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The cytosolic subunit p67^{phox} of the NADPH-oxidase complex does not bind NADPH

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ABSTRACT

The NADPH-oxidase of phagocytic cells is a multicomponent enzyme that generates superoxide. It comprises a membrane flavocytochrome b_{558} and four cytosolic proteins; p67^{phox}, p47^{phox}, p40^{phox} and Rac. The NADPH-binding site of this complex was shown to be located on the flavocytochrome b_{558}. However, a number of studies have suggested the presence of another site on the p67^{phox} subunit which is the key activating component. Using several approaches like tryptophan quenching fluorescence measurement, inhibition by 2′,3′-dialdehyde NADPH, and free/bound NADPH concentration measurements, we demonstrate that no NADPH binds on p67^{phox}, thus definitively solving the controversy on the number and location of the NADPH-binding sites on this complex.

1. Introduction

Human neutrophils play an essential role in the inflammatory response to kill invading pathogens. Upon stimulation, neutrophils exhibit a burst of cyanide-insensitive oxygen consumption accompanied by a NADPH–dependent production of superoxide anions ($O_2^{\cdot -}$), precursors of toxic reactive oxygen metabolites. $O_2^{\cdot -}$ production is catalysed by a membrane-bound electron transfer complex named NADPH-oxidase (for review see [1,2]). Dysfunction of the phagocyte NADPH-oxidase leads to severe human pathologies like the chronic granulomatous disease (CGD).

The NADPH-oxidase is a highly regulated enzyme complex, dormant in resting cells and active upon cell stimulation. The activation occurs *via* the phosphorylation of cytosolic regulatory components p47^{phox}, p67^{phox}, p40^{phox} and their translocation to the transmembrane heterodimer, the flavocytochrome b_{558} (Cytb_{558}) in the presence of the small G protein Rac1/2. The Cytb_{558} is composed of two subunits, p22^{phox} and gp91^{phox}. The cytosolic cofactors p47^{phox}, p67^{phox} are absolutely required for the enzymatic activity in vivo [1,3]. In vitro, the, so-called "cell-free system", comprised of p67^{phox}, p47^{phox}, Rac in its GTP bound form and the Cytb_{558}, is able to produce $O_2^{\cdot -}$ upon addition of NADPH (for review see [4]). In vitro, p47^{phox} can be omitted if the concentration of p67^{phox} is raised up to several micromolar [5]. The canonical view is that the NADPH-oxidase catalyses hydride transfer from NADPH to FAD and successive electron transfers from FAD to the catalytic centre (two hemes). The NADPH-binding site was first proposed to be located on another component than Cytb_{558}, hypothesis based on studies of fractions isolated from X-linked-CGD patient's neutrophils (lacking the gp91^{phox} subunit). In these fractions, NADPH-binding proteins were detected and the oxidase activation property (i.e. $O_2^{\cdot -}$ production) was inhibited by covalent binding of NADPH analogs (NADPH-dialdehyde) [6]. In addition, it was found that the oxidase cytosolic subunits bound to a 2′,5′-ADP (a NADPH analog) agarose gel and could be eluted with ATP, GTP and NADPH solutions [7]. Curiously, after labelling of the neutrophil fractions with radioactive nucleotides, the radioactivity was found on various size proteins ranging from 32 to 66 kDa [6,8–10].

In the early 90s, the publication of the first hint of the presence of the catalytic NADPH (and FAD) binding site on the Cytb_{558} strongly suggesting that the C-terminus of gp91^{phox} is a member of the FNR family of reductase [11] clearly weakened the previous hypothesis. This proposal was strengthened by labelling studies from other groups showing a predominant labelling by a radioactive NADPH analog on a glycosylated membrane-bound protein of about 80–100 kDa [12,13] and by functional studies [14] showing that NADPH dependent $O_2^{\cdot -}$ production can be elicited in a cell-free system containing Cytb_{558} in the total absence of cytosolic

Abbreviations: CGD, chronic granulomatous disease; GST, glutathione-S-transferase; MF, neutrophil membrane fraction

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components. However, more recent studies on recombinant p67^{phox} gave controversial results. Using radioactive NADPH-dialdehyde for protein labelling and tryptophan fluorescence quenching measurements, it was proposed that the recombinant p67^{phox} protein contains a NADPH-binding site essential for enzyme activity, with a K_d close to 7 μM [15].

Altogether, these results maintain a doubt on the presence of multiple NADPH-binding site in the oxidase complex, as underlined in several recent reviews [2,16,17]. An assessment of the binding site on p67^{phox} subunit is essential since it would be the primary catalytic event within the enzyme, all other steps depending on substrate binding. The location of the nucleotide binding site is reanalysed here on bovine and human p67^{phox} recombinant proteins in order to eliminate a possible species peculiarity and with different approaches to allow the detection of a large range of K_d value from 10^{-7} to 10^{-4} M.

2. Materials and methods

2.1. Materials

All the chemicals were obtained from Sigma–Aldrich; the Q-Sepharose Fast-Flow (FF), DEAE Sepharose-FF, SP-Sepharose-HP, Glutathione-Sepharose-4B gels were from GE-Healthcare-Bioscience; the His-Select Nickel Affinity gel was from Sigma–Aldrich.

2.2. Recombinant protein productions and purifications

All the constructs used to produce the recombinant proteins from the NADPH-oxidase are listed in Table S1 (Supplementary data). Except the His-p67^{phox} protein which was expressed in *Escherichia coli* BL21(DE3)pLysS and induced only 6 h (30 °C), all recombinant proteins were induced in BL21(DE3) overnight at 30 °C. The glutathione-S-transferase (GST)-tagged proteins (p67^{phox} and Rac1) were purified on Q-Sepharose-FF chromatography followed by a Glutathione-Sepharose-4B affinity column (elution with 50 mM Tris, 10 mM reduced glutathione, pH 8.0). The His-p67^{phox} was purified as above except that it was purified on a Nickel Affinity gel (elution with 150 mM imidazole). The His-p47^{phox} was purified through the SP-Sepharose chromatography and purified further through a Nickel Affinity gel.

The fusion-tags could be removed by the use of either thrombin or Factor-Xa. Since the human and bovine p67^{phox} proteins have an additional cleavage site for these endoproteases, only the bovine p67^{phox} was subjected to limited digestion with protease Factor-Xa (2 h at room temperature; 40 units of protease/mg of protein).

Protein concentrations were estimated using the Bicinchoninic acid protein assay with BSA as standard. All isolated proteins were subjected to 10% BisTris-NuPAGE SDS gels (Invitrogen), stained with Coomassie Brilliant Blue (Fig. 1).

The intact nicotinamide nucleotide transhydrogenase and its NADH-binding domain were purified as described in [18,19].

2.3. Purification of membrane fraction from neutrophils (MF)

The membrane fraction was obtained after bovine neutrophils purification from blood as described in [20]. The yield was between 5 and 20 mg of membrane protein from 10 l of blood.

2.4. Tryptophan fluorescence spectroscopy measurement

The tryptophan fluorescence signals of bovine and human p67^{phox} were recorded in a 1 × 1 cm quartz cuvette on a Spex-Fluorolog1681 spectrofluorimeter at 25 °C with an excitation and emission wavelengths of 280 nm and 340 nm, respectively. The

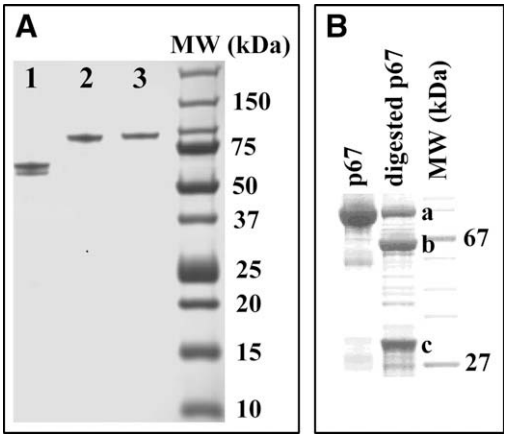

Fig. 1. SDS–PAGE electrophoresis of recombinant purified cytosolic p67^{phox} and GST-cleaved bovine GST-His-p67^{phox}. (A) Each lane was loaded with 0.5–1.5 μg of human His-p67^{phox} (lane 1), human GST-p67^{phox} (lane 2) and bovine GST-His-p67^{phox} (lane 3), molecular weight marker (dual-color, Biorad). (B) Undigested and Factor-Xa digested bovine GST–His-p67^{phox} (a: GST-tagged p67^{phox}, b: p67^{phox} and c: GST-tag) and the molecular weight marker (Ozyme).

titrations were achieved by successive additions of 5 μl aliquots of 3 mM NADPH (or NADH when indicated). A titration in identical conditions was performed on a bovine serum albumin (BSA) sample (0.5 μM), a well-known non-NADPH-binding protein. This was used to correct the fluorescence signals from inner filtering effect [21] and dilution. To validate this method, negative and positive controls were performed with tryptophanyl solution (8 μM) titrated with NADPH and NADH-binding domain of transhydrogenase (2.5 μM) with NADH, respectively. Indeed, transhydrogenase has a K_d value for NADH (20 μM) in the same range as the one expected for the NADPH-oxidase complex [18].

2.5. Estimation of K_d by unbound and bound NADPH concentration measurements

Four milligrams of p67^{phox} were mixed with 2 ml of Glutathione-Sepharose gel for 1 h. The gel was washed twice briefly by centrifugation (at 10 000 rpm) with PBS buffer to remove the unbound proteins and then distributed in four tubes. In each tube, NADPH was added to final concentrations of 0 μM, 36 μM, 71 μM, 135 μM (in 0.5 ml of PBS). After 10 min incubation, the tubes were centrifuged as previously. At this stage, the protein (15–20 μM) is located at the bottom of the tube bound onto the gel and the free NADPH concentration can be determined from an aliquot taken on top of the tube. The NADPH fluorescence of the diluted supernatant (v:v 1:20) was recorded (λ_{ex} = 340 nm, λ_{em} = 460 nm). Control experiments, in the absence of proteins, were performed in parallel. The levels of fluorescence in the absence of NADPH as well as the concentration of the bound protein were checked. A positive control, showing that dinucleotides can bind to gel-trapped proteins was realised with a His-tagged nicotinamide nucleotide transhydrogenase bound on Nickel resin. We found that all the binding sites were occupied at the NADPH concentrations used in these experiments, which is in agreement with a K_d for NADPH of 1 μM.

It should be noted that some purified protein preparations displayed an intrinsic NADPH oxidation activity without addition of any oxidant. When this reaction was observed, it occurred extremely slowly. However, it could cause artefacts in the estimation of free NADPH concentration essentially because of the high level of protein concentration used (15–20 μM). We evaluated this undesirable NADPH oxidation by measuring spectrophotometrically the NADPH absorption decrease at 340 nm for several hours