Biochimica et Biophysica Acta 1798 (2010) 1179–1188

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

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

Mariano A. Ostuni [a], Leila B. Lamanuzzi [a], Tania Bizouarn [a], Marie-Claire Dagher [b], Laura Baciou [a,*]

[a] Laboratoire de Chimie-Physique, UMR8000, CNRS-Université Paris-Sud, 91405 Orsay, France
[b] Centre Diagnostic et Recherche CGD, TIM-C TheREx, UMR 5525-CNRS, Université Joseph-Fourier, CHU Grenoble, Grenoble, France

ARTICLE INFO

Article history:
Received 13 November 2009
Received in revised form 22 January 2010
Accepted 12 February 2010
Available online 18 February 2010

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} (Cytb_{558}) which consists of a heterodimer of the gp91^{phox} and p22^{phox} subunits. The Cytb_{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 Cytb_{558} (rCytb_{558}). We expressed the gp91^{phox} and p22^{phox} 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^{phox} undergoes co-translational glycosylation. Spectroscopic analyses showed that the rCytb_{558} displayed comparable spectral properties to neutrophil Cytb_{558}. In contrast to rCytb_{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} (Cytb_{558}) and four cytosolic subunits (p40^{phox}, p47^{phox}, p67^{phox}, GTP-binding protein Rac). The activity of the oxidase is controlled by the translocation of these cytosolic subunits to the membrane-bound Cytb_{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. Cytb_{558}, p40^{phox}, p47^{phox}, p67^{phox}, 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 Cytb_{558} has been studied by a variety of immunochemical, biochemical and analytical approaches. It consists of two non-covalently linked proteins, p22^{phox} and gp91^{phox} in a molar ratio of 1:1 [14]. Gp91^{phox} is anchored in the membrane by a series of six hydrophobic transmembrane segments and p22^{phox} can potentially form two to four membrane-spanning domains [15–17]. The hydrophilic part in the C-terminal portion of p22^{phox} is essential for

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

* Corresponding author. Laboratoire de Chimie-Physique, UMR 8000-CNRS, Bât. 350, Université Paris-Sud, F-91405 Orsay cedex, France. Tel.: +33 1 69157711; fax: +33 1 69156188.
E-mail address: laura.baciou@u-psud.fr (L. Baciou).

0005-2736/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamem.2010.02.016

recruitment of the cytosolic partners and oxidase activity [15,18–20]. The gp91^{phox} 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^{phox}, based on sequence homology with the ferredoxin-NADP$^+$ reductase family, proposed that this portion is the subject of important protein–protein interactions [27]. In neutrophils, gp91^{phox} is detected as a highly mannosylated 65-kDa monomer while the mature gp91^{phox}, heavily glycosylated [28] migrates as a broad band on SDS-PAGE around 91-kDa.

A deeper knowledge of the overall Cytb_{558} 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_{558}. Although neutrophils are the cells with the highest natural abundance of the membrane Cytb_{558}, 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_{558} 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_{558} was still purified in an insufficient amount. Several attempts to express the entire Cytb_{558} in an *E. coli* system were not successful (personal unpublished results), only production of the truncated C-terminal gp91^{phox} 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_{558}. In this work, we monitor the quantity and the quality of the rCytb_{558} 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_{558} 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, Cyt*c*, 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_{558} and expression in *P. pastoris*

2.2.1. Gp91^{phox} and p22^{phox} cDNA subcloning

We designed a construct for a large-scale production of the heterodimeric Cytb_{558} 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^{phox} and p22^{phox} 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^{phox} (CYBB cDNA) and p22^{phox} (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^{phox} protein at the N-terminus generating a Flag-tagged gp91^{phox}. 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^{phox} and p22^{phox} 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^{-5}% 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 $OD_{600nm} = 1.0$. 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_{558} (rCytb_{558})

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