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Mariano A. Ostuni, Leila Lamanuzzi, Tania Bizouarn, Marie-Claire Dagher, Laura Baciou. Expression of functional mammal flavocytochrome b558 in yeast: Comparison with improved insect cell system. *Biochimica et Biophysica Acta: Biomembranes*, 2010, 1798 (6), pp.1179-1188. <10.1016/j.bbamem.2010.02.016>. <hal-05452317>

**HAL Id: hal-05452317**

**<https://hal.science/hal-05452317v1>**

Submitted on 10 Jan 2026

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# Expression of functional mammal flavocytochrome $b_{558}$ in yeast: Comparison with improved insect cell system

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## ARTICLE INFO

### Keywords:

NADPH-oxidase  
Flavocytochrome  $b_{558}$   
Heterologous expression  
Membrane protein  
Proteoliposome  
Superoxide generation

## ABSTRACT

Activity of phagocyte NADPH-oxidase relies on the assembly of five proteins, among them the transmembrane flavocytochrome  $b_{558}$  (Cyt $b_{558}$ ) which consists of a heterodimer of the gp91<sup>phox</sup> and p22<sup>phox</sup> subunits. The Cyt $b_{558}$  is the catalytic core of the NADPH-oxidase that generates a superoxide anion from oxygen by using a reducing equivalent provided by NADPH via FAD and two hemes. We report a novel strategy to engineer and produce the stable and functional recombinant Cyt $b_{558}$  (rCyt $b_{558}$ ). We expressed the gp91<sup>phox</sup> and p22<sup>phox</sup> subunits using the baculovirus insect cell and, for the first time, the highly inducible *Pichia pastoris* system. In both hosts, the expression of the full-length proteins reproduced native electrophoretic patterns demonstrating that the two polypeptides are present and, that gp91<sup>phox</sup> undergoes co-translational glycosylation. Spectroscopic analyses showed that the rCyt $b_{558}$  displayed comparable spectral properties to neutrophil Cyt $b_{558}$ . In contrast to rCyt $b_{558}$  produced in the insect cells with higher yield, the enzyme expressed in yeast displayed a superoxide dismutase-sensitive NADPH-oxidase activity, indicating a superoxide generation activity. It was also blocked by an inhibitor of the respiratory burst oxidase, diphenylene iodonium (DPI). As in neutrophil NADPH-oxidase, activation occurred by the interactions with the soluble regulatory subunits suggesting comparable protein-protein contact patterns. We focus on the stability and function of the protein during solubilisation and reconstitution into liposomes. By comparing oxidase activities in different membrane types, we confirm that the lipid-protein environment plays a key role in the protein function.

## 1. Introduction

NADPH-oxidase is well known as a major source for non-mitochondrial superoxide radical production in phagocytes (neutrophils) generated by exposure to microorganisms or inflammatory mediators [1,2]. Superoxide is scavenged rapidly into reactive oxygen species (ROS) which play a critical role in the killing of invading microorganisms [3]. The NADPH-oxidase is a multicomponent complex which consists of a catalytic membrane flavocytochrome  $b$ , referred to as flavocytochrome  $b_{558}$  (Cyt $b_{558}$ ) and four cytosolic subunits (p40<sup>phox</sup>, p47<sup>phox</sup>, p67<sup>phox</sup>, GTP-binding protein Rac). The activity of the oxidase is

controlled by the translocation of these cytosolic subunits to the membrane-bound Cyt $b_{558}$  to form the active enzyme [4,5]. This activation process is tightly regulated and involves phosphorylation events correlated to specific protein-protein interactions [6,7]. Mutations in anyone of the five subunits, i.e. Cyt $b_{558}$ , p40<sup>phox</sup>, p47<sup>phox</sup>, p67<sup>phox</sup>, or Rac result in a dysfunctional oxidase as observed in the human genetic disorder chronic granulomatous disease (CGD) characterized by an inability to fight against bacterial and fungal infections due to the absence of ROS production [8–12]. In addition to the well-known phagocyte NADPH-oxidase, studies over the last decade disclosed the existence of other members of the NADPH-oxidase family, the so-called Nox family [5,13]. Identified in many organisms (fungi, plants, mammals...), they have a broad tissue distribution and are proposed to have many physiological roles (hormone synthesis, vascular tone control) and thereby, to be implicated in several human diseases.

The membrane Cyt $b_{558}$  has been studied by a variety of immunochemical, biochemical and analytical approaches. It consists of two non-covalently linked proteins, p22<sup>phox</sup> and gp91<sup>phox</sup> in a molar ratio of 1:1 [14]. Gp91<sup>phox</sup> is anchored in the membrane by a series of six hydrophobic transmembrane segments and p22<sup>phox</sup> can potentially form two to four membrane-spanning domains [15–17]. The hydrophilic part in the C-terminal portion of p22<sup>phox</sup> is essential for

*Abbreviations:* rCyt $b_{558}$ , recombinant cytochrome  $b_{558}$ ; PL, proteoliposome; DMSO, dimethylsulphoxide; SOD, superoxide dismutase; Cyt $c$ , cytochrome  $c$ ; PMSF, phenylmethanesulfonyl fluoride; DPI, diphenylene iodonium; DDM, n-dodecyl  $\beta$ -D-maltoside; OG, octyl  $\beta$ -D-glucopyranoside; SM, sucrose monolaurate;  $\delta$ -ALA,  $\delta$ -aminolevulinic acid; DOPC, dioleoylphosphatidylcholine; EPC, egg phosphatidylcholine; NADPH, reduced  $\beta$ -nicotinamide adenine dinucleotide phosphate; PNGase-F, peptide N-glycosidase F; DTT, dithiothreitol; FAD, flavin adenine dinucleotide

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recruitment of the cytosolic partners and oxidase activity [15,18–20]. The gp91<sup>phox</sup> subunit is the true redox component. It is associated to two hemes [21,22] and contains in its carboxyl terminal region the FAD and NADPH binding sites [23–26]. Modeling of the C-terminal cytosolic region of gp91<sup>phox</sup>, based on sequence homology with the ferredoxin-NADP<sup>+</sup> reductase family, proposed that this portion is the subject of important protein–protein interactions [27]. In neutrophils, gp91<sup>phox</sup> is detected as a highly mannosylated 65-kDa monomer while the mature gp91<sup>phox</sup>, heavily glycosylated [28] migrates as a broad band on SDS-PAGE around 91-kDa.

A deeper knowledge of the overall Cytb<sub>558</sub> structure and a more detailed understanding of the interactions among the NADPH-oxidase complex subunits are restricted by the relatively poor availability of purified native Cytb<sub>558</sub>. Although neutrophils are the cells with the highest natural abundance of the membrane Cytb<sub>558</sub>, it is notoriously difficult to use traditional methods to purify amounts of native membrane proteins that are sufficient for comprehensive functional analysis or crystallization attempts [29,30]. The use of natural sources also excludes the possibility of creating genetically modified proteins. This latter problem was overcome by successfully generating functional recombinant Cytb<sub>558</sub> produced in mammalian heterologous expression system [31–33] and in insect cells [34]. Despite large efforts to scale-up the mammalian cell culture [35] and to subsequently improve the purification strategies [29], the recombinant Cytb<sub>558</sub> was still purified in an insufficient amount. Several attempts to express the entire Cytb<sub>558</sub> in an *E. coli* system were not successful (personal unpublished results), only production of the truncated C-terminal gp91<sup>phox</sup> was possible [36,37]. Cell-free expression, which eliminates most central problems associated with the conventional cellular production of membrane protein, managed to synthesize the truncated form with an incomplete function [38,39].

To overcome this bottleneck, we followed two strategies. First, we introduced several modifications to the previously published baculovirus/insect cell expression procedure in order to increase the yield. Second, for the first time, we investigated the methylotrophic yeast *Pichia pastoris* expression system as a valuable tool for high-yield production of Cytb<sub>558</sub>. In this work, we monitor the quantity and the quality of the rCytb<sub>558</sub> such as its membrane localization, glycosylation, heterodimer formation and redox cofactor association. Moreover, we provide evidence on the integrity of the membrane protein complex including its function and correct assembly with its cytosolic partners. In particular, rCytb<sub>558</sub> incorporated into liposomes showed native-like NADPH-oxidase activity highlighting the effect of membrane lipids on the functioning of the membrane complex.

## 2. Materials and methods

### 2.1. Materials

δ-ALA, SOD, reduced β-NADPH, Cytc, arachidonic acid, PMSF, Tween 20, DDM, OG, yeast extract, yeast nitrogen base, peptone, dextrose, biotin, glycerol, DMSO, ampicillin, anti-Flag M2 antibody were from Sigma. Sucrose Monolaurate (SM) was purchased from AppliChem. Reagents for DNA extraction and purification were from Qiagen. Peptide N-Glycosidase (PNGase-F), restriction enzymes and other DNA modifying enzymes were purchased from New England Biolabs. Oligonucleotides were obtained from Eurogentec. The BacToBac and mutagenesis system, anti-Flag tag monoclonal antibody and Flag resin were purchased from Invitrogen. StrepTactin column and StrepTactin peroxidase were from IBA (Goettingen).

### 2.2. Cloning of the cDNA of bovine Cytb<sub>558</sub> and expression in *P. pastoris*

#### 2.2.1. Gp91<sup>phox</sup> and p22<sup>phox</sup> cDNA subcloning

We designed a construct for a large-scale production of the heterodimeric Cytb<sub>558</sub> in the methylotrophic yeast *P. pastoris* (Fig. 1A;

see also Supplementary data). The expression vector was the pAO815 vector, usually used as a multicopy integration plasmid (Invitrogen). The pAO815/NOX plasmid containing the entire expression cassette (gp91<sup>phox</sup> and p22<sup>phox</sup> sequences) was constructed as follows. Since it was shown to improve targeting to the membrane [40], the α-factor secretion signal sequence from *Saccharomyces (S.) cerevisiae* was fused to the N-terminal of both bovine subunits gp91<sup>phox</sup> (CYBB cDNA) and p22<sup>phox</sup> (CYBA cDNA) coding regions. To facilitate immunological detection and purification, the DNA encoding the Flag epitope (DYKDDDDK) was fused to the DNA encoding the gp91<sup>phox</sup> protein at the N-terminus generating a Flag-tagged gp91<sup>phox</sup>. A Kex2 cleavage site was introduced between the α-factor and the Flag tag.

#### 2.2.2. Membrane protein complex expression in yeast

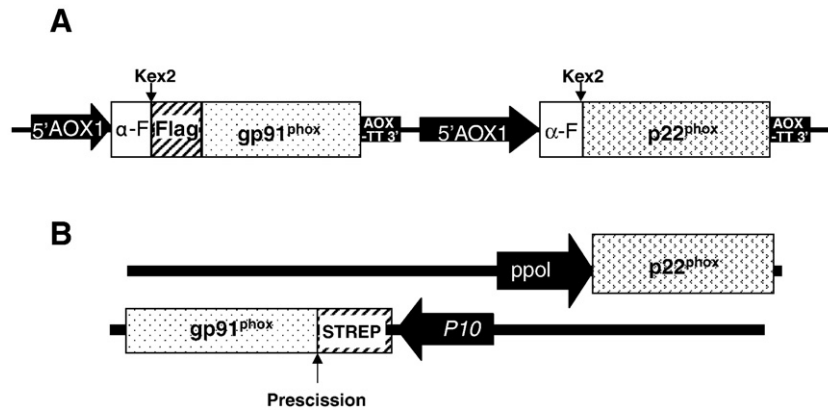
Competent *P. pastoris* SMD1168 strain (*his4 pep4*) was transformed with either the pAO815/NOX expressing plasmid (containing the entire expression cassette with the gp91<sup>phox</sup> and p22<sup>phox</sup> sequences) or with the pAO815 control empty plasmid using the EasyComp™ Kit, following manufacturer's instructions (Invitrogen). For transformation into *P. pastoris*, the plasmid was linearized with AatII restriction enzyme. Positive clones were selected on histidine-deficient media (MD plates (1.34% (w/v) yeast nitrogen base without amino acids, 0.4 mg/l biotin, 2% (w/v) dextrose, 1.5% (w/v) agar)). Selected clones were spread onto YPD plates containing 0.05 mg/ml ampicillin. To screen for protein expression, ampicillin resistant clones of SMD1168/pAO815/NOX were grown for an initial small-scale production. After overnight culture in BMGY media (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6, 1.34% yeast nitrogen base, 4 × 10<sup>-5</sup>% biotin, 1% glycerol) supplemented with 0.05 mg/ml ampicillin, cells were pelleted by centrifugation (10 min at 3500 rpm) and resuspended in BMMY medium (similar to BMGY but glycerol was substituted by 1% methanol) supplemented with ampicillin at an OD<sub>600nm</sub> = 1.0. Cells were grown in baffled culture flasks at 30 °C with shaking at 200 rpm. Methanol (1%, v/v final concentration) was supplied every 24 h to maintain the expression induction. When mentioned, the incubation medium was supplemented with 0.3 mM δ-ALA and 2% DMSO. A control culture was performed using the same protocol but with yeast transformed with the pAO815 control plasmid. Cells were harvested by centrifugation after one, two or three days and stored at –80 °C.

#### 2.2.3. Preparation of the yeast membrane fraction

Cell pellets were thawed and resuspended in 50 mM sodium phosphate, pH 7.4, 1 mM PMSF, 1 mM EDTA and 5% glycerol. An equivalent volume of glass beads were added and cells were disrupted by 8 cycles of 30 s vortexing/30 s ice bath. The clear supernatant containing the crude cell lysate was separated from the cell debris and unbroken cells by centrifugation at 500 × g for 10 min at 4 °C. The membrane fractions were collected by centrifugation at 100,000 × g for 120 min and resuspended in 50 mM Tris/HCl pH 8, 120 mM NaCl, 10% glycerol, 1 mM PMSF. The protein concentration was determined using the Pierce BCA protein assay (Thermo Scientific) with BSA as standard.

#### 2.2.4. Detergent solubilisation and purification of recombinant Cytb<sub>558</sub> (rCytb<sub>558</sub>)

Several detergents (DDM, OG or SM) were added to the membrane protein fraction from yeast in a detergent/protein ratio of 3 (w/w), and the mixed solutions were incubated for 1 h at 4 °C in a roller shaker. The solubilised protein extracts were incubated overnight at 4 °C in a roller shaker with the anti-Flag/sepharose resin (Sigma) pre-equilibrated with 50 mM Tris/HCl pH 7.5, 120 mM NaCl, and rinsed twice with 20 volumes of the same buffer supplemented with 0.5% detergent. Then, the resin was rinsed twice and elution was



**Fig. 1.** A. Schematic drawing of the *P. pastoris* expression cassette for heterologous production of rCytb<sub>558</sub>. AOX1, Alcohol oxidase 1 gene promoter;  $\alpha$ -F, coding region for the prepropeptide of the *S. cerevisiae* mating type factor  $\alpha$ ; Flag, coding region for the Flag tag; Kex2, coding region for Kex2 protease cleavage site. B. Schematic drawing of the baculovirus expression cassette in the Baculovirus transfer vector pFASTBac Dual. ppol, Polyhedrin promoter; p10, p10 promoter; STREP, Strep Tag II coding sequence. The site of cleavage by protease Precission is indicated by an arrow.

performed by incubating the resin for 1 min with 3 $\times$  Flag peptide (150  $\mu$ g/ml in 50 mM Tris/HCl pH 7.5, 120 mM NaCl, 1% detergent).

### 2.2.5. Reconstitution of rCytb<sub>558</sub> in liposomes

A stock solution of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) or egg phosphatidylcholine (EPC) from Avanti Polar Lipids was diluted in phosphate buffer solution (PBS, pH 7.4), and mixed with the purified rCytb<sub>558</sub> at a concentration corresponding to a lipid-to-protein ratio of 5:1 (w/w). The resulting micellar protein-lipid-detergent mixture was stirred for 15 min at room temperature before addition of Bio-Beads SM2 (Biorad), which removed the detergent and induced vesicle formation [41]. Vesicle formation was followed by absorbance at 400 nm. Bio-Beads were subsequently removed and the solution containing the proteoliposomes (PL) was kept at  $-20^{\circ}\text{C}$ , until use.

### 2.3. Cloning of the human gp91<sup>phox</sup> and p22<sup>phox</sup> cDNA and expression of rCytb<sub>558</sub> in insect cells

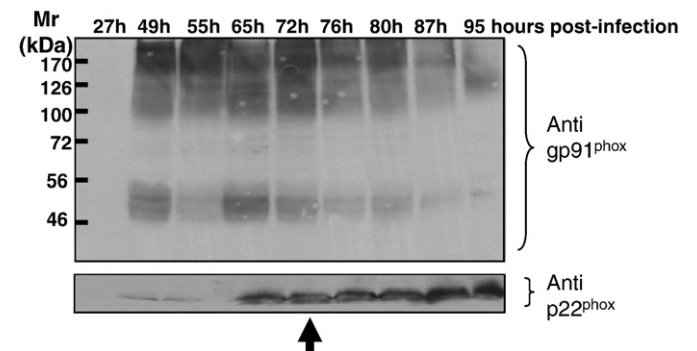
In order to improve the expression level obtained in a previous study [34], both gp91<sup>phox</sup> and p22<sup>phox</sup> cDNAs were cloned in the same pFAST Bac Dual baculovirus transfer vector under the control of polyhedrin and the p10 promoters, respectively (Fig. 1B). P22<sup>phox</sup> cDNA was amplified by PCR and inserted into the BamH1 site downstream of the polyhedrin promoter. In order to improve the ATG context (Kozak consensus sequence), the GATCCATG sequence was mutated to GAACCATG, thus eliminating the BamH1 site. Gp91<sup>phox</sup> cDNA was cloned from a dibutyl-AMPc differentiated HL60 library by classical procedures described in [42]. In order to suppress sequences that may induce unwanted transcription [43], five TAAG or CTTA sites were mutated. The sequence was verified. A strep tag II sequence MSA/WSHPQFEK was added to the N-terminus by PCR amplification. A cleavage site for PreScission protease was introduced between the tag and the protein. The construct was introduced into the Xho1 site downstream of the polyhedrin promoter.

Recombinant baculovirus was made by transposition following the BacToBac protocol (Invitrogen). After 3 cycles of amplification, recombinant baculoviruses were used to infect insect cells for protein expression. The amount of virus and the duration of expression were monitored using antibodies directed to the C-terminus of both subunits (Fig. 2). P22<sup>phox</sup> appeared only after 65–72 h of infection whereas the expression of gp91<sup>phox</sup> decreased after 72 h. The optimal culture conditions were therefore chosen to be 65–72 h. Expression was performed at high multiplicity of infection (MOI) (1–10 infectious viral particle/cell). At 24 h post-infection, hemin (5  $\mu$ M in 0.1 N NaOH) was added to the culture medium.

At 65–72 h post-infection, insect cells were harvested by centrifugation for 5 min at 5000 $\times$ g, washed and resuspended in relaxation buffer (Hepes 20 mM, pH 7.4 KCl 100 mM). The cells supernatant was further subjected to centrifugation at 30,000 $\times$ g to harvest cell debris, then to an ultracentrifugation at 300,000 $\times$ g for 45 min. The resulting pellet that contained extra cellular viral particles (ECV) liberated from the infected cells was suspended in relaxation buffer by a gentle sonication in the presence of DFP 1 mM and leupeptin 10  $\mu$ g/ml. Insect cells were suspended in 4 volumes of relaxation buffer in the presence of protease inhibitors and subjected to sonication for 2 min with 40 W output and 1 s bursts using a Branson sonifier. Unbroken cells were eliminated by a 10 min centrifugation at 5000 $\times$ g, and then the homogenate was layered onto a 10/30% (w/v) sucrose gradient and centrifuged in a Beckman SW41 rotor at 35,000 $\times$  rpm for 30 min. The upper (on top of the 30% sucrose layer) and the lower (at the 10/30% interface) membrane fractions had similar properties and were pooled. For the quantification of Cytb<sub>558</sub>, the fractions were clarified with OG (40 mM).

### 2.4. Electrophoretic and immunoblot analyses

Samples were analyzed by electrophoresis under denaturing conditions using 10% SDS ClearPAGE precast gel (VWR International). Gels were stained with Coomassie blue. For immunoblot analyses, unstained proteins were transferred to a nitrocellulose membrane Hybond ECL (GE Healthcare), and probed with either the rabbit anti-gp91<sup>phox</sup> or anti-p22<sup>phox</sup> polyclonal antiserum (1:1000 dilution) or mouse anti-Flag monoclonal antibody (1:3000). Anti-gp9<sup>phox</sup> and



**Fig. 2.** Optimization of rCytb<sub>558</sub> expression in insect cells. The presence of gp91<sup>phox</sup> and p22<sup>phox</sup> was monitored at various times post-infection on a crude cell lysate by western blotting using antibodies directed to the C-terminus of both proteins. An optimal infection time of 65–72 h (indicated by an arrow) was chosen.

anti-p22<sup>phox</sup> antibodies are raised against C-termini of proteins from human origin (cross-reacting with proteins from bovine origin). Immune complexes were detected using the ECL kit (GE Healthcare).

### 2.5. Deglycosylation by PNGase-F digestion

In order to check the glycosylation state of the protein expressed in *P. pastoris*, enzymatic deglycosylation was performed by incubating 10 µg of neutrophil membrane proteins (5 mg/ml) or 10 µg of OG-solubilised protein with 1–2 U of the peptide N-glycosidase F (PNGase-F), at 37 °C overnight, followed by western blot analysis.

### 2.6. Preparation of subcellular fractions from bovine neutrophils

Routinely, neutrophil membrane was purified from 8–10 l bovine blood as described in [44]. Between 5 and 8 mg of plasma membrane proteins and 300 mg cytosolic proteins (per 10 l of blood cells) were usually obtained.

### 2.7. Spectral analysis of Cytb<sub>558</sub>

All spectral assays were carried out using an Uvikon 943 double-beam spectrophotometer (Kontron Instruments). The sodium dithionite-reduced *minus* oxidized difference spectra were recorded between 400 and 600 nm. The Cytb<sub>558</sub> heme was quantified by measuring the absorbance difference between the peak at 427 nm and the hollow at 411 nm using a millimolar extinction coefficient of  $\Delta\epsilon_{427-411} = 200 \text{ mM}^{-1} \text{ cm}^{-1}$  [45]. The values were consistent with those obtained by measuring the peak at 558 nm using  $\Delta\epsilon_{\text{red-ox}} = 21 \text{ mM}^{-1} \text{ cm}^{-1}$ .

### 2.8. NADPH-oxidase activity assay

The superoxide production was measured spectrophotometrically at 550 nm as the rate of SOD-inhibitable Cytc reduction as previously described [44]. 1.5–40 µg of *P. pastoris* membrane fraction or reconstituted PLs (corresponding to 1.5–6 pmol of rCytb<sub>558</sub>) were incubated for 5 min at 25 °C with purified recombinant cytosolic factors [bovine p67<sup>phox</sup> (7.5 µg) and p47<sup>phox</sup> (8 µg), and human RacQ61L (4 µg)] in the presence of 10 mM MgSO<sub>4</sub> and 30 µg of arachidonic acid (1 ml final volume). The recombinant cytosolic proteins were produced and obtained as described in [46]. Then, 50 µM Cytc was added and the reaction was initiated by the addition of 200 µM NADPH. The optimal amount of arachidonic acid was checked for each experimental condition (see S2/B). The membrane fractions (3–4 pmol of the native Cytb<sub>558</sub>) isolated from bovine neutrophils were used as control for the oxidase activity in the same experimental conditions, except 40 µg arachidonic acid was added. For Sf9 cells and ECV, the oxidase activity was assayed with 30 µg of membrane fractions, 300 µg of bovine neutrophil cytosol and 30 µg arachidonic acid. The amount of superoxide was calculated using a molar absorption coefficient ( $\Delta\epsilon$  of the reduced minus oxidized form of Cytc) of  $21 \text{ mM}^{-1} \text{ cm}^{-1}$  [47]. Control experiments were performed in the presence of 350 U/ml of SOD or 100 µM DPI.

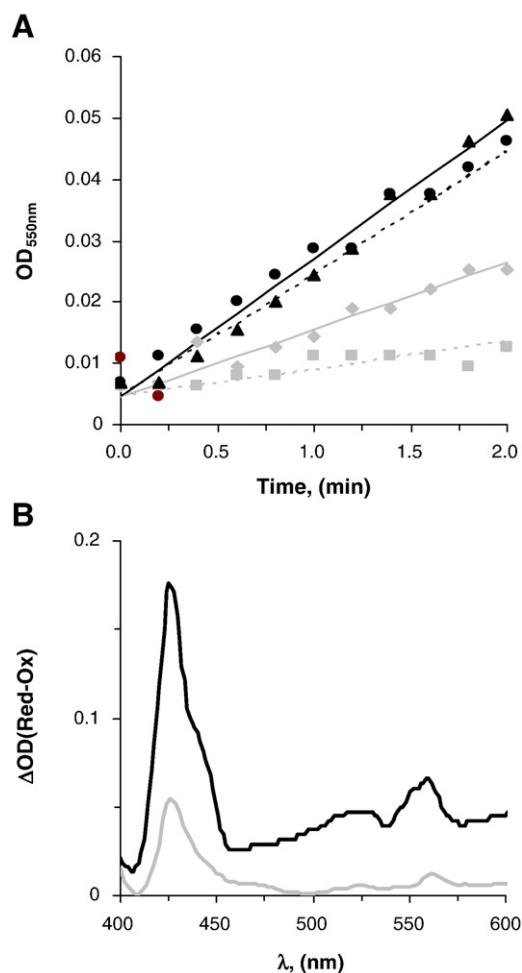
## 3. Results

### 3.1. Cytb<sub>558</sub> production in insect cells

The feasibility of Cytb<sub>558</sub> expression in insect cells was previously shown in two publications. Katkin et al. [48] obtained expression of the two subunits in insect cells but no activity could be measured. In the following paper by Rotrosen et al., the authors modified the cloning vectors using only the coding regions (untranslated regions can be deleterious [43]) and after supplementing the culture with  $\delta$ -ALA instead of hemin, they could reconstitute an oxidase activity,

although their Cytb was expressed at a low level and was poorly glycosylated [34]. We tried to improve the yield of Cytb<sub>558</sub> expression. First we took advantage of the BacToBac expression system and of the dual expression plasmid to make sure that the two subunits are expressed in the same insect cell infected by a single baculovirus (instead of co-infection). Indeed, it was shown that the Cytb<sub>558</sub> stability is dependent on the co-expression of gp91<sup>phox</sup> and p22<sup>phox</sup> [49,50]. Second, we performed extensive mutagenesis of the gp91<sup>phox</sup> cDNA in order to suppress any unwanted transcription initiation sites that might have reduced the stability of the mRNA. Third, we modified the ATG context of p22<sup>phox</sup> cDNA to add an A at position –3'. Fourth we introduced a Strep tag II at the N-terminus to facilitate detection and purification using a Streptactin column.

rCytb<sub>558</sub> was produced at concentrations up to 0.25 nmol/mg of protein in the insect cell membranes, as determined by the dithionite-reduced *minus* oxidized spectrum (Fig. 3B, black trace). Contrary to previous studies, gp91<sup>phox</sup> was expressed in a highly glycosylated form with a broad smear between 100 and 180 kDa, as detected by the anti gp91<sup>phox</sup> antibody (Fig. 2). The Strep tag II at the N-terminus was either hidden or cleaved since it was impossible to detect or purify the protein with the Strep tag II reagent Streptactin. A control protein with a Strep tag II at the C-terminus could be easily detected and



**Fig. 3.** Spectral and functional characterization of rCytb<sub>558</sub> expressed in the membranes from baculovirus infected insect cells. A. NADPH-oxidase activity. Two types of membranes are compared; the plasma membrane (black lines) of infected cells and the extracellular viral particles (ECV) (grey lines). In this assay, 30 µg of each membrane fractions are mixed with 300 µg of bovine neutrophil cytosol in the presence of 30 µg arachidonic acid in a final volume of 1 ml. Cytc reduction was recorded at 550 nm with SOD (triangles or squares) and without (circles or diamonds) SOD. Panel B shows the corresponding spectra (Sf9 membrane, black lines and ECV, grey lines, respectively) obtained with hemin supplementation.

purified (data not shown). In our hands, hemin was more efficient than  $\delta$ -ALA in increasing the peaks at 426 and 558 nm up to 10 times. Unfortunately, when we assessed the oxidase activity of insect cell membranes, we found that the reduction of Cyt<sub>c</sub> in the presence of neutrophil cytosol was SOD-insensitive (Fig. 3A, black traces). This was confirmed with recombinant cytosolic factors (data not shown). Such a diaphorase activity was not mentioned in the previous papers. It had however been described in rabbit neutrophil membranes after purification [51].

We made another attempt to obtain an active rCyt<sub>b558</sub>. Baculoviruses acquire their envelope from the insect cell membranes. It was previously shown that functional  $\beta$ 2-adrenergic receptors could be recovered from extracellular baculovirus particles (ECV) [52]. We therefore collected baculovirus particles from the expression medium. A typical Cyt<sub>b558</sub> spectrum was found in these particles (Fig. 3B, grey trace). Moreover, in the ECV fraction, a significant amount of SOD-sensitive NADPH-oxidase activity could be detected upon addition of bovine cytosolic components (Fig. 3A, grey trace), indicating that the human protein is expressed in an active form and that the activation mechanism of the NADPH-oxidase is devoid of species features as was previously observed [46]. However, recovery of ECV by ultracentrifugation from 1 l of culture is tedious and the yield remains low (see Table 1). For this reason, we decided not to invest further efforts into reconstitution of the oxidase activity and abandoned this approach. We chose to focus on the yeast expression system never tested up to now.

### 3.2. Cyt<sub>b558</sub> production in yeast

The protease deficient SMD1168 is growing more slowly than other *P. pastoris* strains and, thus, this reduces synthesis rates to a level that better matches the endoplasmic reticulum folding capacity. We expected in this way to improve functional expression of membrane proteins. Crude cell lysates of eleven selected clones were subjected to Western blot analysis with anti-Flag and anti-p22<sup>phox</sup> antibodies concomitantly (Fig. 4). Membrane fractions isolated from bovine neutrophils (bMF) and a cell lysate from *P. pastoris* transformed with empty pAO815 vector (V) were used as positive and negative control, respectively. To exclude possible nonspecific recognition of polyclonal antibodies, we focused only on bands that were absent in the empty vector control but present in the *P. pastoris* clones.

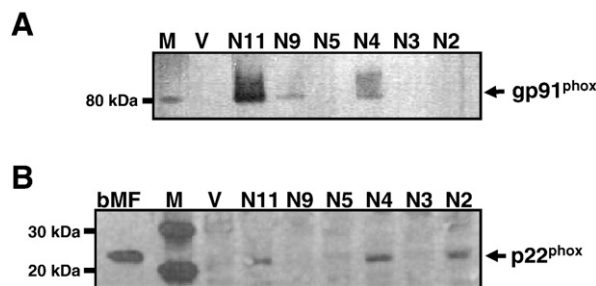
As shown in the immunodetection blot using the monoclonal antibody anti-Flag, the N4, N9 and N11 clones displayed a signal with an apparent molecular mass of >80-kDa in good agreement with the expected size of a glycosylated gp91<sup>phox</sup>. The signal in N9 was not very pronounced. In the N11 and N4 clones, signal intensities were

**Table 1**

Cyt<sub>b558</sub> content membrane fraction isolated from bovine neutrophils, from yeast and insect cells. Membrane fractions were purified as described in the Materials and methods section. Data from yeast cells from the 72 h-methanol-induced N11 clone, Baculovirus infected insect cells and the extra cellular viral particles (ECV) are shown. The amount of Cyt<sub>b558</sub> was quantified from sodium dithionite-reduced minus oxidized difference spectra and the total membrane protein from BCA protein assay (standard deviation less than 15%). A molecular weight of 80,000 Da for Cyt<sub>b558</sub> was used for calculation.

	Bovine neutrophils	<i>P. pastoris</i>	Baculovirus Sf9 insect cells	Baculovirus ECV
$\mu$ g of Cyt <sub>b558</sub> /mg of total membrane protein	71	3.4	40	20
Total membrane protein content (mg/l) <sup>a</sup>	0.9	390	160	20
Cyt <sub>b558</sub> yield (mg/l) <sup>a</sup>	0.064	1.4	6.4	0.40
Cyt <sub>b558</sub> yield (nmol heme/l) <sup>a</sup>	0.8	16.8	80	5

<sup>a</sup> Values refer to 1 l of bovine blood, *P. pastoris* culture and Sf9 insect cell culture.



**Fig. 4.** Analysis of protein expression in crude extracts of 24 h-methanol-induction cultures of independent transformed SMD1168 yeast cells. 50  $\mu$ g of proteins were loaded on a SDS/PAGE under reducing conditions (5 mM DTT) and analyzed by immunoblotting against (A) anti-Flag antibody (1:1000) and (B) anti-p22<sup>phox</sup> antibody (1:1000) as described in the Materials and methods section. The yeast clones were transformed with a pAO815 vector either empty (V) or harboring the Nox expression cassette (N-). M = molecular weight marker (Magic Marker, Invitrogen). Purified native bovine membrane fraction (bMF), containing 0.3  $\mu$ g of Cyt<sub>b558</sub>, were included as positive control.

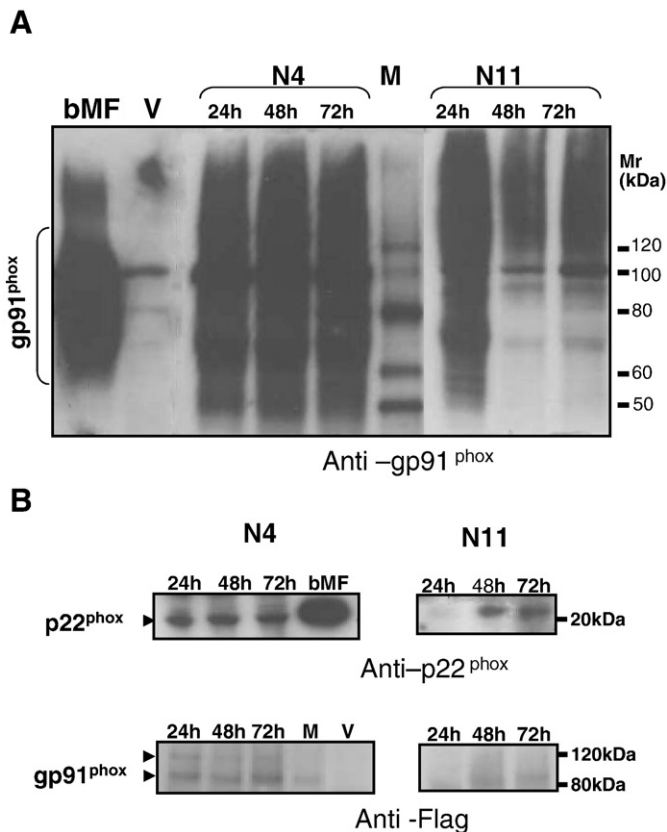
significantly higher suggesting a better expression level, particularly in N11. As expected, anti-Flag antibody was not reactive against a lysate from a yeast clone with the empty vector. In N4, N9 and N11, we looked for the concomitant band corresponding to the p22<sup>phox</sup> protein in the yeast clones by using polyclonal anti-p22<sup>phox</sup> antibody. The 22-kDa protein band is detected in the N4 and N11 clones indicating the presence of the p22<sup>phox</sup> protein. The 22-kDa band was also present in the N2 clone, but since no signal was detected for the large gp91<sup>phox</sup> subunit, this clone was no longer considered in this study. The N9 clone did not seem to express p22<sup>phox</sup>. The clones with a dissociated expression of gp91<sup>phox</sup> and p22<sup>phox</sup> might be useful in the future to study heterodimer formation. Considering the behavior of the different clones, it was clear that the N4 and N11 clones were the more amenable clones for larger-scale production and for subsequent purification and characterization of correct folding and assembly.

To improve the expression level of both subunits, we varied the methanol-induction time (24, 48 and 72 h) (Fig. 5). A hyperglycosylated signal using anti-gp91<sup>phox</sup> was observed for N4 compared to N11 (Fig. 5A) which rendered the interpretation very difficult. The use of the anti-Flag and anti-p22<sup>phox</sup> antibodies (Fig. 5B) was more informative and showed that, in N4, the production of gp91<sup>phox</sup> concomitantly to p22<sup>phox</sup> was satisfactory and stable. However, the strong signal described above might indicate, together with the expected entire glycosylated protein, the presence of N-terminal truncated gp91<sup>phox</sup> protein. In N11, bands detected by anti-p22<sup>phox</sup> and anti-Flag antibodies appeared more distinctly for a longer induction period suggesting that the production and/or assembly needs a longer time. We have also studied the effects of different additives like  $\delta$ -ALA and DMSO to the growth media (Fig. S1/A and /B).  $\delta$ -ALA was added to facilitate heme synthesis which might be a rate-limiting step. DMSO enhances protein expression but also principally, stabilizes the glycosylated product on a long induction time (96 h). DMSO acts probably by stabilizing the mannose produced in *P. pastoris* but DMSO may also be implicated in other cellular aspects like simply membrane protein stabilization and protection against proteolysis [53,54]. The addition of the heme precursor and of DMSO to the growth media enhanced and stabilized the expression of Flag-gp91<sup>phox</sup> and p22<sup>phox</sup>.

Thus, protein expression was considered optimal with a methanol-induction period of 72 h in the presence of  $\delta$ -ALA and DMSO.

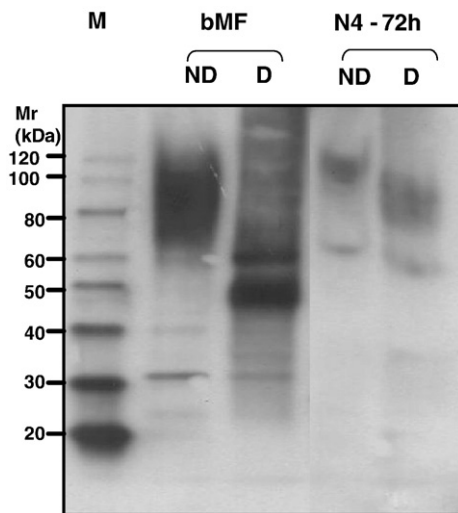
### 3.3. Post-translational modifications analysis

The presence of a broad smear detected with both the anti-Flag and the anti gp91<sup>phox</sup> antibodies is consistent with a highly glycosylated protein. *P. pastoris* is able to add both O-linked and N-linked



**Fig. 5.** Cell lysates from 24, 48 and 72 h-methanol-induced cultures of N4 and N11 *P. pastoris* clones. 50  $\mu$ g of proteins were loaded on a SDS/PAGE gel in reducing conditions (5 mM DTT) and analyzed by immunoblot. A: With anti-gp91<sup>phox</sup> and B: with anti-Flag and anti-p22<sup>phox</sup> antibodies. M = molecular weight marker (Magic Marker). Native bovine membrane fraction (bMF) and the 72 h-cell lysate of the empty vector pAO815 clone (V) are included as positive and negative controls, respectively.

carbohydrate molecules to the proteins, therefore differently from the sugar added by mammal cells where they are all N-linked. To assess the glycosylated state of gp91<sup>phox</sup>, we show in Fig. 6 the comparison of the native and recombinant gp91<sup>phox</sup> before and after deglycosylation using



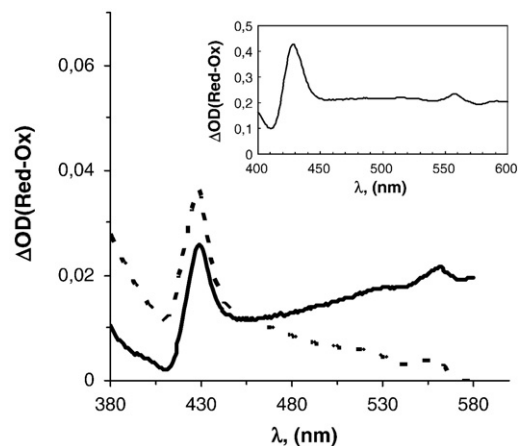
**Fig. 6.** Deglycosylation of native and recombinant Cytb<sub>558</sub>. Bovine and *P. pastoris* (N4 clone, 72 h-methanol induction) membrane fractions were incubated overnight at 37 °C with (D) or without (ND) PNGase-F (1–2 enzyme units) and analyzed by anti-gp91<sup>phox</sup> antibody immunodetection. *P. pastoris* membranes were pretreated with 1.5% octyl-glucoside (OG).

PNGase-F that cleaves N-linked oligosaccharides. The broad band of glycosylated neutrophil gp91<sup>phox</sup> can be observed in the immunodetection blot using anti-gp91<sup>phox</sup> antibody. Deglycosylated proteins from neutrophils migrate as a major band of apparent Mr of 50-kDa which is in agreement with previously reported results (50–58 kDa) describing the deglycosylated polypeptide in native phagocyte [28] or using *in vitro* biosynthesis of the gp91<sup>phox</sup> or transgenic PLB-985 or COS-T cell lines expressing the gp91<sup>phox</sup> [39,50,55]. After deglycosylation, the recombinant protein expressed in yeast migrates to lower molecular weight than the glycosylated protein. We observed a shift of the 70-kDa band to about 60-kDa, which is a size expected for non-glycosylated recombinant gp91<sup>phox</sup> polypeptide. Concomitantly the highly glycosylated form (120-kDa) migrates to lower molecular weight too. This result indicates that the membrane rCytb<sub>558</sub> is a glycoprotein with only N-linked sugars.

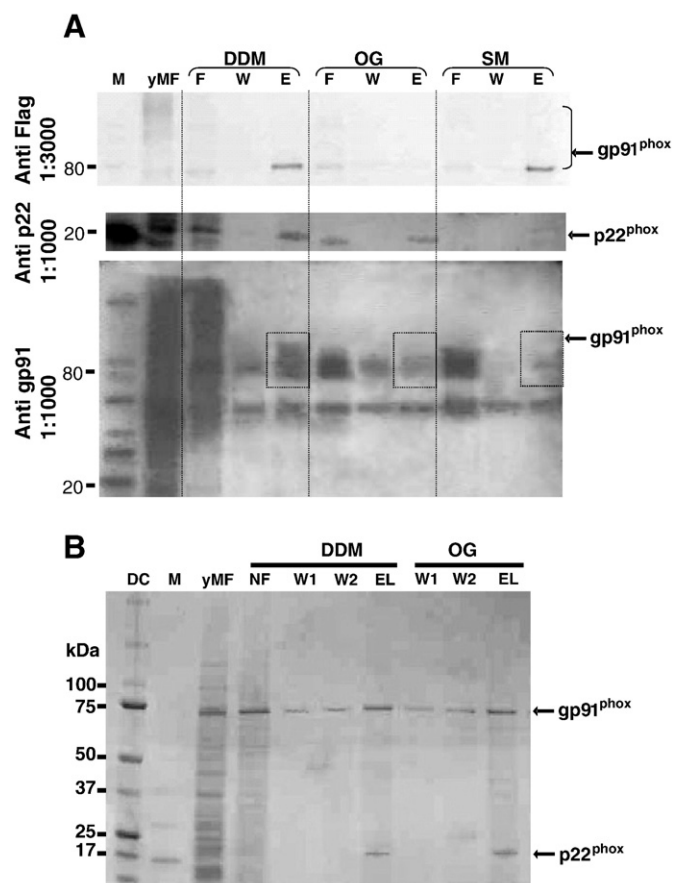
#### 3.4. Folding and dimerization analysis of the rCytb<sub>558</sub> produced in yeast

To further characterize the folding of the flavocytochrome expressed in *P. pastoris* N11 clone, we analyzed dithionite-reduced minus oxidized difference spectra of yeast membrane fractions (Fig. 7). N11 membrane extracts solubilised in the presence of 1.5% DDM were also analyzed. The difference spectra show that the recombinant proteins presented the characteristic peaks of the Cytb<sub>558</sub> from bovine neutrophils i.e., a Soret band at 427 nm and  $\alpha$  band at 558 nm (Fig. 7, inset). Table 1 summarizes the amount of rCytb<sub>558</sub> obtained in yeast and compares the amount extracted from bovine blood or insect cells. We usually obtained, from 1 l of culture, 6–9 g wet weight of cells yielding to 1.4 mg/l of rCytb<sub>558</sub>.

It was shown that transgenic cell lines expressing gp91<sup>phox</sup> in the absence of p22<sup>phox</sup> subunit exhibit a heme spectrum similar to neutrophil Cytb<sub>558</sub> [56]. Therefore, to confirm that the spectral properties of the crude membranes are associated to the gp91<sup>phox</sup>/p22<sup>phox</sup> dimer and not to gp91<sup>phox</sup> alone, we solubilised the membrane rCytb<sub>558</sub> with different detergents (DDM, OG or SM (1.5%)) and purified it on the Flag affinity column. The flow-through, washing and elution fractions were tested with the anti-Flag, anti-p22<sup>phox</sup> and anti-gp91<sup>phox</sup> antibodies (Fig. 8A). After elution from the Flag affinity column, DDM and OG seemed to be efficient detergents for solubilising in terms of total and stable rCytb<sub>558</sub> complex, as demonstrated by a simultaneous presence of bands corresponding to gp91<sup>phox</sup> and p22<sup>phox</sup> subunits. The good purity of the purified rCytb<sub>558</sub> is shown by the SDS-PAGE gel analysis (Fig. 8B). Thus, the Flag tag present on the N-Terminal of gp91<sup>phox</sup> protein allowed co-purification of both subunits.



**Fig. 7.** Spectral analysis of rCytb<sub>558</sub>. Dithionite-reduced minus oxidized spectra of membrane fractions isolated from N11 transformed clone (1.5 mg of membrane protein/ml) at 72 h of methanol induction (black lines) and solubilised with 1.5% DDM (dashed lines). Inset: dithionite-reduced minus oxidized difference spectra from bovine membrane fraction.

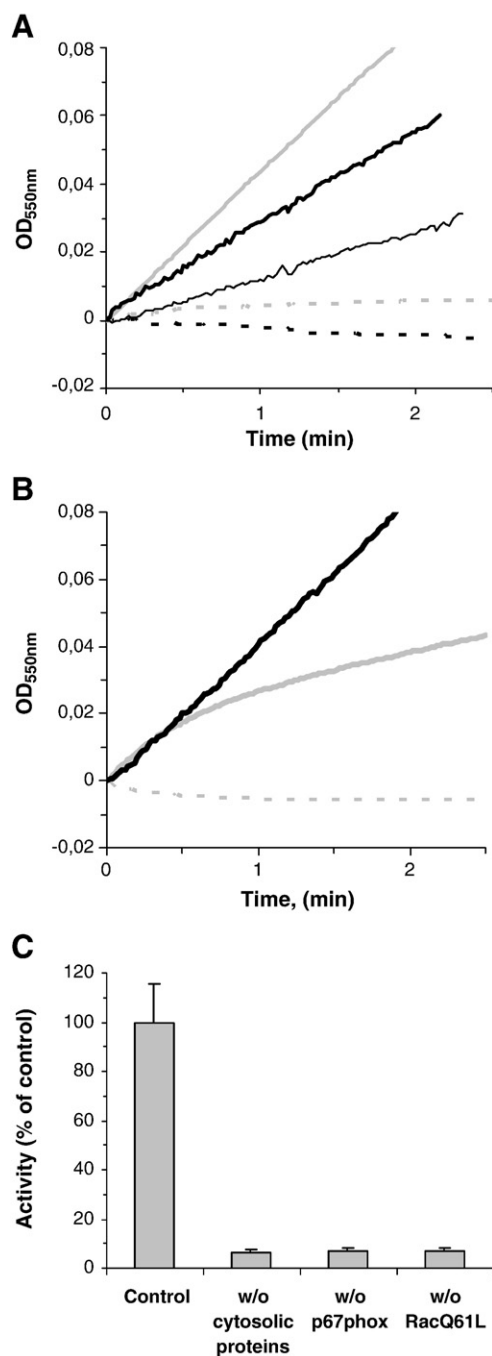


**Fig. 8.** Analyses of the chromatography purification fractions. (A) Immunoblot of the chromatography purification fractions. Immunoblots of flow-through (F), washing (W) and elution (E) chromatography fractions from DDM, OG and SM solubilised membranes from N11 clone were performed using anti-Flag monoclonal, anti-p22<sup>phox</sup> polyclonal, or anti-gp91<sup>phox</sup> polyclonal antibodies. Nonpurified *P. pastoris* membranes (yMF) are included. (B) SDS-PAGE of the chromatography purification fractions. Blue Coomassie stained gel of flow-through (F), washing (W1 and W2, two successive washing steps) and elution (E) chromatography fractions from DDM and OG solubilised membranes from N11 clone. Molecular weight markers (Dual Color (DC) and Magic marker (M)) and nonpurified *P. pastoris* membranes (yMF) are included.

### 3.5. Functional rCytb<sub>558</sub> from *P. pastoris* and reconstitution in proteoliposomes

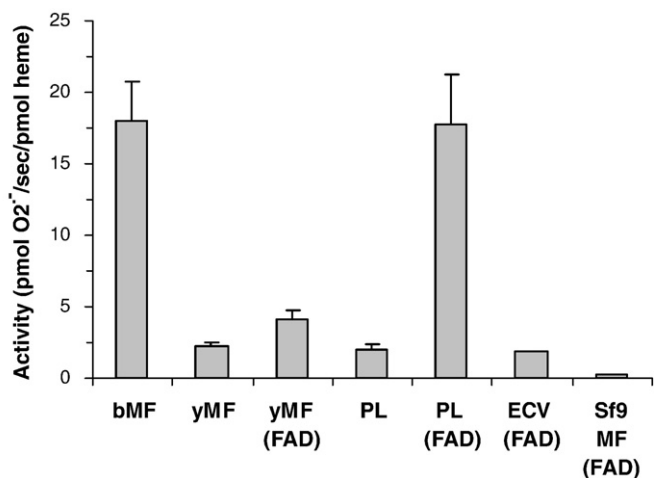
The next fundamental question was whether the rCytb<sub>558</sub> expressed in yeast is in an active form. To address this question, we used a cell-free system as previously described [46] to examine its ability to generate superoxide. Therefore, we measured the oxidase activities of the yeast membrane fractions (Fig. 9A), of DOPC-PLs (Fig. 9B) and EPC-PLs (data not shown) and compared them with the oxidase activity of bovine neutrophil membranes. The superoxide generation rates are presented in Fig. 9 and the values are collected in Fig. 10.

As shown in Fig. 8A, similarly to the flavocytochrome in neutrophil membranes, rCytb<sub>558</sub> in yeast membranes has a SOD- and DPI-sensitive NADPH-dependent cytochrome c reductase activity. Yet, the level of oxidase activity in yeast membrane ( $2.3 \pm 0.3$  pmol O<sub>2</sub><sup>-</sup>/s/pmol heme) is ~13% of that produced by neutrophil membrane fractions ( $18.0 \pm 2.7$  pmol O<sub>2</sub><sup>-</sup>/s/pmol heme) in identical experimental conditions (Fig. 10). Addition of external FAD increased the activity two-fold (~23% of native membranes). Any attempt to increase the level of oxidase activity (such as optimization of activator concentration or cytosolic proteins) failed. Nevertheless, the superoxide which was generated in the cell-free assay with the yeast membrane fractions resulted from the direct activation of the enzyme by the addition of the



**Fig. 9.** Cell free NADPH-oxidase activity of recombinant Cytb<sub>558</sub>. (A) DPI-sensitive NADPH-oxidase activity of membrane fractions from *P. pastoris* (black lines) and bovine neutrophils (grey lines), dotted lines represent the activity in the presence of 100 μM DPI, thin black line represents the *P. pastoris* membrane fraction activity without the addition of FAD. Activity measurements were assayed with recombinant p67<sup>phox</sup>, p47<sup>phox</sup> and Rac cytosolic proteins and in the presence of arachidonic acid (as described in the Materials and methods section). (B) SOD-sensitive NADPH-oxidase activity of membrane fractions from DOPC reconstituted rCytb<sub>558</sub> with (black lines) or without (grey lines) FAD (50 μM). Dotted lines represent the activity in the presence of 350 U/ml SOD. (C) Dependence of the NADPH oxidase activity on the addition of the cytosolic subunits. SOD-sensitive NADPH-oxidase activity of Cytb<sub>558</sub> in membrane fraction from *P. pastoris* was measured in the absence of any cytosolic subunit and in the absence of p67<sup>phox</sup> or RacQ61L. Control is performed in the presence of p67<sup>phox</sup>, p47<sup>phox</sup> and RacQ61L recombinant proteins. In all experiments, arachidonic acid was added at the appropriate concentration (see Material and methods section).

activator (arachidonic acid) and the regulatory cytosolic proteins (p47<sup>phox</sup>, p67<sup>phox</sup> and Rac). A control experiment performed in the absence of any cytosolic proteins or in the presence of an incomplete set



**Fig. 10.** Superoxide generating activity of *Cytb*<sub>558</sub> from N11 *P. pastoris* clone, bovine neutrophils and Baculovirus infected insect cells. The superoxide production was measured spectrophotometrically as the rate of cytochrome *c* reduction at 550 nm as described in the Materials and methods section and in supplementary Fig. S2A. In the reaction mixture, the *Cytb*<sub>558</sub> heme content was of 5.6, 3.3, 9 and 15 pmol in *P. pastoris*, bovine neutrophil, Sf9 and ECV membrane fractions, respectively. 1.5 pmol of *Cytb*<sub>558</sub> heme reconstituted in DOPC-PLs was used. After 5 min incubation with the bovine cytosol or recombinant cytosolic factors in the presence of 10 mM MgSO<sub>4</sub> and the appropriate amount of arachidonic acid (Fig. S2B), the reaction was initiated by the addition of 200 μM NADPH.

of cytosolic proteins shows only background activity (Fig. 9C). These data confirm that, although the activity level is lower than in neutrophil membranes, the NADPH-oxidase activity in yeast membranes results from specific interactions with the recombinant cytosolic factors.

Since it is believed that the lipid environment is essential to reconstitute NADPH-oxidase activity [57], we further decided to remove r*Cytb*<sub>558</sub> from the yeast membrane environment and to reconstitute it into liposomes of either DOPC or EPC. The oxidase activity obtained with PLs was also low compared to that obtained with the bovine membrane fractions even after optimization of the arachidonic acid concentration (Fig. S2/B). We hypothesized that a possible loss of FAD occurred during the protein expression and/or during the purification process as it is commonly observed after purification of *Cytb*<sub>558</sub> from neutrophils [58]. Indeed, after addition of exogenous FAD, the oxidase activity was increased to a rate ( $17.8 \pm 2.7$  pmol O<sub>2</sub><sup>-</sup>/s/pmol heme) comparable to that of native flavocytochrome (Fig. 10). Meanwhile, we noticed that r*Cytb*<sub>558</sub> incorporated into DOPC (monounsaturated) liposomes has a superoxide capacity higher by about 10–15% compared to that in egg phosphatidylcholine liposomes.

These results show that the r*Cytb*<sub>558</sub> produced in yeast, either in the yeast membrane and in PLs, is in a stable form and can be activated by the cytosolic proteins and generate superoxide. *P. pastoris* is thus a very convenient source of r*Cytb* in an active form. Moreover, addition of negatively charged phospholipids enhances the activity. There might be differences in lipid composition between yeast and bovine neutrophil membranes that could be overcome by exogenous lipids.

#### 4. Discussion

The *Cytb*<sub>558</sub> complex is the catalytic component of the NADPH-oxidase which generates superoxide during the phagocyte respiratory burst. Given the importance of the phagocyte NADPH-oxidase in host defence, many of the biochemical features of its assembly, structure, function and biosynthesis have been the object of extensive study. The *Cytb*<sub>558</sub> currently isolated from both neutrophils and mammalian expressing cell types is obtained in limited amounts, therefore insufficient for structural studies or for biophysical investigation. To our knowledge, the work depicted in this report deals with the first

heterologous expression of *Cytb*<sub>558</sub> in yeast, and provides an improvement in the overproduction of r*Cytb*<sub>558</sub>, compared to previous works.

In the present study, we show that we were also able to significantly increase the yield of r*Cytb*<sub>558</sub> in the insect cell expression system. However the production of active protein remained anecdotal since it was unfortunately localized in extracellular baculovirus particles. Therefore, in the current study, we have mainly examined the quality and the integrity of the recombinant protein produced in yeast with respect to its localization, the subunits' co-expression and the heme binding. We have shown in the present work that the anti-gp91<sup>phox</sup> and anti-p22<sup>phox</sup> antibodies raised against the C-terminus of the human proteins (but cross-reacting with proteins from bovine origin) recognized the flavocytochrome *b*<sub>558</sub> produced in *P. pastoris*. Thus, both membrane subunits were co-expressed successfully. It is likely that most of the heterodimer is addressed to the cytoplasmic membrane. However, we cannot exclude that, in the highly optimized expression conditions, recombinant proteins accumulated at the level of yeast organelle (such as the Golgi apparatus or the endoplasmic reticulum) as suggested by the increase of unprocessed p22<sup>phox</sup> (30 kDa protein band; α-factor sequence not cleaved) in the presence of DMSO in the culture media (Fig. S1/B). Incomplete processing might be due to the glycosylation pattern of the gp91<sup>phox</sup> which hinders the proteolytic site of the Kex2 protease on the associated p22<sup>phox</sup> subunit in the assembled *Cytb*<sub>558</sub>. This cleavage occurs in the Golgi apparatus and, thus, would suggest that both subunits are associated already at that organelle level. Similar conclusions have been drawn from the studies of *Cytb*<sub>558</sub> biosynthesis in neutrophils [55,59]. Nevertheless, upon addition of DMSO, the level of unprocessed p22<sup>phox</sup> increases suggesting a limiting step of the processing mechanism due certainly to a retention of the over-expressed protein in the endoplasmic reticulum. Indeed DMSO was proposed to help in membrane proliferation [60] and in up-regulating amino-acid synthesis [61] in *S. cerevisiae*.

It was proposed in several studies that heme incorporation is a prerequisite step in the heterodimer formation [50,59]. Rotrosen et al., in producing recombinant cytochrome *b*<sub>558</sub> in Sf9 insect cells, co-infected with baculovirus vectors for p22<sup>phox</sup> and gp91<sup>phox</sup> expression, reported that heme precursor supplementation was required for optimal expression of r*Cytb*<sub>558</sub> [34]. Our results support this observation since we noticed that the addition of δ-ALA increased the signal corresponding to glycosylated gp91<sup>phox</sup> (Fig. S1/A).

The r*Cytb*<sub>558</sub> produced in *P. pastoris* was subjected to post-translational modifications as illustrated by the glycosylation pattern. Treatment of solubilised proteins with peptide N-glycosidase F which removes all linked carbohydrates resulted in disappearance of the 120 kDa band and the appearance of a 60 kDa band. We did not notice any effect of the presence of p22<sup>phox</sup> on the maturation of gp91<sup>phox</sup> since several *P. pastoris* clones that transformed with the vector for gp91<sup>phox</sup> expression alone underwent heavy glycosylation even in the absence of p22<sup>phox</sup> partner (data not shown). Further investigations have to be done to describe more precisely the *Cytb*<sub>558</sub> maturation process in yeast.

The centrepiece of the work reported here is that the membrane heterodimer has comparable properties to the natural one. It displays reduced *minus* oxidized absorbance spectra identical to that of neutrophil flavocytochrome, indicating a native heme environment. Most importantly, it is capable of generating superoxide upon activation by the cytosolic regulatory factors in the presence of the amphiphilic reagent, arachidonic acid. Although low compared to native neutrophils which, in our hands, achieve in cell-free assays a turnover in the range of 18 pmol O<sub>2</sub><sup>-</sup>/s/pmol heme, the oxidase activity measured in yeast membrane fraction was SOD- and DPI-sensitive.

However, the incorporation of the detergent solubilised membrane complex into liposomes containing negatively charged lipids completely restored the oxidase activity to a native-like level upon

addition of external FAD in agreement with previous work [62]. Several possible explanations can be given for the different activities measured in the yeast membranes and the liposomal preparation. Since the activity could be restored after purification and reconstitution in liposomes, one can easily exclude topological reasons due to the Flag tag in the N-terminus of gp91<sup>phox</sup>. Thus, the presence of the small tag does not hinder the regulatory subunits' interactions with the membrane complex. Also, the localization and the importance of the glycosylation in yeast cells may prevent the activation by arachidonic acid. However, the activator-dose effect (Fig. S2/B), comparable to that for a neutrophil membrane, would argue against this hypothesis. Alternatively, the rate of O<sub>2</sub><sup>-</sup> generation may be determined by different rate-limiting steps which might differ depending on the membrane environment. It is well-known that lipids might affect the activities of membrane proteins [63]. Indeed, the membrane phospholipid content has a strong impact on membrane fluidity which could be a prerequisite for oxidase activity. Phosphatidylcholine and phosphatidylethanolamine are proposed to be the major phospholipids present in the membrane from *P. pastoris* grown on methanol [64]. This is close to the lipid composition of EPC liposomes which contain a high content of mono- and polyunsaturated fatty acids. Because egg PC and DOPC possess an identical polar head group (i.e., phosphocholine), the slight difference in superoxide generation rates seemed attributable to a difference in the non-polar region of these phospholipids. However, besides the lipid composition of the membranes, another aspect of membrane composition is important in this regard such as the presence of proteins in the yeast membranes. In PLs, the significantly high mass ratio of lipid to protein at 5:1 might facilitate either activation by the arachidonic acid or interactions with the regulatory soluble subunits. In contrast to DOPC or EPC liposomes, the lipid and/or protein environment in *P. pastoris* membranes might, therefore, not stabilize the oxidase in an active form and might impede conformational changes associated with the activation process of the flavocytochrome. These constraints would be eliminated when the complex is reconstituted in liposomes. Also, numerous studies have postulated that structural changes in flavocytochrome are key steps in oxidase activation. In particular, it was proposed that, in B cells, the lipid environment could partly block the protein in its inactive state to explain a decreased activity compared to neutrophils [65]. The dependence of the arrangement of the flavocytochrome within the lipid bilayer is interesting regarding its enzymatic specificity. Further investigations of the differences in lipid composition between the membranes mentioned here might be useful to better understand the implication of the protein-lipid interactions in the heterodimerization, synthesis and activity of the flavocytochrome b<sub>558</sub>.

## 5. Conclusion

Taken as a whole, our results show that the rCytb<sub>558</sub> produced in yeast demonstrated a capacity of producing superoxide, modulated to some extent by the membrane environment. Neither the Flag tag nor the yeast high glycosylation seemed to alter the functioning of Cytb<sub>558</sub> which was able to interact with the cytosolic subunits in a similar manner as the native one. This indicates that the activation process is probably not altered and principally, that the protein-protein interactions between membrane and cytosolic components crucial for efficient activation are conserved. With the yeast expression system, we have obtained a valuable rCytb<sub>558</sub> of the NADPH-oxidase complex harboring an affinity tag which should facilitate further characterization of this enzyme that may also serve as a relevant model for other members of the NOX family.

Finally, we demonstrate that the membrane environment of the flavocytochrome can directly govern its capacity of superoxide production.

## Acknowledgements

We are very grateful to Dr. M. Quinn (Montana State University, USA) and Dr. Marie-José Stasia (CHU Grenoble, France) for providing bovine and human gp91<sup>phox</sup> and p22<sup>phox</sup> cDNA. We thank Dr. Gérard Brandolin (CEA, Grenoble) for the anti-gp91<sup>phox</sup> and anti-p22<sup>phox</sup> polyclonal antibodies.

This work was supported by the ANRjc grant from the French National Agency for Research [JCJC06-137200], by the Centre Nationale de la Recherche Scientifique (CNRS), and by the Université Paris-Sud-11.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbamem.2010.02.016.

## References

- [1] A.W. Segal, How neutrophils kill microbes, *Annu. Rev. Immunol.* 23 (2005) 197–223.
- [2] B.M. Babior, NADPH oxidase, *Curr. Opin. Immunol.* 16 (2004) 42–47.
- [3] B.M. Babior, Oxidants from phagocytes: agents of defense and destruction, *Blood* 64 (1984) 959–966.
- [4] M.T. Quinn, K.A. Gauss, Structure and regulation of the neutrophil respiratory burst oxidase: comparison with nonphagocyte oxidases, *J. Leukoc. Biol.* 76 (2004) 760–781.
- [5] W.M. Nauseef, Biological roles for the NOX family NADPH oxidases, *J. Biol. Chem.* 283 (2008) 16961–16965.
- [6] J. El-Benna, P.M. Dang, M.A. Gougerot-Pocidallo, J.C. Marie, F. Braut-Boucher, p47phox, the phagocyte NADPH oxidase/NOX2 organizer: structure, phosphorylation and implication in diseases, *Exp. Mol. Med.* 41 (2009) 217–225.
- [7] C. Massenet, S. Chenavas, C. Cohen-Addad, M.C. Dagher, G. Brandolin, E. Pebay-Peyroula, F. Fieschi, Effects of p47phox C terminus phosphorylations on binding interactions with p40phox and p67phox. Structural and functional comparison of p40phox and p67phox SH3 domains, *J. Biol. Chem.* 280 (2005) 13752–13761.
- [8] D.R. Ambruso, C. Knall, A.N. Abell, J. Panepinto, A. Kurkchubasche, G. Thurman, C. Gonzalez-Aller, A. Hiester, M. deBoer, R.J. Harbeck, R. Oyer, G.L. Johnson, D. Roos, Human neutrophil immunodeficiency syndrome is associated with an inhibitory Rac2 mutation, *Proc. Natl. Acad. Sci. U. S. A.* 97 (2000) 4654–4659.
- [9] D.A. Williams, W. Tao, F. Yang, C. Kim, Y. Gu, P. Mansfield, J.E. Levine, B. Petryniak, C.W. Derrow, C. Harris, B. Jia, Y. Zheng, D.R. Ambruso, J.B. Lowe, S.J. Atkinson, M.C. Dinauer, L. Boxer, Dominant negative mutation of the hematopoietic-specific Rho GTPase, Rac2, is associated with a human phagocyte immunodeficiency, *Blood* 96 (2000) 1646–1654.
- [10] A.G. Kurkchubasche, J.A. Panepinto, T.F. Tracy Jr., G.W. Thurman, D.R. Ambruso, Clinical features of a human Rac2 mutation: a complex neutrophil dysfunction disease, *J. Pediatr.* 139 (2001) 141–147.
- [11] A.J. Thrasher, N.H. Keep, F. Wientjes, A.W. Segal, Chronic granulomatous disease, *Biochim. Biophys. Acta* 1227 (1994) 1–24.
- [12] C. Meischl, D. Roos, The molecular basis of chronic granulomatous disease, *Springer Semin. Immunopathol.* 19 (1998) 417–434.
- [13] J.D. Lambeth, NOX enzymes and the biology of reactive oxygen, *Nat. Rev. Immunol.* 4 (2004) 181–189.
- [14] T.M. Wallach, A.W. Segal, Stoichiometry of the subunits of flavocytochrome b558 of the NADPH oxidase of phagocytes, *Biochem. J.* 320 (Pt 1) (1996) 33–38.
- [15] Y. Zhu, C.C. Marchal, A.J. Casbon, N. Stull, K. von Lohneysen, U.G. Knaus, A.J. Jesaitis, S. McCormick, W.M. Nauseef, M.C. Dinauer, Deletion mutagenesis of p22phox subunit of flavocytochrome b558: identification of regions critical for gp91phox maturation and NADPH oxidase activity, *J. Biol. Chem.* 281 (2006) 30336–30346.
- [16] A.R. Davis, P.L. Mascolo, P.L. Bunker, K.M. Sipes, M.T. Quinn, Cloning and sequencing of the bovine flavocytochrome b subunit proteins, gp91-phox and p22-phox: comparison with other known flavocytochrome b sequences, *J. Leukoc. Biol.* 64 (1998) 114–123.
- [17] R.M. Taylor, J.B. Burritt, D. Baniulis, T.R. Foubert, C.I. Lord, M.C. Dinauer, C.A. Parkos, A.J. Jesaitis, Site-specific inhibitors of NADPH oxidase activity and structural probes of flavocytochrome b: characterization of six monoclonal antibodies to the p22phox subunit, *J. Immunol.* 173 (2004) 7349–7357.
- [18] I. Dahan, I. Issaeva, Y. Gorzalczany, N. Sigal, M. Hirschberg, E. Pick, Mapping of functional domains in the p22(phox) subunit of flavocytochrome b(558) participating in the assembly of the NADPH oxidase complex by "peptide walking", *J. Biol. Chem.* 277 (2002) 8421–8432.
- [19] M.C. Dinauer, E.A. Pierce, R.W. Erickson, T.J. Muhlebach, H. Messner, S.H. Orkin, R. A. Seger, J.T. Curnutte, Point mutation in the cytoplasmic domain of the neutrophil p22-phox cytochrome b subunit is associated with a nonfunctional NADPH oxidase and chronic granulomatous disease, *Proc. Natl. Acad. Sci. U. S. A.* 88 (1991) 11231–11235.
- [20] K.J. Biberstine-Kinkade, L. Yu, N. Stull, B. LeRoy, S. Bennett, A. Cross, M.C. Dinauer, Mutagenesis of p22(phox) histidine 94. A histidine in this position is not required for flavocytochrome b558 function, *J. Biol. Chem.* 277 (2002) 30368–30374.

- [21] A.R. Cross, J. Rae, J.T. Curnutte, Cytochrome b-245 of the neutrophil superoxide-generating system contains two nonidentical hemes. Potentiometric studies of a mutant form of gp91phox, *J. Biol. Chem.* 270 (1995) 17075–17077.
- [22] M.T. Quinn, M.L. Mullen, A.J. Jesaitis, Human neutrophil cytochrome b contains multiple hemes. Evidence for heme associated with both subunits, *J. Biol. Chem.* 267 (1992) 7303–7309.
- [23] A.W. Segal, I. West, F. Wientjes, J.H. Nugent, A.J. Chavan, B. Haley, R.C. Garcia, H. Rosen, G. Scrace, Cytochrome b-245 is a flavocytochrome containing FAD and the NADPH-binding site of the microbicidal oxidase of phagocytes, *Biochem. J.* 284 (Pt 3) (1992) 781–788.
- [24] J. Doussi re, G. Brandolin, V. Derrien, P.V. Vignais, Critical assessment of the presence of an NADPH binding site on neutrophil cytochrome b558 by photoaffinity and immunochemical labeling, *Biochemistry* 32 (1993) 8880–8887.
- [25] P. Ravel, F. Lederer, Affinity-labeling of an NADPH-binding site on the heavy subunit of flavocytochrome b558 in particulate NADPH oxidase from activated human neutrophils, *Biochem. Biophys. Res. Commun.* 196 (1993) 543–552.
- [26] D. Rotrosen, C.L. Yeung, T.L. Leto, H.L. Malech, C.H. Kwong, Cytochrome b558: the flavin-binding component of the phagocyte NADPH oxidase, *Science* 256 (1992) 1459–1462.
- [27] W.R. Taylor, D.T. Jones, A.W. Segal, A structural model for the nucleotide binding domains of the flavocytochrome b-245 beta-chain, *Protein Sci.* 2 (1993) 1675–1685.
- [28] A.M. Harper, M.F. Chaplin, A.W. Segal, Cytochrome b-245 from human neutrophils is a glycoprotein, *Biochem. J.* 227 (1985) 783–788.
- [29] C.I. Lord, M.H. Riesselman, J.M. Gripenrot, J.B. Burritt, A.J. Jesaitis, R.M. Taylor, Single-step immunoaffinity purification and functional reconstitution of human phagocyte flavocytochrome b, *J. Immunol. Methods* 329 (2008) 201–207.
- [30] R.M. Taylor, A.J. Jesaitis, Immunoaffinity purification of human phagocyte flavocytochrome b and analysis of conformational dynamics, *Methods Mol. Biol.* 412 (2007) 429–437.
- [31] I. de Mendez, T.L. Leto, Functional reconstitution of the phagocyte NADPH oxidase by transfection of its multiple components in a heterologous system, *Blood* 85 (1995) 1104–1110.
- [32] M.O. Price, L.C. McPhail, J.D. Lambeth, C.H. Han, U.G. Knaus, M.C. Dinauer, Creation of a genetic system for analysis of the phagocyte respiratory burst: high-level reconstitution of the NADPH oxidase in a nonhematopoietic system, *Blood* 99 (2002) 2653–2661.
- [33] L. Zhen, A.A. King, Y. Xiao, S.J. Chanock, S.H. Orkin, M.C. Dinauer, Gene targeting of X chromosome-linked chronic granulomatous disease locus in a human myeloid leukemia cell line and rescue by expression of recombinant gp91phox, *Proc. Natl. Acad. Sci. U. S. A.* 90 (1993) 9832–9836.
- [34] D. Rotrosen, C.L. Yeung, J.P. Katkin, Production of recombinant cytochrome b558 allows reconstitution of the phagocyte NADPH oxidase solely from recombinant proteins, *J. Biol. Chem.* 268 (1993) 14256–14260.
- [35] R.M. Taylor, D. Baniulis, J.B. Burritt, J.M. Gripenrot, C.I. Lord, M.H. Riesselman, W.S. Maaty, B.P. Bothner, T.E. Angel, E.A. Dratz, G.F. Linton, H.L. Malech, A.J. Jesaitis, Analysis of human phagocyte flavocytochrome b(558) by mass spectrometry, *J. Biol. Chem.* 281 (2006) 37045–37056.
- [36] C.H. Han, Y. Nisimoto, S.H. Lee, E.T. Kim, J.D. Lambeth, Characterization of the flavoprotein domain of gp91phox which has NADPH diaphorase activity, *J. Biochem.* 129 (2001) 513–520.
- [37] Y. Nisimoto, H. Ogawa, K. Miyano, M. Tamura, Activation of the flavoprotein domain of gp91phox upon interaction with N-terminal p67phox (1–210) and the Rac complex, *Biochemistry* 43 (2004) 9567–9575.
- [38] B. Marqu s, L. Liguori, M.H. Paquet, A. Villegas-Mendez, R. Rothe, F. Morel, J.L. Lenormand, Liposome-mediated cellular delivery of active gp91(phox), *PLoS ONE* 2 (2007) e856.
- [39] T.M. Wallach, A.W. Segal, Analysis of glycosylation sites on gp91phox, the flavocytochrome of the NADPH oxidase, by site-directed mutagenesis and translation in vitro, *Biochem. J.* 321 (Pt 3) (1997) 583–585.
- [40] H.M. Weiss, W. Haase, H. Michel, H. Reilander, Expression of functional mouse 5-HT<sub>5A</sub> serotonin receptor in the methylotrophic yeast *Pichia pastoris*: pharmacological characterization and localization, *FEBS Lett.* 377 (1995) 451–456.
- [41] J.J. Lacap re, F. Delavoie, H. Li, G. Peranzi, J. Maccario, V. Papadopoulos, B. Vidic, Structural and functional study of reconstituted peripheral benzodiazepine receptor, *Biochem. Biophys. Res. Commun.* 284 (2001) 536–541.
- [42] A. Fuchs, M.C. Dagher, P.V. Vignais, Mapping the domains of interaction of p40phox with both p47phox and p67phox of the neutrophil oxidase complex using the two-hybrid system, *J. Biol. Chem.* 270 (1995) 5695–5697.
- [43] D. O'Reilly, L. Miller, V. Luckow, *Baculovirus Expression Vectors, a Laboratory Manual*, WH Freeman and Company, New York, 1992.
- [44] F. Morel, J. Doussi re, M.J. Stasia, P.V. Vignais, The respiratory burst of bovine neutrophils. Role of a b type cytochrome and coenzyme specificity, *Eur. J. Biochem.* 152 (1985) 669–679.
- [45] D.R. Light, C. Walsh, A.M. O'Callaghan, E.J. Goetzl, A.I. Tauber, Characteristics of the cofactor requirements for the superoxide-generating NADPH oxidase of human polymorphonuclear leukocytes, *Biochemistry* 20 (1981) 1468–1476.
- [46] L. Baciou, M. Erard, M.C. Dagher, T. Bizouarn, The cytosolic subunit p67phox of the NADPH-oxidase complex does not bind NADPH, *FEBS Lett.* 583 (2009) 3225–3229.
- [47] B. van Gelder, E.C. Slater, The extinction coefficient of cytochrome c, *Biochim. Biophys. Acta* 58 (1962) 593–595.
- [48] J.P. Katkin, H.L. Malech, T.L. Leto, Baculovirus mediated expression of human phagocytic cell oxidase cytochrome b558 in sf9 insect cells, *Inflammation* 16 (1992) 393–410.
- [49] C.A. Parkos, M.C. Dinauer, A.J. Jesaitis, S.H. Orkin, J.T. Curnutte, Absence of both the 91kD and 22kD subunits of human neutrophil cytochrome b in two genetic forms of chronic granulomatous disease, *Blood* 73 (1989) 1416–1420.
- [50] L. Yu, L. Zhen, M.C. Dinauer, Biosynthesis of the phagocyte NADPH oxidase cytochrome b558. Role of heme incorporation and heterodimer formation in maturation and stability of gp91phox and p22phox subunits, *J. Biol. Chem.* 272 (1997) 27288–27294.
- [51] F. Laporte, J. Doussi re, P.V. Vignais, Respiratory burst of rabbit peritoneal neutrophils. Transition from an NADPH diaphorase activity to an  $\cdot\text{O}_2(-)$ -generating oxidase activity, *Eur. J. Biochem.* 194 (1990) 301–308.
- [52] T.P. Loisel, H. Ansanay, S. St-Onge, B. Gay, P. Boulanger, A.D. Strosberg, S. Marullo, M. Bouvier, Recovery of homogeneous and functional beta 2-adrenergic receptors from extracellular baculovirus particles, *Nat. Biotechnol.* 15 (1997) 1300–1304.
- [53] N. Andre, N. Cherouati, C. Prual, T. Steffan, G. Zeder-Lutz, T. Magnin, F. Pattus, H. Michel, R. Wagner, C. Reinhart, Enhancing functional production of G protein-coupled receptors in *Pichia pastoris* to levels required for structural studies via a single expression screen, *Protein Sci.* 15 (2006) 1115–1126.
- [54] A.K. Shukla, W. Haase, C. Reinhart, H. Michel, Heterologous expression and comparative characterization of the human neuromedin U subtype II receptor using the methylotrophic yeast *Pichia pastoris* and mammalian cells, *Int. J. Biochem. Cell Biol.* 39 (2007) 931–942.
- [55] L. Yu, F.R. DeLeo, K.J. Biberstine-Kinkade, J. Renee, W.M. Nauseef, M.C. Dinauer, Biosynthesis of flavocytochrome b558. gp91(phox) is synthesized as a 65-kDa precursor (p65) in the endoplasmic reticulum, *J. Biol. Chem.* 274 (1999) 4364–4369.
- [56] L. Yu, M.T. Quinn, A.R. Cross, M.C. Dinauer, Gp91(phox) is the heme binding subunit of the superoxide-generating NADPH oxidase, *Proc. Natl. Acad. Sci. U. S. A.* 95 (1998) 7993–7998.
- [57] S. Shpungin, I. Dotan, A. Abo, E. Pick, Activation of the superoxide forming NADPH oxidase in a cell-free system by sodium dodecyl sulfate. Absolute lipid dependence of the solubilized enzyme, *J. Biol. Chem.* 264 (1989) 9195–9203.
- [58] B.M. Babior, R.S. Kipnes, Superoxide-forming enzyme from human neutrophils: evidence for a flavin requirement, *Blood* 50 (1977) 517–524.
- [59] F.R. DeLeo, J.B. Burritt, L. Yu, A.J. Jesaitis, M.C. Dinauer, W.M. Nauseef, Processing and maturation of flavocytochrome b558 include incorporation of heme as a prerequisite for heterodimer assembly, *J. Biol. Chem.* 275 (2000) 13986–13993.
- [60] Y. Murata, T. Watanabe, M. Sato, Y. Momose, T. Nakahara, S. Oka, H. Iwahashi, Dimethyl sulfoxide exposure facilitates phospholipid biosynthesis and cellular membrane proliferation in yeast cells, *J. Biol. Chem.* 278 (2003) 33185–33193.
- [61] W. Zhang, D.L. Needham, M. Coffin, A. Rooker, P. Hurban, M.M. Tanzer, J.R. Shuster, Microarray analyses of the metabolic responses of *Saccharomyces cerevisiae* to organic solvent dimethyl sulfoxide, *J. Ind. Microbiol. Biotechnol.* 30 (2003) 57–69.
- [62] J. Li, R.J. Guillory, Purified leukocyte cytochrome b558 incorporated into liposomes catalyzes a cytosolic factor dependent diaphorase activity, *Biochemistry* 36 (1997) 5529–5537.
- [63] A.G. Lee, How lipids affect the activities of integral membrane proteins, *Biochim. Biophys. Acta* 1666 (2004) 62–87.
- [64] T. Wriessnegger, E. Leitner, M.R. Beleggrats, E. Ingolic, G. Daum, Lipid analysis of mitochondrial membranes from the yeast *Pichia pastoris*, *Biochim. Biophys. Acta* 1791 (2009) 166–172.
- [65] M.H. Paquet, A.W. Coleman, J. Burritt, F. Morel, NADPH oxidase of Epstein-Barr virus immortalized B lymphocytes. Effect of cytochrome b(558) glycosylation, *Eur. J. Biochem.* 268 (2001) 5197–5208.