Stoichiometry of the subunits of flavocytochrome b_{558} of the NADPH oxidase of phagocytes

Timothy M. WALLACH and Anthony W. SEGAL*

Department of Medicine, University College London, The Rayne Institute, 5 University Street, London WC1E 6JJ, U.K.

Flavocytochrome b_{558}, the membrane-spanning component of the NADPH oxidase system of phagocytic cells, is composed of two subunits, p22^{phox} and gp91^{phox} (where *phox* stands for phagocyte oxidase). The stoichiometry of the subunits has been determined for purified flavocytochrome b_{558} by: (1) densitometry of Coomassie Blue-stained proteins separated by SDS/PAGE, (2) aromatic absorbance at 280 nm by the subunits after separation by gel filtration under denaturing conditions, (3) cross-linking studies with bis[sulphosuccinimidyl]suberate, where the molecular mass of the cross-linked complex was determined by Western blotting, and (4) radiolabelling of pure flavocytochrome b_{558} on lysine residues with ^{125}I-labelled Bolton–Hunter reagent (*N*-succinimidyl-3-(4-hydroxy-5-[^{125}I]iodophenyl)propionate), followed by SDS/PAGE and determination of the radioactivity on each subunit. The ratio of p22^{phox} to gp91^{phox} in the purified flavocytochrome b_{558} was related back to that in the neutrophil membrane by quantitative Western and dot-blotting to ensure that the stoichiometry was maintained during purification. These measurements showed that the two subunits were present in neutrophil membranes in a molar ratio of 1:1.

INTRODUCTION

Phagocytic cells play a key role in the immune system, engulfing and sequestering bacteria in a phagocytic vacuole into which degradative enzymes and the superoxide anion, O_2^-, are released [1]. When the system is defective, little or no superoxide is produced, microbial killing and digestion are not completed and the individual is severely immunocompromised, leading to the condition known as chronic granulomatous disease (CGD) [2].

The NADPH oxidase system has a number of protein components. p22^{phox} and gp91^{phox} (where *phox* stands for phagocyte oxidase) together form a membrane-spanning electron transport chain called flavocytochrome b_{558} [3]. This complex is regulated by the cytosolic proteins p47^{phox}, p67^{phox}, p40^{phox} and p21*rac* [4]. Another component, Rap1A, might be associated with the flavocytochrome b_{558} [5], which transfers electrons from cytosolic NADPH via FAD and haem cofactors to molecular O_2 to form O_2^-, which is released into the lumen of the vacuole.

The NADPH-binding site and FAD prosthetic group are localized on gp91^{phox} [6,7]. There is an apparent FAD-to-haem ratio of 1:2 [6,8], and accumulating evidence for the involvement of two haem centres in electron transport [9–12]. In particular, recent midpoint potential analysis of a non-functional flavocytochrome b_{558} from a CGD patient with an Arg^{54} → Ser mutation in gp91^{phox} showed that the mutant contained two non-identical haems. This led to the reanalysis of normal flavocytochrome b_{558} in which the midpoint potential had previously been determined as −245 mV, and two haem centres with midpoint potentials of −225 and −265 mV were identified [12].

Although not disulphide-linked, the flavocytochrome b_{558} subunits co-purify, and are mutually dependent for stability in the cell because the loss of one due to a genetic lesion in CGD is associated with the absence of the other [13,14]. Separation of the subunits by high concentrations of salt, or by a denaturant such as SDS, results in the loss of the haem spectrum [15]. Until now the subunit stoichiometry of flavocytochrome b_{558} has not been conclusively determined. There have been suggestions that pure flavocytochrome b_{558} could be monomeric [10,16], which were possibly due to proteolysis of the missing subunit. Cross-linking and hydrodynamic studies pointed towards a heterodimer but were unable to exclude the possibility of a trimer [15,17]. A recent communication in abstract form described an approach that used N-terminal sequencing on purified material that showed a 1:1 subunit stoichiometry [18]. However, this value would be affected if a proportion of the subunits were blocked. In none of these approaches was the composition of the complex in the purified material related back to the composition of the complex in the original neutrophil membrane, despite the possibility of partial enrichment of either p22^{phox} or gp91^{phox} during purification [10,15,19]. This study was conducted to determine the stoichiometry of the subunits by the application of a variety of techniques, and to compare the stoichiometry in the pure material with that in neutrophil membrane.

EXPERIMENTAL

Preparation of neutrophil membranes and pure flavocytochrome b_{558}

Neutrophils were prepared as described in [16] and neutrophil membrane as outlined in [20], but using nitrogen cavitation (after equilibration at 2.8 MPa (400 lb/in^2) for 30 min at 4 °C) to break the cells. Membranes were washed in 1 M NaCl before detergent extraction. Flavocytochrome b_{558} was purified from a chronic myeloid leukaemia source as described in [20] but with the addition of a sucrose-density centrifugation step [21]. The protease inhibitors di-isopropyl fluorophosphate (1 mM), Na 7-amino-1-chloro-3-L-tosylamidoheptan-2-one (1 mg/ml), pepstatin (1 mg/ml), leupeptin (1 mg/ml) and PMSF (1 mM) were added to all the solutions.

Electrophoresis

SDS/PAGE was performed by the method of Laemmli [22] with 7% or 11% (w/v) gels on the Hoefer Mighty Small II system.

Abbreviations used: Bolton–Hunter reagent, *N*-succinimidyl-3-(4-hydroxy-5-[^{125}I]iodophenyl)propionate; BS^3: bis[sulphosuccinimidyl]suberate; CGD, chronic granulomatous disease; *phox*, phagocyte oxidase.

* To whom correspondence should be addressed.

Samples were incubated at 37 °C for 30 min in SDS sample buffer. Protein molecular mass standards were from Pharmacia and Bio-Rad. Gels were stained in 0.2 % (w/v) Coomassie Brilliant Blue R250/45 % (v/v) methanol/10 % (v/v) acetic acid and destained in 25 % (v/v) methanol/10 % (v/v) acetic acid.

Densitometry

Coomassie Blue-stained gels were dried on transparent cellophane and the stain intensity was quantified with a scanning densitometer (Sharp-JX330 with ImageMaster software).

Gel filtration

Flavocytochrome b_{558} was separated into its component subunits by denaturing gel filtration in the presence of the reducing agent dithiothreitol (as described in [21]) with a TSK 3000 column on a Hewlett Packard HPLC (HP 1050) system equipped with a diode array detector recording at 280 nm.

Cross-linking

Purified flavocytochrome b_{558} was exchanged into phosphate buffer [140 mM NaCl/10 mM KCl/0.5 % Triton X-100/10 mM NaH_2PO_4 (pH 7.2)]. For this, 0.1 mg of pure material was diluted with 10 vol of phosphate buffer, bound to a 1.0 ml heparin–agarose column, washed with phosphate buffer and eluted in 1 ml of phosphate buffer containing 0.64 M NaCl. A 10 mM stock solution of the water soluble cross-linker bis-[sulphosuccinimidyl]suberate (BS^3) was prepared in 5 mM sodium citrate buffer (pH 5.0). Various amounts of the BS^3 stock were added to 20 μl (2 μg) aliquots of flavocytochrome b_{558}. The reaction mixtures were incubated at room temperature for 30 min and cross-linking was terminated by the addition of 2 μl of 0.5 M Tris buffer (pH 7.0). Cross-linked materials were subjected to SDS/PAGE and the components were identified by Western blotting.

Iodination

Bolton–Hunter reagent {*N*-succinimidyl-3-(4-hydroxy-5-[^{125}I]iodophenyl)propionate} (1 μCi) was evaporated to dryness in a glass tube. Purified flavocytochrome b_{558} was prepared as described for the cross-linking experiments except that flavocytochrome b_{558} bound to heparin agarose was washed with distilled water and eluted with 0.6 M NaCl in distilled water. The purified material was then precipitated with 3 vol. of acetone at −20 °C for 30 min and centrifuged at 12000 ***g*** at 4 °C for 20 min. The acetone was decanted and residual traces were removed under vacuum in a Speedi-vac. The pellet was resuspended in 200 μl of phosphate buffer (100 mM NaH_2PO_4, pH 8.5) and 50 μl (5 μg) of flavocytochrome b_{558} was transferred to the iodination tube. The mixture was incubated at room temperature for 3 min. SDS [20 % (w/v); 5 μl] was added and the reaction was allowed to progress for a further 12 min. Iodinated materials were separated by SDS/PAGE and ^{125}I incorporation into the protein bands was quantified in unstained dried gels with a Fuji Bas1000 PhosphorImager.

Western blotting

Proteins were transferred from the polyacrylamide gel to reinforced 0.45 μm (pore size) nitrocellulose paper at 4–8 mA/cm^2 for 1 h with a semi-dry system (Pharmacia LKB 2117 Multiphor II) and transfer buffer comprising 200 mM glycine, 0.1 % SDS, 10 % (v/v) methanol and 25 mM Tris/HCl, pH 8.9. Blots were rinsed in distilled water and protein molecular mass standards were revealed by reversible staining in 0.2 % Ponceau S/3 % (w/v) trichloroacetic acid/3 % (w/v) sulphosalicylic acid. The nitrocellulose was blocked in blocking solution consisting of Tris-buffered saline [200 mM NaCl/50 mM Tris/HCl (pH 7.4)] with 0.05 % Tween-20, 3 % (w/v) fat-free milk powder and 1 % (w/v) BSA for 1 h. Blots were probed with primary antibody overnight and then rinsed in Tris-buffered saline with 0.05 % Tween-20. They were developed with a horseradish peroxidase-conjugated secondary antibody (in accordance with the Amersham enhanced chemiluminescence kit) or an alkaline phosphatase-conjugated secondary antibody (Promega). For quantification, blots were incubated in ^{125}I-labelled Protein A (0.5 μCi/ml blocking solution) for 1 h, then rinsed and air-dried; the bound radioactivity was quantified with a Fuji Bas1000 PhosphorImager.

Dot-blotting

Dot-blots were prepared with a Schleicher and Schuell Minifold I (96 sample wells) dot-blotter with 0.2 μm (pore size) reinforced nitrocellulose paper. Samples were diluted and denatured in 0.1 % SDS/200 mM glycine/25 mM Tris/HCl (pH 8.9) before blotting, and were quantified as described for Western blots.

Antibodies to the subunits of flavocytochrome b_{558}

These were diluted in blocking solution and incubated with the blots overnight. Primary antibodies were: MC2 (a rabbit polyclonal antibody raised against full-length recombinant $p22^{phox}$ expressed in *Escherichia coli*), Pep37 (a rabbit polyclonal antibody raised against a synthetic peptide corresponding to the 30 C-terminal amino acids of $gp91^{phox}$) and MAB48 (a mouse monoclonal antibody against $gp91^{phox}$, a gift from A. J. Verhoeven).

RESULTS

Densitometry

Pure flavocytochrome b_{558} was separated into its component subunits by SDS/PAGE and stained with Coomassie Blue. Although Coomassie staining is non-specific and depends in part on the hydrophobicity of a given protein, the amino acid compositions of the two subunits are fairly similiar. Therefore it was assumed that they should stain equivalently, in which case staining intensity would be proportional to the amount of protein present.

The amount of Coomassie stain bound to each subunit was quantified by densitometry, with two approaches. In the first a contour line was drawn around the bands giving relative staining intensities (intensity × mm^2) of 2.61 for $p22^{phox}$ and 7.57 for $gp91^{phox}$, a ratio of 1:2.9. In the second, the areas under the lane profile were calculated and gave relative staining intensities (intensity × mm) of 0.38 for $p22^{phox}$ and 1.17 for $gp91^{phox}$, a ratio of 1:3.1 (see Figure 1).

The molecular masses of $p22^{phox}$ and $gp91^{phox}$ predicted from their amino acid sequences are 20.8 and 65.2 kDa respectively, giving a relative protein content of 1:3.1. Thus, the Coomassie Blue staining indicated a subunit stoichiometry of 1:1.

Gel filtration

Pure flavocytochrome b_{558} was denatured by SDS in the presence of dithiothreitol, and the subunits were separated by gel filtration chromatography. The A_{280} trace of the eluate showed two clear peaks. The eluate fractions were analysed by SDS/PAGE and

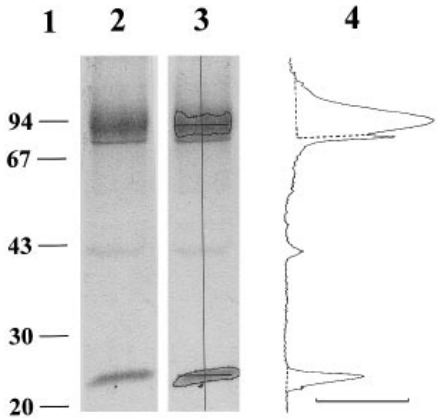


Figure 1 Densitometry of flavocytochrome b_{558}

Pure flavocytochrome b_{558} was fractionated into gp91^{phox} and p22^{phox} components by SDS/PAGE [11% (w/v) gel] and stained with Coomassie Blue (lane 2). The stain intensity was quantified with a scanning densitometer by two approaches: (1) by drawing contour lines around each band (indicated in lane 3) and (2) by integration of the densitometer absorbance trace (shown in lane 4). The horizontal bar represents 0.2 absorbance units. The positions of protein molecular mass standards are marked at the left in kDa.

Coomassie staining to demonstrate that the peaks corresponded to gp91^{phox} and p22^{phox} elution (Figure 2). The Western blot (Figure 2, inset) confirms that the species with apparent molecular masses of approx. 42 and 64 kDa were aggregated p22^{phox}. The A_{280} peak areas corresponding to each subunit were integrated to give relative absorbance values of 21450 units² for p22^{phox} and 61750 units² for gp91^{phox}.

Molar absorption coefficients at 280 nm for each of the two subunits were determined with the ProtParam program (http://expasy.hcuge.ch/sprot/protparam.html; [23]) on the basis of their tryptophan, tyrosine and cysteine content. Because the proteins were examined under reducing conditions, the contribution from cysteine was eliminated. The molar absorption coefficient of p22^{phox} was calculated to be 36370 $M^{-1} \cdot cm^{-1}$ and that of gp91^{phox} to be 128660 $M^{-1} \cdot cm^{-1}$.

From the Beer–Lambert law, the concentration of a protein is proportional to A_{280} divided by the absorption coefficient of that protein. From this, the concentrations of p22^{phox} and gp91^{phox} were determined as 0.59 and 0.48 (relative units) respectively, a stoichiometry of 1.2:1.

Cross-linking

Pure flavocytochrome b_{558} was cross-linked and the complex size relative to original subunit sizes monitored by Western blotting. In each case a calibration curve was prepared from the Ponceau-stained markers run with each gel.

Figure 3(a) shows the result from an 11% (w/v) polyacrylamide gel blotted for 1 h at 4 mA/cm² (gel front, 30 kDa region) and 8 mA/cm² (67 kDa, top of gel). Different blotting conditions were used to maximize transfer to the nitrocellulose. The blot was probed for p22^{phox} with MC2. From the standard curve (Figure 3b), p22^{phox} underwent a mean mass shift from 22 to 105 kDa.

Figure 3(c) shows the same samples run on a 7% (w/v) polyacrylamide gel and blotted for 1 h at 8 mA/cm². The blot was probed for gp91^{phox} with MAB48. gp91^{phox} is a 65.2 kDa protein that runs anomalously and as a broad band on SDS/PAGE due to heavy glycosylation. From the calibration curve

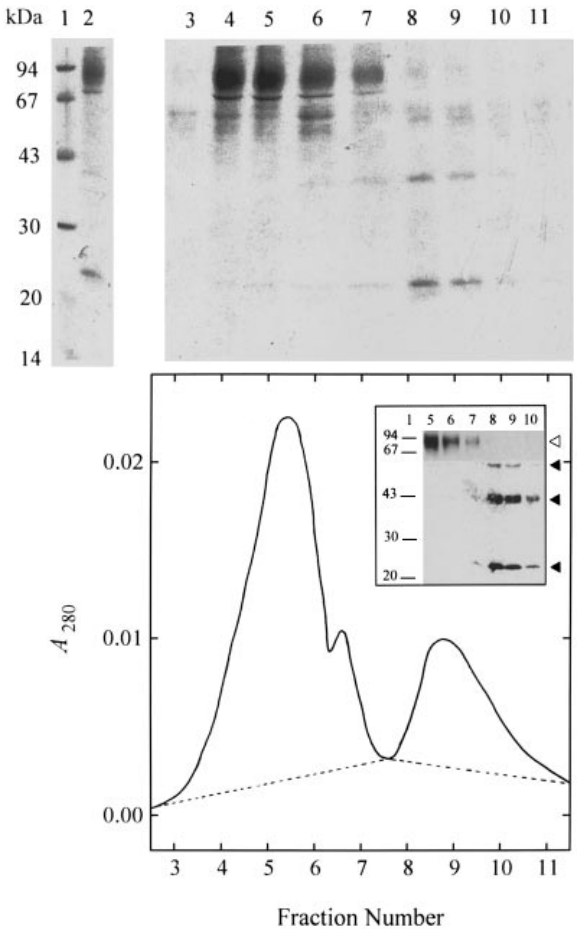


Figure 2 A_{280} analysis of gel filtration of flavocytochrome b_{558}

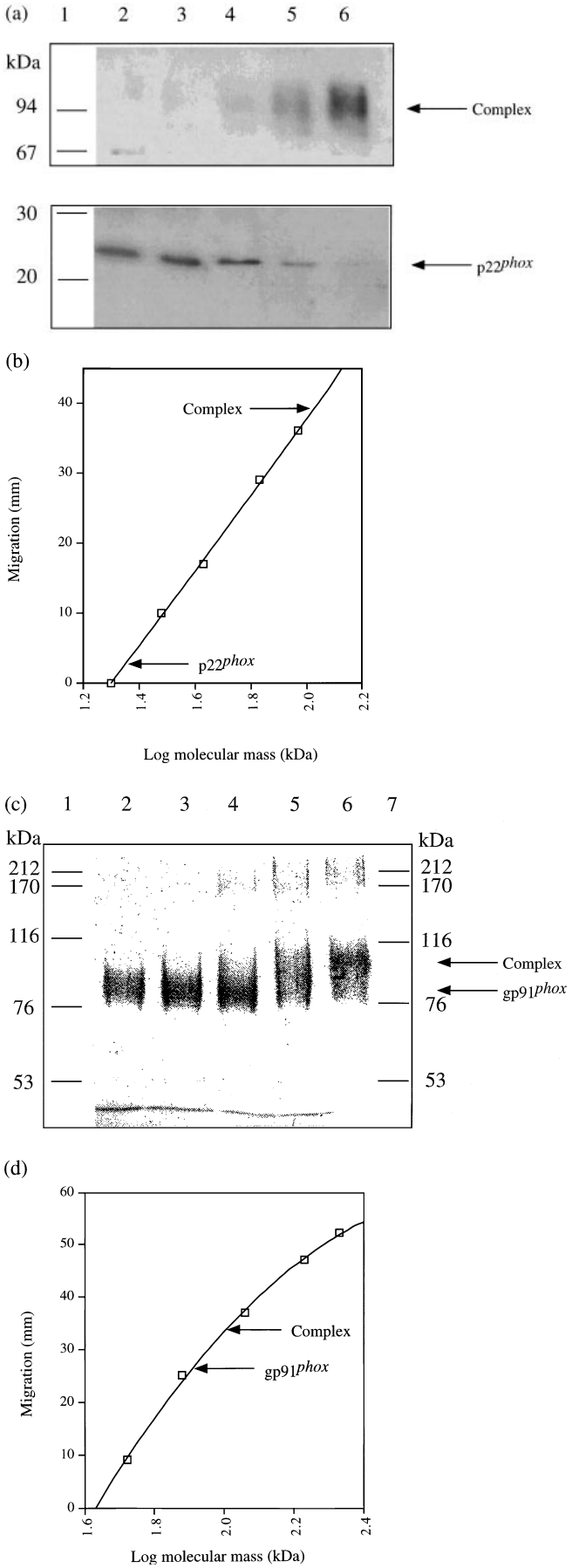
Pure flavocytochrome b_{558} was subjected to denaturing gel-filtration chromatography in the presence of SDS and dithiothreitol. The fractions collected were precipitated with acetone and analysed by SDS/PAGE and Coomassie staining (upper panel). Lane 1, molecular mass standards (kDa); lane 2, start material; lanes 3–11, fractions collected. Lower panel: the A_{280} trace of the eluate corresponding exactly to the Coomassie-stained gel. The areas of the trace integrated to determine the amount of gp91^{phox} present relative to p22^{phox} are marked. Inset: Western blot probed for gp91^{phox} (open arrowhead) and p22^{phox} (filled arrowheads).

(Figure 3d), gp91^{phox} was found to have a mean apparent molecular mass of 82 kDa. The cross-linked complex was found to have a mean apparent molecular mass of 102 kDa.

It has been established that Rap1A is lost from flavocytochrome b_{558} during purification [24]. This excludes the possibility that Rap1A is incorporated into the cross-linked complex. The size increase was equivalent to the formation of a dimer consisting of one molecule each of p22^{phox} and gp91^{phox}. This supports the conclusions drawn from previous work with cross-linkers [17].

Specific incorporation of ^{125}I label

Bolton–Hunter reagent was used to incorporate a ^{125}I label on to lysine residues and the N-terminal group of each subunit. The pure flavocytochrome b_{558} was denatured with SDS to ensure that the subunits were unfolded and target residues were equally accessible for iodination. Duplicate samples were fractionated by SDS/PAGE, one was silver-stained to confirm the identity of the



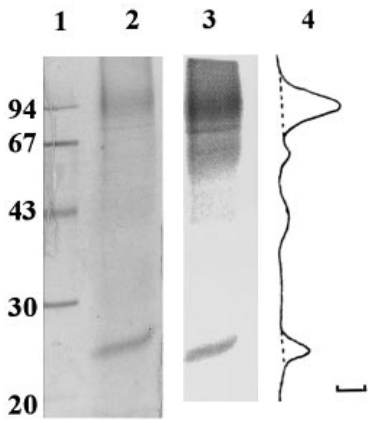


Figure 4 [^{125}I]Bolton–Hunter labelling of flavocytochrome b_{558}

Pure flavocytochrome b_{558} was denatured and labelled with [^{125}I]Bolton–Hunter reagent. Duplicate samples of ^{125}I-labelled material were fractionated by SDS/PAGE. One sample was silver-stained to identify p22^{phox} and gp91^{phox} subunits (lane 2). The other sample was dried without staining and ^{125}I was quantified with a PhosphorImager (lane 3). The contributions to the radioactivity profile from each subunit were integrated (lane 4). The horizontal bar represents 200 PSL (where PSL is a measure of the radiation dose detected by a PhosphorImager plate).

subunits; the other was dried down, without staining, for ^{125}I-label quantification with a PhosphorImager (Figure 4). The ratio of ^{125}I counts obtained from p22^{phox} and gp91^{phox} was 1:3.9. As there are nine lysine residues in p22^{phox} and 34 lysine residues in gp91^{phox}, there are 10 and 35 target groups respectively to be labelled. From this, assuming that the two subunits are radiolabelled at an equivalent specific radioactivity, the stoichiometry of p22^{phox} to gp91^{phox} was determined as 1:1.1.

The p22^{phox} to gp91^{phox} subunit stoichiometry was the same in different preparations of neutrophil membrane samples and also in a sample of the pure flavocytochrome b_{558}

Quantitative blotting was used to compare the amounts of p22^{phox} and gp91^{phox} present in different samples of neutrophil membrane according to the relative amounts of their antigen present.

Six independent preparations of neutrophil membrane, four (A–D) derived from pools of buffy coats each prepared from three normal individuals, and two (E and F) from individual patients with chronic myeloid leukaemia, were diluted to various concentrations and dot-blotted in duplicate. The dot-blots were incubated with antibody to p22^{phox} (MC2) or antibody to gp91^{phox}

Figure 3 Cross-linking of flavocytochrome b_{558}

Pure flavocytochrome b_{558} was cross-linked with various concentrations of the agent BS^3 and the resulting complexes were analysed by Western blotting. A standard curve of $\log_{10}$(molecular mass) against migration was determined from the molecular mass standards in each case. The original and cross-linked positions of p22^{phox} and gp91^{phox} are indicated on the blots and on the standard curves. (**a**) Western blot probed for p22^{phox} with MC2 and revealed with a horseradish peroxidase-conjugated secondary antibody. Lane 1, positions of molecular mass standards; lanes 2–6, material cross-linked with 0, 0.02, 0.05, 0.25 and 0.5 mM BS^3 respectively. (**b**) Standard curve with the mean original and mean cross-linked positions of p22^{phox} marked. (**c**) Western blot probed for gp91^{phox} with MAB48 and revealed with a horseradish peroxidase-conjugated secondary antibody. Lanes 1 and 7, positions of molecular mass standards; lanes 2–6, material cross-linked with 0, 0.02, 0.05, 0.25 and 0.5 mM BS^3 respectively. (**d**) Standard curve with the mean original and mean cross-linked positions of gp91^{phox} marked.

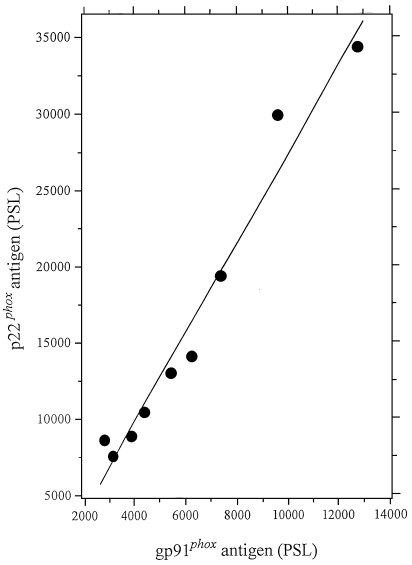


Figure 5 Determination of p22^{phox} and gp91^{phox} antigen ratio in membrane samples by quantitative dot-blotting

A typical example of a linear regression used to determine the p22^{phox} to gp91^{phox} antigen ratio from a dot-blot with a membrane sample. In this case the membrane sample was E and the slope (p22^{phox}/gp91^{phox} antigen ratio) was 2.91 ± 0.21. PSL is a measure of the radiation dose detected by a PhosphorImager plate.

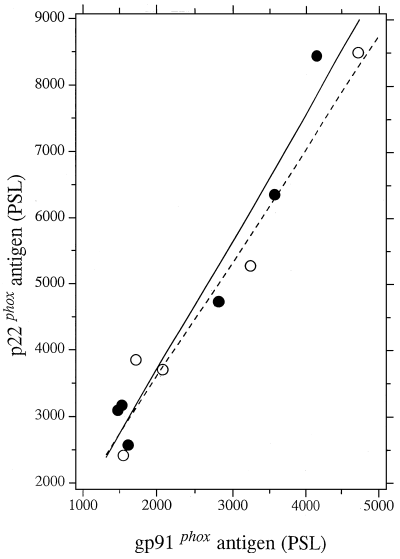


Figure 6 Comparison of p22^{phox} and gp91^{phox} antigen ratios in neutrophil membrane and a sample of pure flavocytochrome b_{558} by quantitative dot-blotting

Comparison of the p22^{phox} antigen to gp91^{phox} antigen ratios in neutrophil membrane sample E (●, solid line) and a pure sample of flavocytochrome b_{558} (○, broken line) from a quantitative dot-blot. Linear regression analysis on the membrane sample gave a p22^{phox}/gp91^{phox} antigen ratio of 1.93 ± 0.22, and on the pure sample a ratio of 1.72 ± 0.21. PSL is a measure of the radiation dose detected by a PhosphorImager plate.

(Pep37), and the amount of antigen was quantified with ^{125}I-labelled Protein A and a PhosphorImager. From these data, graphs were prepared relating p22^{phox} antigen to gp91^{phox} antigen. The slope of the linear regression of each sample gave the antigen ratio (Figure 5). The antigen ratios were found to be consistent between samples A–F (values for each sample are quoted as slopes $\pm$ S.E.M.): 2.56 ± 0.07, 2.70 ± 0.36, 2.08 ± 0.21, 2.35 ± 0.28, 2.91 ± 0.21 and 2.53 ± 0.08 respectively. A statistical test was performed for heterogeneity of slopes [25]. According to this analysis there was no evidence at the 95% confidence level of statistical significance that the slopes were different, indicating a constant amount of p22^{phox} subunits relative to gp91^{phox} subunits in the different membrane samples.

In a separate experiment, quantitative dot-blots were used to compare the amounts of p22^{phox} and gp91^{phox} present in neutrophil membrane with those in pure flavocytochrome b_{558}. The blots were prepared with a sample of the pure flavocytochrome b_{558} used in these studies and membrane sample E (which was the source of the pure material). The ratios of p22^{phox} antigen to gp91^{phox} antigen from the linear regressions of each sample were found to be similar, with slopes of 1.72 ± 0.21 for pure flavocytochrome b_{558} and 1.93 ± 0.22 for membrane sample E (Figure 6). The estimated difference of 0.21 between the slopes was not statistically significant at the 95% confidence level.

From the foregoing it has been shown that the ratio between the two subunits was close to 1:1 in pure flavocytochrome b_{558}. No obvious difference was observed between the slopes of the regressions from the pure flavocytochrome b_{558} and membrane sample E, and the slopes were very different from the slopes that would have been observed had the ratio in the membrane sample been 1:2 or 2:1.

To support this, the ratio of the subunits was also compared on Western blots in which p22^{phox} and gp91^{phox} were first separated by SDS/PAGE. The pure material and two of the membrane samples, A and E, were subjected to electrophoresis on the same gel and blotted on the same nitrocellulose membrane; this experiment was repeated three times, each time with duplicates of each of the samples. The antigen concentrations were determined with the appropriate specific antibody and ^{125}I-labelled Protein A.

The ratios of p22^{phox} antigen to gp91^{phox} antigen from the pure flavocytochrome b_{558} sample in the three experiments were normalized to a subunit stoichiometry of 1:1. The corresponding ratios of the subunits in membrane samples A and E in the three experiments were (1) 1.1:1 and 1.1:1, (2) 0.8:1 and 1.0:1 and (3) 0.9:1 and 1.2:1.

These approaches demonstrated that that the subunit composition of the flavocytochrome b_{558} sample used in the quantitative studies was not altered during purification.

DISCUSSION

We have determined the stoichiometry of the p22^{phox} and gp91^{phox} subunits of flavocytochrome b_{558} by four independent methods to be: (1) 1:1, (2) 1.2:1, (3) 1:1 and (4) 1:1.1. Taken individually, these results are not conclusive, but in combination they are complementary and demonstrate the flavocytochrome b_{558} complex to be a heterodimer consisting of one molecule of each of the subunits.

A key part of this study was relating the subunit stoichiometry in the pure material used back to that in neutrophil membrane. Different preparations of flavocytochrome b_{558} have shown that there is a risk of subunit enrichment or partial proteolysis during the purification. In extreme cases, one subunit has apparently been lost [10,16]. For this study the relative amounts of p22^{phox} antigen and gp91^{phox} antigen in various samples of membrane and in the pure material used were determined by quantitative blotting. This analysis showed that the antigen ratio, and thus

the stoichiometry of the protein subunits, was not altered in the course of the protein purification.

This work provides some evidence for the locations of the two haems in the complex. Resonance Raman and electron spin resonance studies have indicated that the two haems in flavocytochrome b_{558} are each in a low-spin state, with bis-axial histidine ligands [11,26–28]. There have been suggestions from sequence analysis that haem was associated with $p22^{phox}$ [29], which was supported by experimental evidence [10,20]. Other workers have suggested that the haems must be co-ordinated between $p22^{phox}$ and $gp91^{phox}$ [17,30,31]. If the possibility of non-histidine haem ligands is excluded by the spectral studies, then as $p22^{phox}$ has only one invariant histidine residue [30], and the flavocytochrome b_{558} complex is composed of one $p22^{phox}$ subunit and one $gp91^{phox}$ subunit, at least one haem must reside entirely on $gp91^{phox}$ along with the FAD- and NADPH-binding sites. The second haem could be shared between $p22^{phox}$ and $gp91^{phox}$.

We thank Karolyn Shatwell for helpful advice, Chris Frost for guidance on statistical analysis and the Wellcome Trust for financial support.

REFERENCES

1 Segal, A. W., Geisow, M., Garcia, R., Harper, A. and Miller, R. (1981) Nature (London) **290**, 406–409
2 Clark, R. A. (1990) J. Infect. Dis. **161**, 1140–1147
3 Jesaitis, A. J. (1995) J. Immunol. **155**, 3286–3288
4 Thrasher, A. J., Keep, N. H., Wientjes, F. and Segal, A. W. (1994) Biochim. Biophys. Acta **1227**, 1–24
5 Quinn, M. T., Parkos, C. A., Walker, L., Orkin, S. H., Dinauer, M. C. and Jesaitis, A. J. (1989) Nature (London) **342**, 198–200
6 Segal, A. W., West, I., Wientjes, F., Nugent, J. H., Chavan, A. J., Haley, B., Garcia, R. C., Rosen, H. and Scrace, G. (1992) Biochem. J. **284**, 781–788
7 Rotrosen, D., Yeung, C. L., Leto, T. L., Malech, H. L. and Kwong, C. H. (1992) Science **256**, 1459–1462
8 Nisimoto, Y., Otsuka-Murakami, H. and Lambeth, D. J. (1995) J. Biol. Chem. **270**, 16428–16434
9 Iizuka, T., Kanegasaki, S., Makino, R., Tanaka, T. and Ishimura, Y. (1985) J. Biol. Chem. **260**, 12049–12053
10 Yamaguchi, T., Hayakawa, T., Kaneda, M., Kakinuma, K. and Yoshikawa, A. (1989) J. Biol. Chem. **264**, 112–118
11 Hurst, J. K., Loehr, T. M., Curnutte, J. T. and Rosen, H. (1991) J. Biol. Chem. **266**, 1627–1634
12 Cross, A. R., Rae, J. and Curnutte, J. T. (1995) J. Biol. Chem. **270**, 17075–17077
13 Segal, A. W. (1987) Nature (London) **326**, 88–91
14 Parkos, C. A., Dinauer, M. C., Jesaitis, A. J., Orkin, S. H. and Curnutte, J. T. (1989) Blood **73**, 1416–1420
15 Parkos, C. A., Allen, R. A., Cochrane, C. G. and Jesaitis, A. J. (1987) J. Clin. Invest. **80**, 732–742
16 Harper, A. M., Dunne, M. J. and Segal, A. W. (1984) Biochem. J. **219**, 519–527
17 Parkos, C. A., Allen, R. A., Cochrane, C. G. and Jesaitis, A. J. (1988) Biochim. Biophys. Acta **932**, 71–83
18 Huang, J., Hitt, N. and Kleinberg, M. E. (1995) Biochemistry **34**, 16753–16757
19 Segal, A. W. (1993) Immunodeficiency **4**, 167–179
20 Nugent, J. H., Gratzer, W. and Segal, A. W. (1989) Biochem. J. **264**, 921–924
21 Teahan, C., Rowe, P., Parker, P., Totty, N. and Segal, A. W. (1987) Nature (London) **327**, 720–721
22 Laemmli, U.K. (1970) Nature (London) **227**, 680–685
23 Gill, S. C. and von Hippel, P. H. (1989) Anal. Biochem. **182**, 319–326
24 Cross, A. R. and Curnutte, J. T. (1995) J. Biol. Chem. **270**, 6543–6548
25 Armitage, P. and Berry, G. (eds.) (1985) in Statistical Methods in Medical Research, pp. 274–275, Blackwell Scientific, Oxford
26 Fujii, H. and Kakinuma, K. (1992) Biochim. Biophys. Acta **1136**, 239–246
27 Miki, T., Fujii, H. and Kakinuma, K. (1992) J. Biol. Chem. **267**, 19673–19675
28 Fujii, H., Johnson, M. K., Finnegan, M. G., Miki, T., Yoshida, L. S. and Kakinuma, K. (1995) J. Biol. Chem. **270**, 12685–12689
29 Parkos, C. A., Dinauer, M. C., Walker, L. E., Allen, R. A., Jesaitis, A. J. and Orkin, S. H. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 3319–3323
30 Dinauer, M. C., Pierce, E. A., Bruns, G. A., Curnutte, J. T. and Orkin, S. H. (1990) J. Clin. Invest. **86**, 1729–1737
31 Quinn, M. T., Mullen, M. L. and Jesaitis, A. J. (1992) J. Biol. Chem. **267**, 7303–7309

Received 21 May 1996; accepted 16 July 1996