*phox* and p22-*phox* interact in forming cytochrome b_{558}. Previous attempts to measure the stoichiometry of gp91-*phox* to p22-*phox* relied on measurements of molecular mass of purified cytochrome b_{558}. Parkos et al. (1988a) detected a range for cytochrome b_{558} mass between 100 and 135 kDa from hydrodynamic and SDS−PAGE experiments. They concluded that cytochrome b_{558} was either a 1:1 or a 2:1 gp91-*phox*:p22-*phox* heteromer. Nugent et al. (1989) concluded from their hydrodynamic data that cytochrome b_{558} was instead a larger 2:2 or 3:3 p22-*phox*:gp91-*phox* heteromer. Recently, Taylor et al. (1993) suggested from gp91-*phox* modeling data that cytochrome b_{558} is a 2:1 p22-*phox*:gp91-*phox* heterotrimer. Measurements of cytochrome b_{558} molecular mass suffer from several methodological problems. First, estimations were required to account for the effects of lipid and detergent bound to cytochrome b_{558} in equations used to derive the mass of cytochrome b_{558} from hydrodynamic experimental results (Parkos et al., 1988a). Second, gp91-*phox* is extensively and heterogeneously glycosylated which introduced an additional estimated correction in determining the mass of cytochrome b_{558}. Third, the large difference in mass between gp91-*phox* and p22-*phox* required that the molecular mass of cytochrome b_{558} be measured precisely. For instance, the range of cytochrome b_{558} molecular mass (100−135 kDa) determined

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[1] Abbreviations: OG, octyl β-glucopyranoside; SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electropheresis; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); EDTA, (ethylenedinitrilo)tetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; Tris, Tris(hydroxyethyl)aminomethane.

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by Parkos et al. (1988a) is essentially equal to the mass of p22-*phox* itself. In this paper, we report our results determining the stoichiometry of p22-*phox* to gp91-*phox* in cytochrome b_{558} employing a sensitive protein-sequencing approach which was independent of detergent, lipid, and glycosylation confounding effects and which was unaffected by differences in molecular mass between p22-*phox* and gp91-*phox*.

MATERIALS AND METHODS

Purification of Neutrophil Membrane. Granulocytes were collected by pheresis from normal donors under protocols approved by the University of Maryland at Baltimore Institutional Review Board. Donors were pretreated with either dexamethasone (Roxane, Columbus, OH) (12 mg given 24 h prior to pheresis and 10 mg at time of pheresis) or recombinant granulocyte colony stimulating factor (Amgen, Thousand Oaks, CA) (5 μg/kg doses given 48 and 24 h prior to pheresis). Recovery of neutrophils was facilitated by hydroxyethyl−starch infusion and citrate anticoagulation. Granulocytes were typically >85% neutrophils. Erythrocytes were lysed with hypotonic saline, and neutrophils were incubated for 30 min at room temperature with 5 mM diisopropyl fluorophosphate in phosphate-buffered saline or in Hank's buffered saline without magnesium and calcium. Neutrophils were resuspended to 400 × 10^6/mL in relaxation buffer (100 mM KCl, 10 mM PIPES, pH 7.4, 3 mM $MgCl_2$, 3.5 mM NaCl; Kleinberg et al., 1989) containing 1 mM ATP, 1 mM EGTA, and 10 μM PMSF and disrupted by nitrogen cavitation. Nuclei and unbroken cells were removed by centrifugation at 500*g* for 5 min, and granules were removed by centrifugation at 10 000*g* for 20 min. Membranes were isolated from the resulting supernatant by centrifugation at 100 000*g* for 45 min, washed once with relaxation buffer, collected by repeat centrifugation, and resuspended at 1 × 10^9 cell equiv/mL (~2 mg protein/10^9 cell equiv) in 0.34 M sucrose/50% relaxation buffer. Neutrophil membranes were stored at −80 °C until used for cytochrome b_{558} purification.

Purification of Cytochrome b_{558}. A detailed purification method will be described elsewhere. Briefly, neutrophil membrane pellets were extracted with 1 M NaCl for 15 min then washed with phosphate-extraction buffer (0.1 M potassium phosphate, pH 7.4, 0.1 M KCl, 1 mM EDTA, 1 mM sodium azide, 20% v/v glycerol) containing 0.8 mg of cholic acid/mL. The extracted membrane pellet was then solubilized in phosphate-extraction buffer containing 1% Triton X-100 (membrane research grade, Boehringer Mannheim GmbH, Mannheim, Germany) at 1 × 10^9 cell equivalents/mL and passed through a mixed-bed triple column of (diethylamino)ethyl-Sepharose, carboxymethyl-Sepharose, and aminooctyl-agarose (all from Sigma, St. Louis, MO).

The triple-column flow-through containing cytochrome b_{558} was batch-adsorbed to anti-p22-*phox* IgG immobilized to protein G-agarose (Sigma, St. Louis, MO) and incubated overnight at 4 °C. Anti-p22-*phox* IgG was affinity-purified from serum from a goat immunized with the synthetic peptide C-GGPPGGPQVNPIPVTDEVV (corresponding to the carboxyl-terminal 19 amino acids of p22-*phox*) conjugated to maleimide-activated keyhole limpet hemocyanin (Pierce, Rockford, IL). After washing with 1% Triton X-100/phosphate-extraction buffer, the bound cytochrome b_{558} was batch-eluted by incubation of the anti-p22-*phox* IgG/protein G-agarose for 24 h at 4 °C with 2−5 mM C-GGPPGGPQVNPIPVTDEVV synthetic peptide in 1% Triton X-100/phosphate-extraction buffer. Cytochrome b_{558}-containing eluate was dialyzed exhaustively against 1% Triton X-100 in 5% phosphate-extraction buffer to decrease ionic strength and to remove peptide. The dialysate was loaded onto a heparin-agarose (Life Technologies, Gaithersburg, MD) column equilibrated with 1% Triton X-100 in 5% phosphate-extraction buffer and washed with 40 mM octyl β-glucopyranoside (OG) (Sigma, St. Louis, MO) in 5% phosphate-extraction buffer. Cytochrome b_{558} was eluted subsequently with 1 M NaCl/40 mM OG in 5% phosphate-extraction buffer. The amount of purified cytochrome b_{558} was quantitated from the dithionite-reduced vs oxidized difference spectrum using $\epsilon_{428-415\ nm} = 180\ cm^{-1}\ mM^{-1}$ [averaged from Light et al. (1981), Bos et al. (1978), and Capeillere-Blandin et al. (1991)]. Specific activity of purified cytochrome b_{558} was 12.8 nmol of heme/mg of protein (mean of three experiments). Protein content was measured in the bicinchoninic acid assay (Pierce, Rockford, IL). Elution of cytochrome b_{558} with a NaCl gradient from the heparin-agarose column in the final purification step resulted in identical elution profiles for heme, p22-*phox*, and gp91-*phox* (data not shown), indicating that cytochrome b_{558} remained intact throughout purification.

Protein Sequencing of Cytochrome b_{558}. Purified cytochrome b_{558} was buffer-exchanged into 40 mM OG in 5 mM sodium phosphate, pH 7.0, by ultrafiltration on 10 000 molecular weight cutoff membranes (Microcon, Amicon, Beverly, MA). Cytochrome b_{558} (~100 pmol) was adsorbed directly to the polybrene filter in the sequencing chamber of a model 477A Applied Biosystems automated protein sequencer. Cytochrome b_{558} was sequenced by standard methods according to manufacturer's protocols. High sequencing yield was critically dependent on use of membrane research grade Triton X-100, avoidance of Tris-containing buffers, and removal of glycerol from buffer immediately before sequencing.

RESULTS

We found that an existing method for purifying cytochrome b_{558} (Rotrosen et al., 1992) failed to yield consistently pure cytochrome b_{558} suitable for protein sequencing. Therefore, we developed a novel method to purify cytochrome b_{558} from human neutrophil membranes by affinity chromatography using an anti-p22-*phox* antibody immobilized to agarose beads. Neutrophils were isolated from granulocyte pheresis collections of normal volunteers in whom circulating neutrophil counts were boosted by pretreatment with dexamethasone or recombinant granulocyte colony stimulating factor. Cytochrome b_{558} content in neutrophil membranes ranged between 1−2 nmol/mg of membrane protein (2−4 nmol/10^9 cell equiv). Sufficient cytochrome b_{558}-containing flow-through fraction from the triple column was applied to saturate binding to the anti-GGPPGGPQVNPIPVTDEVV IgG/protein G-agarose column. After extensive washing, bound cytochrome b_{558} was eluted completely with synthetic peptide. Cytochrome b_{558} was concentrated by binding to heparin agarose followed by elution with 40 mM OG in 1 M NaCl. Eluted cytochrome b_{558} yielded two bands on Coomassie Blue-stained SDS−PAGE gels (Figure 1) corresponding to gp91-*phox* and p22-*phox* confirmed by immunoblotting duplicate samples with specific anti-gp91-*phox*

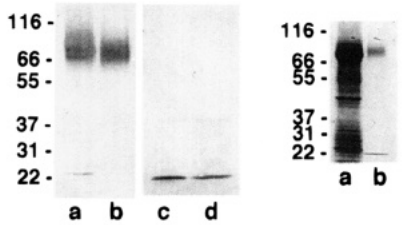

FIGURE 1: Purification of cytochrome b_{558}. Neutrophil membranes were purified as described in Materials and Methods. Left panel, immunoblots of neutrophil membrane (lanes a and c) and purified cytochrome b_{558} (lanes b and d) separated on an 8%−16% SDS−PAGE gel, transferred to polyvinylidene difluoride membrane, and incubated with anti-gp91-*phox* (lanes a and b) and anti-p22-*phox* sera (lanes c and d). Right panel, separation of neutrophil membrane (lane a) and purified cytochrome b_{558} eluted from heparin agarose (lane b) on an 8%−16% SDS−PAGE gel stained with Coomassie Blue.

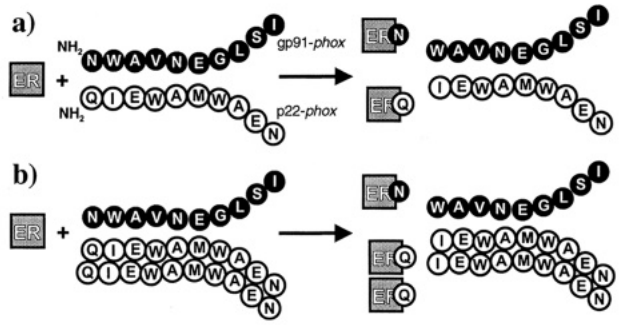

FIGURE 2: Schematic showing simultaneous sequencing of gp91-*phox* and p22-*phox* assuming 1:1 and 2:1 ratios. ER represents Edmans reagent (phenyl isothiocyanate). In the first cycle, glycine is released from both gp91-*phox* and p22-*phox* (not shown). In the second cycle, asparagine (N) is released from gp91-*phox* while glutamine (Q) is released simultaneously from p22-*phox*. (a) For the 1:1 ratio, equal numbers of asparagine and glutamine are released in the second cycle. (b) For the 2:1 ratio, twice as many glutamines as asparagines are released in cycle 2.

(Kleinberg et al., 1989) and anti-p22-*phox* sera (Figure 1). No other bands were seen with Coomassie Blue staining.

In these experiments, we took advantage of previous observations that the amino-termini of both gp91-*phox* and p22-*phox* were not blocked post-translationally (Teahan et al., 1987; Parkos et al., 1988b). We reasoned that gp91-*phox*:p22-*phox* stoichiometry could be determined by simultaneous amino-terminal peptide sequencing of p22-*phox* and gp91-*phox* from highly purified cytochrome b_{558}. We expected each sequencing cycle would yield two amino acids simultaneously released from gp91-*phox* and p22-*phox*, respectively (Figure 2). Assignment of simultaneously released amino acids to gp91-*phox* or p22-*phox* was made after examination of the known primary amino acid sequence for each cytochrome b_{558} subunit. Despite exhaustive dialysis, we also were able to detect residual p22-*phox* synthetic peptide sequence in addition to p22-*phox* and gp91-*phox* sequences. However, the synthetic peptide sequence did not match p22-*phox* or gp91-*phox* amino acid sequences at any positions and therefore did not interfere with analysis. Amounts of synthetic peptide amino acids detected at each cycle were comparable or less than amounts of p22-*phox* and gp91-*phox* amino acids.

A representative result of one of three sequencing experiments is shown in Figure 3 where cytochrome b_{558} was sequenced through at least 25 cycles. The sequences of the first 25 residues of gp91-*phox* and p22-*phox* are shown with

NH_2GNWAVNEGLSIFVILVWLGLNVFLF gp91

NH_2GQIEWAMWANEQALASGLILITGGI p22

	p22	gp91	Ratio (p22/gp91)
E	49	37	1.32
G	74	82	0.90
I	98	109	0.90
W	23	36	0.65
A	91	102	0.89
N	54	47	1.14
L	95	108	0.88
S	30	36	0.83

FIGURE 3: Results of one experiment of simultaneous peptide sequencing of cytochrome b_{558}. A 100 pmol amount of purified cytochrome b_{558} was sequenced by automated Edman degradation. Amino acid sequences for first 25 positions of gp91-*phox* and p22-*phox* are shown above the table. The first column lists identities of eight amino acids found in the first 25 positions of both gp91-*phox* and p22-*phox*. The second and third columns show picomole amounts of individual amino acids released from p22-*phox* and gp91-*phox*, respectively. Picomole amounts were averaged for multiple occurrences of the same amino acid in positions 1−25 of gp91-*phox* and/or p22-*phox* (positions 1, 18, and 20 of both p22-*phox* and gp91-*phox* excluded from analysis). The fourth column lists calculated p22-*phox*/gp91-*phox* ratios for each of the eight common amino acids. Ratios ranged from 0.65 to 1.32 in this experiment. This experiment resulted in the largest span of ratios between amino acids for the three experiments summarized in Table 1.

corresponding picomoles of amino acid detected for each sequencing cycle. Glycines in cycle 1 and leucines in cycles 18 and 20 were excluded from analysis because they were present in identical positions in both p22-*phox* and gp91-*phox*. For p22-*phox*, the mean quantity was 66.5 pmol for simultaneously released amino acids averaged over 25 cycles. For gp91-*phox*, the mean was 74.1 pmol resulting in a p22-*phox*/gp91-*phox* ratio of 0.92. Two other experiments gave values of 0.92 and 0.95. These values are closer to the theoretical value of 1.0 for a 1:1 $\alpha_n\beta_n$ p22-*phox*:gp91-*phox* heteromer rather than to the theoretical p22-*phox*/gp91-*phox* ratio of 2.0 expected for a 2:1 $\alpha_{2n}\beta_n$ p22-*phox*/gp91-*phox* heteromer.

Closer examination of the amount of amino acid released in each sequencing cycle shows that the yields for individual amino acids vary (Figure 3). For instance, only 23.3 and 23.4 pmol of p22-*phox* tryptophan were measured in sequencing cycles 5 and 8, respectively. In contrast, recoveries were higher for nonlabile amino acids such as leucine and alaninc. These differences in amounts of detected amino acids depend on a number of factors including lability of individual amino acids to degradation and efficiency of derivatization of released amino acids and detection by HPLC.

To correct for potential confounding factors introduced by differences in sequencing yields for individual amino acids, we compared recoveries of identical amino acids common to both p22-*phox* and gp91-*phox*. p22-*phox* and gp91-*phox* share eight amino acids (asn, ala, glu, gly, ile, leu, ser, trp) in the first 25 positions. We compared the relative quantities released for each of these eight amino acids in three separate experiments (Table 1). Amounts were averaged when an amino acid was found more than once in the first 25 positions of p22-*phox* or gp91-*phox*. For example, isoleucine is found at positions 11 and 14 in gp91-

Table 1: Ratio of p22-*phox* to gp91-*phox* Amino Acids Released Simultaneously with Peptide Sequencing of Cytochrome b_{558}[a]

amino acid	p22-*phox*/gp91-*phox* ratio
glutamic acid	1.20 ± 0.15
glycine	0.86 ± 0.03
isoleucine	0.86 ± 0.03
tryptophan	0.81 ± 0.08
alanine	0.98 ± 0.04
asparagine	1.09 ± 0.05
leucine	0.94 ± 0.03
serine	0.92 ± 0.05
mean of eight common amino acids	0.96 ± 0.05
all amino acids	0.93 ± 0.01

[a] Summary results of three cytochrome b_{558}-sequencing experiments are presented in the above table. Cytochrome b_{558} was purified from three separate lots of human neutrophil membranes and sequenced by automated Edman degradation as described in Materials and Methods. Identities of eight amino acids found in the first 25 amino-terminal residues of both p22-*phox* and gp91-*phox* are listed in the first column. Amounts of p22-*phox* and gp91-*phox* amino acids released with each sequencing cycle were quantitated. Shown in the second column are ratios of p22-*phox*/gp91-*phox* calculated for the eight common amino acids as well as for all amino acids present in p22-*phox* and gp91-*phox* residues 1−25 (positions 1, 18, and 20 excluded from analysis). Means ± standard errors were calculated from p22-*phox*/gp91-*phox* ratios from each of the three experiments.

phox and at positions 3, 19, 21, and 25 in p22-*phox*. The averaged amounts of isoleucine released during simultaneous sequencing gave a p22-*phox*/gp91-$phox_{ILE}$ ratio of 0.90. Likewise, p22-*phox*/gp91-*phox* ratios for the other seven common amino acids ranged from 0.81 to 1.20; values close to 1.0 and consistent with a 1:1 $\alpha_n\beta_n$ p22-*phox*:gp91-*phox* heteromeric structure for cytochrome b_{558}.

DISCUSSION

Despite intense interest in the NADPH oxidase, little is known about the structure of cytochrome b_{558}. Much of the difficulty in studying the cytochrome b_{558} structure is due to its integral membrane protein character, heavy glycosylation, problems with obtaining large amounts of highly purified cytochrome b_{558}, and inability to overexpress recombinant cytochrome b_{558} in suitable cell lines. In this paper, we purified cytochrome b_{558} from human neutrophils by a novel method which yielded cytochrome b_{558} sufficiently pure for amino acid sequencing. Our purpose was to determine the stoichiometry of gp91-*phox* and p22-*phox* subunits in cytochrome b_{558}. We view this as an important initial step in further study of the structure of cytochrome b_{558}.

The amino-termini of gp91-*phox* and p22-*phox* are not blocked post-translationally in *vivo* thereby allowing direct amino acid sequencing of purified cytochrome b_{558}. We quantitated simultaneous release of the first 25 amino acids of gp91-*phox* and p22-*phox* during Edman degradation sequencing cycles. As expected, two amino acids per sequencing cycle were detected (not including the additionally detected sequence of the p22-*phox* synthetic peptide used for affinity purification of cytochrome b_{558}). Examination of the sequences of dual amino acids released in each cycle revealed two peptide sequences with exact matches to the corresponding reported amino-terminal peptide sequences of gp91-*phox* and p22-*phox*.

We performed three independent purifications of cytochrome b_{558} followed by amino acid sequencing. Our data are best interpreted as showing a 1:1 p22-*phox*:gp91-*phox* stoichiometry consistent with an $\alpha_n\beta_n$ cytochrome b_{558} heteromer. The mean ratio of p22-*phox*/gp91-*phox* amino acids was 0.90, which was close to the predicted ratio of 1.0 for 1:1 p22-*phox*:gp91-*phox* stoichiometry. Comparison of quantities of individual p22-*phox* versus gp91-*phox* amino acids showed without exception for the eight common amino acids a ratio significantly closer to the predicted 1.0 for 1:1 stoichiometry rather than the 2.0 ratio expected for 2:1 p22-*phox*:gp91-*phox* stoichiometry.

The quaternary structure of cytochrome b_{558} has been examined in detail in two previous publications both relying primarily on hydrodynamic methods. Parkos et al. (1988a) measured a molecular mass for the cytochrome/detergent complex as 202 kDa in Triton X-100 and 188 kDa in OG−sucrose gradients. Corrections for bound detergent/lipid yielded an estimated molecular mass for cytochrome b_{558} of 100−127 kDa. These values were compared to M_r's between 110 000 and 135 000 for cross-linked cytochrome b_{558} estimated from mobilities on SDS−PAGE gels. They concluded that the minimal cytochrome b_{558} unit consisted of one gp91-*phox* and one or two p22-*phox* subunits. Nugent et al. (1989) also examined the subunit stoichiometry of cytochrome b_{558} in sedimentation−equilibrium studies in $^1H_2O/^2H_2O$ mixtures in Triton N-101 and OG detergents. In Triton N-101, they found a range of masses for cytochrome b_{558} and estimated the molecular mass of 350 kDa for the smallest species of cytochrome b_{558}. In OG, they found a molecular mass of only 22 kDa which they interpreted as evidence for dissociation in OG of gp91-*phox* and p22-*phox* with the heme retained by p22-*phox*.

Conclusions drawn from the studies of Parkos et al. (1988a) and Nugent et al. (1989) quoted above are limited by inherent experimental limitations. Both studies required assumptions or indirect measurements to account for the degree of detergent and phospholipid binding. Parkos et al. (1988a) estimated that detergent/lipid made up half of the molecular mass of cytochrome b_{558} complex detected in their hydrodynamic experiments. Both studies also made assumptions to account for the effect of glycosylation on measurements of molecular mass. As can be seen in SDS−PAGE gels in Figure 1, the degree and heterogeneity of glycosylation leads to a broad smear in the gp91-*phox* band (Kleinberg et al., 1989) giving an indication of the degree of uncertainty that gp91-*phox* glycosylation introduces into determination of cytochrome b_{558} molecular mass. That the studies of Parkos et al. (1988a) and Nugent et al. (1989) differ so markedly also suggests that observed molecular masses for cytochrome b_{558} may be sensitive to details of the methods used.

Our method of determining p22-*phox*:gp91-*phox* stoichiometry through simultaneous amino acid sequencing of pure cytochrome b_{558} is independent of detergent/lipid binding to the cytochrome and of the extent of gp91-*phox* glycosylation. In addition, our method is not affected by differences in molecular mass between p22-*phox* and gp91-*phox*. On the basis of amino acid sequencing, the difference between expected experimental results for 1:1 stoichiometry versus 2:1 stoichiometry is 100% (p22-*phox*:gp91-*phox* ratios of 1.0 versus 2.0). On the basis of molecular mass, the difference between expected experimental results for 1:1 versus 2:1 stoichiometry is at best 20% (110 kDa versus 130 kDa for $\alpha\beta$ 1:1 versus $\alpha_2\beta_1$ 2:1 p22-*phox*:gp91-*phox* ratios, respec-

tively). Given the previously mentioned problems accounting for detergent/lipid binding and glycosylation, it is unlikely that a direct measure of molecular mass of cytochrome b_{558} would be sufficiently precise to determine p22-*phox*:gp91-*phox* stoichiometry.

Although our data supports a p22-*phox*:gp91-*phox* ratio of 1:1, we cannot differentiate between $\alpha\beta$ and $\alpha_n\beta_n$ (where $n = 2$ or 3, as suggested by data of Nugent et al. (1989)] cytochrome b_{558} heteromers. A 1:1 heteromeric structure implies some properties for the electron transfer mechanism of cytochrome b_{558}. Purified cytochrome b_{558} has a heme content of ~20 nmol of heme/mg of protein, which implies the presence of two hemes per each $\alpha\beta$ cytochrome b_{558} unit (Parkos et al., 1987). Absorbance, Raman resonance, and electron paramagnetic resonance spectroscopies suggest that cytochrome b_{558} hemes are tightly bound with histidine imidazole coordination to iron in both axial positions (Hurst et al., 1991; Ueno et al., 1991; Miki et al., 1992). Quinn et al. (1992) detected retention of heme bound to both gp91-*phox* and p22-*phox* separated by lithium dodecyl sulfate–PAGE at 4 °C. They proposed two possibilities to account for this finding. If cytochrome b_{558} is an $\alpha\beta$ heterodimer, then one cytochrome heme may be coordinated to the single invariant p22-*phox* histidine and to another histidine in gp91-*phox*. Then the second heme must necessarily be ligated to two other gp91-*phox* histidines. However, if cytochrome b_{558} is actually an $\alpha_2\beta$ cytochrome b_{558} heterotrimer, then each of the two hemes could be ligated to each of two p22-*phox* subunits and two histidines in gp91-*phox*. Our data indicates that only the first model is feasible. If cytochrome b_{558} is a higher order $\alpha_n\beta_n$ heteromer, then it is possible that hemes may be alternatively ligated to p22-*phox* and gp91-*phox*.

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