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Assembly of phagocyte NADPH oxidase: A concerted binding process?

Gilda Karimi [a], Chantal Houée Levin [a], Marie Claire Dagher [b], Laura Baciou [a], Tania Bizouarn [a,c,*]

[a] Laboratoire de Chimie Physique, Université Paris Sud, UMR8000, CNRS, Orsay F-91405, France
[b] Laboratoire TIMC-IMAG, Equipe TheREx, UMR5525, 38710 La Tronche, France
[c] Laboratoire de Chimie Physique, bat 350, Université Paris Sud, 91405 Orsay cedex, France

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ABSTRACT

Background: The phagocyte NADPH-oxidase is a multicomponent enzyme that generates superoxide anions. It comprises a membrane redox component flavocytochrome b_{558} and four cytosolic proteins ($p67^{phox}$, $p47^{phox}$, $p40^{phox}$ and Rac) that must assemble to produce an active system. In this work we focused on the spatio-temporal control of the activation process of phagocyte NADPH oxidase.
Methods: A wide range of techniques including fast kinetics with a stopped-flow apparatus and various combinations of the activating factors was used to test the order of assembly and the role of the $p47^{phox}$–$p67^{phox}$ complex.
Results: The data presented here are consistent with the absence of a catalytic role of the $p47^{phox}$–$p67^{phox}$ interacting state and support the idea of independent binding sites for the cytosolic proteins on the flavocytochrome b_{558} allowing random binding order. However, the formation of the active complex appears to involve a synergistic process of binding of the activated cytosolic subunits to cytochrome b_{558}. All partners should be in the vicinity for optimal assembly, a delay or the absence of one of the partners in this process seems to lead to a decrease in the efficiency of the catalytic core.
Conclusion and general significance: The activation and assembly of the NADPH oxidase components have to be achieved simultaneously for the formation of an efficient and optimal enzyme complex. This mechanism appears to be incompatible with continuous fast exchanges of the cytosolic proteins during the production of superoxide ion in the phagosome.

1. Introduction

Neutrophils are essential actors of the first line of defence in the fight against micro-organisms. In order to destroy the engulfed pathogens, they produce massive amounts of reactive oxygen species (ROS) on their surface. This ROS production is generated from the superoxide ions formed by a reduction of molecular oxygen with NADPH as an electron donor, catalysed by the NADPH oxidase, a multi-component membrane enzyme complex. This complex consists of six proteins, four cytosolic proteins, namely $p40^{phox}$, $p47^{phox}$, $p67^{phox}$ and Rac and two membrane proteins, $gp91^{phox}$ (also called Nox2) and $p22^{phox}$, that form the flavocytochrome b_{558} (cyt b_{558}). Because of the noxious properties of the ROS, their production has to be very finely controlled. This is achieved by a precise spatial and temporal regulation [1–3]. In the absence of pathogens, the neutrophils adopt a resting state in which the NADPH oxidase enzyme is inactive through a separate localisation in the cell of the soluble and the membrane partners. The first ones are maintained in the cytosol and the last ones in specific granules and plasma membrane. All these subunits associate in a stimulus-dependent manner to form the active state of the enzyme. Binding studies between the different soluble proteins performed in vitro suggested that three of the cytosolic proteins are preassembled as a trimer $p40^{phox}$–$p47^{phox}$–$p67^{phox}$ [4–6]. This heterotrimer could be the predominant form that, during the activation process, goes to the membrane to interact with cyt b_{558} since $p67^{phox}$ is unable to translocate to the membrane without $p47^{phox}$ [7–9]. Rac, the fourth cytosolic protein participating in the formation of the NADPH oxidase complex would translocate on its own to the membrane [10,11]. The activation process starts by the binding on receptors of soluble or particulate stimuli that promotes the phosphorylation of most of the components of the NADPH oxidase, $p47^{phox}$ being the most affected by this event. Actually, phosphorylation of $p47^{phox}$ produces conformational changes leading to the exposure of two SH3 motifs, a proline-rich region, a PX domain and an auto-inhibitory domain [12–15]. This structural change leads to interactions of $p47^{phox}$ with both cyt b_{558} and phosphoinositides present in the phagosomal membrane. Experiments done with $p67^{phox}$-N terminal segment (1–242) suggested that there was a need to unmask some region of $p67^{phox}$ to render it fully active [13]. However there is no strong evidence that these changes of $p67^{phox}$ conformation are induced by

Abbreviations: AA, arachidonic acid; FRET, Forster resonance energy transfer; GST, glutathione S transferase; ITC, isothermal titration calorimetry; MF, membrane fraction of human neutrophils; Nox, NADPH oxidase; Phox, phagocyte oxidase; PBS, phosphate buffer saline; PMSF, phenylmethylsulfonyl fluoride; PX domain, phagocyte oxidase domain; ROS, reactive oxygen species; SH3, Src homology 3

* Corresponding author. Tel.: +33 1 69153016.
E-mail address: tania.bizouarn@u-psud.fr (T. Bizouarn).

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phosphorylation and a recent work of El Benna's group showed that p67^{phox} is constitutively partially phosphorylated [16]. It is therefore unclear whether modifications of this protein conformation occur during the activation of the neutrophil.

The role of each protein is also very different. It is well established that cyt b_{558} contains all the redox machinery to support the superoxide anion production, namely the binding sites for NADPH, FAD and two hemes. While p47^{phox} is described as the adaptor for the translocation to the cyt b_{558}, it seems to have a weak role in the activity per se [1–3]. Recent studies suggest that it may even stay only a short time bound on the cyt b_{558} at the beginning of the activation process and disassemble before the end of the superoxide production [17]. On the other hand, p67^{phox} has a crucial role in the initiation and maintenance of the activation of the cyt b_{558} and therefore p67^{phox} is called the activator protein [1–3,18].

The aim of our study is to better understand the mechanism of assembly and activation of the NADPH oxidase complex and more specifically the interaction between p47^{phox} and p67^{phox}. Despite all published works about the assembly it is still not clear whether it is an ordered or random process. We studied in particular the relationship between the duration of assembly and the resulting specific activity of the assembled enzyme. In addition we addressed the stability of the p47^{phox}–p67^{phox} complex by measuring its dissociation constant in the resting and active states. The understanding of the system has been greatly assisted by the use of the cell free system, which helps understand the role of each component independently from the others [19]. Moreover it is a unique way for the acquisition of precise kinetic data. We used an array of techniques including fast kinetics with a stopped-flow apparatus that allows recording of the superoxide production a few milliseconds after mixing the components of the cell-free system. It is well known that in the cell free system, the presence of arachidonic acid allows some components to adopt the active form [12,13,19]. Thus the role of this molecule in the assembly has been also considered.

2. Materials and methods

2.1. Materials

Competent BL21(DE3) cells were purchased from Life Technologies, France. Chromatographies were carried out using SP-Sepharose, Q-Sepharose, Glutathione HP-Sepharose, and Ni-Sepharose resins from GE Healthcare, France. Arachidonic acid and equine heart cytochrome *c* were from Sigma-Aldrich, France and NADPH from ACROS, France.

2.2. Protein and plasmid preparations

The plasmids coding for the human cytosolic proteins, pET15b-Hisp67^{phox}, pET15b-Hisp47^{phox} and pGEX2T-GSTRac1Q61L were provided by Dr. M.C. Dagher, Grenoble, France; and the plasmid pGEX6P-p47^{phox}-ΔCter (aa 1–342) was provided by Pr. F. Fieschi, Grenoble, France [20]. The GTP like form of Rac1 obtained by the mutation Q61L was used in all the experiments presented in this paper. Rac1Q61L was renamed Rac for simplification. All the plasmids were checked for their sequence and used for transformation of *Escherichia coli* BL21(DE3). Purification of p47^{phox}, p67^{phox} and Rac1Q61L was performed mainly as previously described [21]. Briefly, after a first step through an ion exchange column, the fusion protein was purified by affinity chromatography on Ni-Sepharose or Glutathione-Sepharose. The protein was then dialysed overnight against a phosphate buffer (30 mM sodium phosphate, pH 7.5, 100 mM NaCl) and stored at −80 °C. The concentration was established using BCA assay (Pierce) and by the absorbance at 280 nm using a NanoDrop2000 spectrophotometer (Thermo Scientific, France), the extinction coefficient being estimated from the amino acid sequences (Expasy, SIB Bioinformatics Resources Portal). The purity was controlled by SDS gel (10% Bis–Tris Nupage, Invitrogen) using Coomassie Blue reagent and quantified by the ImageJ software. SDS gels in the presence and the absence of β-mercaptoethanol allowed distinguishing between monomeric and dimeric forms of proteins.

2.3. Gel filtration chromatography

The gel exclusion chromatography was performed on a Superdex 75 Hiload 16/60 column (GE Healthcare, France) equilibrated with buffer (30 mM sodium phosphate, pH 7.5, 150 mM NaCl, 2 mM dithiothreitol and 1 mM PMSF). The volume of the injected solution was 500 μl and contained an equimolar concentration of proteins ranging between 20 and 25 μM when a mix of proteins was used. The column was run at 4 °C with a flow rate of 0.5 ml/min. The sample was eluted with an equilibration buffer. The MW protein standard mix (MWND-500, Sigma) was used to calibrate the column: proteins of 132, 66, 45 and 29 kDa were eluted at 47–48 ml, 53–54 ml 59–60 ml and 65–66 ml respectively.

2.4. Neutrophil membrane preparation

The neutrophils were purified from the human blood as described in Ref. [22]. Briefly, 500 ml of blood was sedimented in 2% dextran solution, then the neutrophils were separated from lymphocytes by centrifugation on Ficoll solution and the red cells were removed after their lysis by centrifugation. After sonication, neutrophil membranes and cytosol were separated by ultra-centrifugation for 1 h at 200000 ×*g*. The membrane fraction was re-solubilised in a lysis buffer (20 mM sodium phosphate, pH 7.4, 340 mM sucrose, 7 mM $MgSO_4$, 0.2 mM leupeptin and 1 mM PMSF) by a brief sonication and stored at −80 °C in aliquots. The differential spectrum of reduced minus oxidized cytochrome b_{558} was recorded from each preparation and the amount of heme was quantified using an extinction coefficient of 200 $mM^{-1}\cdot cm^{-1}$ at the Soret band (427 *minus* 411 nm).

2.5. Measurement of the superoxide production in cell-free assays

Unless indicated otherwise, the membrane fraction (2.5 nM cyt b_{558}), p67^{phox} (250 nM), p47^{phox} (200 nM) and Rac (500 nM) were incubated in the presence of 26 μM arachidonic acid (AA) in 500 μl phosphate buffer saline supplemented with 10 mM $MgSO_4$ for 5 min at room temperature (25 °C). The production was initiated by the addition of NADPH (250 μM) and the rate of $O_2^{\bullet-}$ was quantified by cytochrome *c* (50 μM) reduction. The rate was measured at 550 nm in a Thermo Evolution 500 spectrophotometer, with an extinction coefficient ε of 21000 M^{-1} cm^{-1} (reduced *minus* oxidized). In the absence of an incubation period, a lag appears before obtaining the full superoxide production rate, which is assumed to reflect a phase of assembly of the Nox complex. For the study of this lag the following protocol was used. The proteins and arachidonic acid were added, as quickly as possible, to a final volume of 500 μl of PBS buffer supplemented with 10 mM $MgSO_4$ in the order indicated in the figure legend and superoxide anion production was measured immediately after the last component was added.

2.6. Determination of enzymatic parameters

The enzymatic parameters, EC_{50} and $V_{max,}$ were determined by non-linear least square fitting of the curves of superoxide rate of production vs. component concentration using the following expression.

$$V = \frac{V_{max}[P]}{EC50 + [P]} \quad (1)$$

where [P] is the concentration of the considered protein (p67^{phox}, p47^{phox} or Rac).