RESEARCH COMMUNICATION

The mechanism of activation of NADPH oxidase in the cell-free system: the activation process is primarily catalytic and not through the formation of a stoichiometric complex

Andrew R. CROSS^{*1}, Richard W. ERICKSON[†] and John T. CURNUTTE[†]

^{*}Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, CA 92037, U.S.A., and [†]Department of Immunology, Genentech Inc., South San Francisco, CA 94080, U.S.A.

It is commonly assumed that activation of the superoxide-generating NADPH oxidase requires the formation of a stable complex between flavocytochrome b_{-245} (the gp91^{phox}/p22^{phox} heterodimer) and the cytosolic cofactors p47^{phox}, p67^{phox} and Rac2. This association is thought to convert flavocytochrome b_{-245} , which contains the NADPH-binding site, flavin and haem centres, from an inactive into an active state. Here we provide evidence that, in the cell-free system, this activation process does not necessarily require the formation of a stable stoichiometric

complex between the *phox* proteins. To explain this data we propose the hypothesis that p67^{phox} (and possibly Rac2), are capable of activating flavocytochrome b_{-245} in a catalytic fashion, where a single molecule of p67^{phox} (or Rac2) is capable of activating multiple flavocytochrome b_{-245} molecules.

Key words: flavocytochrome *b*, neutrophil, *phox* proteins, superoxide.

INTRODUCTION

NADPH oxidase is a central component of the host resistance to infection. The function of this enzyme is to provide a defence against microbial pathogens by the generation of damaging oxygen species derived from the primary product of the enzyme, superoxide [1]. NADPH oxidase is principally expressed in phagocytic blood cells and normally exists in an inactive state [2]. After appropriate exposure of these cells to soluble or particulate stimuli, the enzyme is rapidly converted into an active form. The active enzyme involves the participation of at least six gene products. Two of these, gp91^{phox} and p22^{phox}, together form a membrane-bound flavocytochrome, flavocytochrome b_{-245} (also known as (flavo-)cytochrome b_{558} or (flavo-)cytochrome b_{559}). Previous work from this and other laboratories has shown that flavocytochrome b_{-245} contains the NADPH-binding site, one molecule of FAD and two molecules of haem [3–6]. These features provide all the necessary machinery to support the generation of superoxide anion (O_2^-) at the extracellular face of the cell membrane (or the intraphagosomal space) at the expense of cytosolic NADPH. Three of the other proteins involved, namely p47^{phox}, p67^{phox} and Rac2, are required to convert the normally dormant oxidase into an active state [7,8]. A further protein, p40^{phox}, is also implicated in oxidase activity, but the role of this component is poorly understood at present. The activity of the oxidase is regulated in part by the physical separation of the cytosolic components (p40^{phox}, p47^{phox}, p67^{phox} and Rac2) from flavocytochrome b_{-245} in the membrane. During the activation process, the cytosolic factors translocate to the membrane where they associate with flavocytochrome b_{-245} [9,10]. Our understanding of NADPH oxidase has been greatly assisted by the development of a cell-free system for oxidase activation that employs anionic amphiphiles to stimulate oxidase activation [11–13]. It is thought that these amphipathic agents mimic the

normal cellular processes of protein phosphorylation and/or secondary messenger lipid generation. As in the whole cell, activation in the cell-free system involves the association of the cytosolic factors with flavocytochrome b_{-245} [14,15]. This phenomenon has led to the general assumption that conversion of the resting enzyme to the active one involves a stoichiometric assembly of the *phox* proteins at the membrane to form an active complex [16,17]. Here we present data from simple titration experiments that strongly suggest that in the cell-free system, at low ratios of p67^{phox} (and to a lesser extent Rac2), these proteins are capable of activating flavocytochrome b_{-245} in a catalytic fashion, rather than through the formation of a stable complex of fixed stoichiometry.

EXPERIMENTAL

Purification, relipidation and refluination of flavocytochrome b_{-245}

Flavocytochrome b_{-245} was purified, refluinated and relipidated using phosphatidylcholine type IIS (Sigma, St. Louis, MO, U.S.A.) as described previously [18]. Flavocytochrome b_{-245} concentration was determined from dithionite-reduced minus oxidized difference spectra using an absorption coefficient of 21.6 mM⁻¹/mol of haem (10.8 mM⁻¹/mol of protein) [19]. The preparation was judged to be fully active based on the turnover number (120–150 mol of O_2^- /s per mol of haem).

Recombinant *phox* proteins

Recombinant p40^{phox}, p47^{phox}, p67^{phox} and Rac2 were expressed and purified to homogeneity as assessed by silver staining of PAGE gels as described previously [20]. Concentrations of the recombinant proteins were determined using the Coomassie Plus protein assay (Pierce, Rockford, IL, U.S.A.). These values were checked by measuring the UV absorbance of the purified proteins and comparing the absorbance at 280 nm with the theoretical

Abbreviations used: GTP[S], guanosine 5'-[γ-thio]triphosphate; SOD, superoxide dismutase.

¹ To whom correspondence should be sent at the following address: Department of Molecular and Experimental Medicine, CAL-1, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, U.S.A. (e-mail scross@scripps.edu).

absorption coefficient calculated using Protein Sequence Analysis software (Ira W. Palmer, Gaithersburg, MD, U.S.A.). The activity of the recombinant proteins was comparable (on a molar basis) with the equivalent amount of neutrophil cytosol.

Cell-free O_2^- assay

Superoxide dismutase (SOD)-inhibitable rates of O_2^- production were measured in duplicate or triplicate in 96-well microtitre plates as described previously [21]. The final volume of 150 μ l contained 200 nM FAD, 10 μ M GTP[S], 166 μ M NADPH and 100 μ M cytochrome *c*. Each well was paired with an identical well supplemented with 300 units of SOD. In most cases, purified flavocytochrome b_{-245} was used at concentrations between 0.15 and 0.3 pmol/well (1–2 nM). Under these conditions, p40^{phox} was added at 10 pmol/well, p47^{phox} 15 pmol/well, p67^{phox} 10 pmol/well and Rac2 at 4 pmol/well. In experiments employing higher flavocytochrome b_{-245} concentrations (0.6 pmol/well), the concentration of the recombinant proteins was increased to 20 pmol/well (p40^{phox} and p67^{phox}), 30 pmol/well (p47^{phox}) and 8 pmol/well (Rac2). For each assay, the desired mixture was incubated for 5 min in the presence of 80 μ M SDS before initiating the reaction with NADPH and following the time course of cytochrome *c* reduction at 550 nm. When required, recombinant proteins were diluted in silicone-treated polypropylene tubes to prevent loss of protein by absorption to surfaces at high protein dilutions.

RESULTS AND DISCUSSION

Effect of p40^{phox} on NADPH oxidase activity

Reports from other laboratories have suggested that p40^{phox} could have both inhibitory [22] or stimulatory [23] effects on the activation of NADPH oxidase. In keeping with our previous observations, in the cell-free system employed here, we did not observe significant effects of p40^{phox} when added in the range of 0–40 pmol/well [20].

Concentration-dependence of NADPH oxidase activity on p47^{phox}

The concentration-dependence of cell-free NADPH oxidase activity on p47^{phox} is shown in Figure 1. Recombinant p47^{phox} was titrated into mixtures of the other *phox* components and the maximum rate of O_2^- was determined as described in the Experimental section. Even at low cytochrome concentrations, approximately a 20-fold excess of p47^{phox} was needed to reach 90% of the maximum activity. Analysis of the curves fitted to a one-site binding equation (hyperbola) using GraphPad Prism (GraphPad Software Inc., San Diego, CA), showed that, over a fourfold change in flavocytochrome b_{-245} concentration (1–4 nM, 0.15–0.6 pmol/well), the concentration of p47^{phox} required to give half the maximum activity remained relatively constant (13.6–24 nM). These values are approximately one-third of those reported by Freeman and Lambeth [24] and most likely reflect differences in the experimental systems (our replacement of purified flavocytochrome b_{-245} for neutrophil membranes and Rac2 in place of Rac1). The V_{max} rates ranged from 11.8 to 15.6 nmol of O_2^- /min per pmol of flavocytochrome b_{-245} [catalytic-centre activity ('turnover number') 100–130 mol of O_2^- /s per mol of haem] in the presence of 20 pmol of p47^{phox} per well. The concentrations of p67^{phox} and Rac2 used here were too low to observe p47^{phox}-independent oxidase activity described elsewhere [20,24].

Figure 1 Concentration-dependence of NADPH oxidase activity on p47^{phox}

NADPH oxidase activity was determined in the cell-free system as described in the Experimental section. The reaction mixtures contained either 0.15 pmol (■), 0.3 pmol (▲) or 0.6 pmol (▼) of flavocytochrome b_{-245} and an excess of p67^{phox} and Rac2. Each data point is representative of one experiment of three performed in duplicate. The curves are the best fits to a one-site binding (hyperbola) equation calculated using GraphPad prism. The error bars represent the S.E.M.

Concentration-dependence of NADPH oxidase activity on p67^{phox}

The concentration-dependence of cell-free NADPH oxidase activity on p67^{phox} is shown in Figure 2. Recombinant p67^{phox} was titrated into mixtures of the other *phox* components and the maximum rate of O_2^- was determined as described in the Experimental section. In contrast with the p47^{phox} curve (Figure 1), the dependence of oxidase activity on p67^{phox} is much sharper, showing saturation at close to 1:1 stoichiometry of p67^{phox}: flavocytochrome b_{-245} . Analysis of these curves gave rise to an unexpected conclusion. Half-maximal activities were obtained at 0.043 pmol of p67^{phox} with 0.15 pmol of flavocytochrome b_{-245} ; 0.054 pmol of p67^{phox} with 0.3 pmol of flavocytochrome b_{-245} ; and 0.17 pmol of p67^{phox} with 0.6 pmol of flavocytochrome b_{-245} . Thus, at low molar ratios of p67^{phox} to flavocytochrome b_{-245} , a disproportionate amount of oxidase activity was obtained. This effect is best illustrated in Figure 2(B) using the activity curve for 0.3 pmol of flavocytochrome b_{-245} as an example. If p67^{phox} is assumed to bind perfectly to the flavocytochrome b_{-245} –p47^{phox}–Rac2 complex in a stoichiometric fashion (i.e. every molecule of p67^{phox} added binds rapidly and completely to a molecule of the flavocytochrome b_{-245} complex and activates it), then it would be expected that there would be a linear dependence on the p67^{phox} added. This is shown by the broken line through the origin intersecting with the calculated V_{max} value (3.56 nmol of O_2^- /min per 0.3 pmol of flavocytochrome b_{-245}). If the binding of p67^{phox} is less than perfect, the experimental activity curve will fall to the right of the theoretical line. In fact, the opposite is the case, and over the lower part of the curve, the experimental values greatly exceed the expected values. Simple calculation from the data indicates that 10 fmol of flavocytochrome b_{-245} , if activated with 10 fmol of p67^{phox} through a mechanism requiring the formation of a stoichiometric complex, should produce a maximum of 0.119 nmol of O_2^- /min under these conditions. In fact, however, the addition of 10 fmol of p67^{phox} is capable of activating sufficient flavocytochrome b_{-245} to produce 0.667 nmol of O_2^- /min, the equivalent of 56 fmol of fully activated flavocytochrome b_{-245} (i.e. a 5.6-fold excess over the p67^{phox} added).

Figure 2 Concentration-dependence of NADPH oxidase activity on p67^{phox}

NADPH oxidase activity was determined in the cell-free system as described in the Experimental section. (A) The reaction mixtures contained either 0.15 pmol (■), 0.3 pmol (▲) or 0.6 pmol (▼) of flavocytochrome *b*₋₂₄₅ and an excess of p47^{phox} and Rac2. Each data point is representative of one experiment of three performed in duplicate. The curves are the best fits to a one-site binding (hyperbola) equation calculated using GraphPad prism. The error bars represent the S.E.M. (B) shows a detail of the curve from (A) for the data generated using 0.3 pmol of flavocytochrome *b*₋₂₄₅. The broken line represents the theoretical maximum binding relationship of 0.3 pmol of p67^{phox} to 0.3 pmol of flavocytochrome *b*₋₂₄₅. See the text for details.

Similar calculations and conclusions can be made for the experiments employing lower (0.15 pmol) and higher (0.6 pmol) concentrations of flavocytochrome *b*₋₂₄₅. The most plausible explanation for these observations is that one molecule of p67^{phox} is capable of activating several (in this case at least five) flavocytochrome *b*₋₂₄₅ molecules. We hypothesize that, at low ratios of p67^{phox} to flavocytochrome *b*₋₂₄₅ (and the other *phox* proteins), p67^{phox} can associate with a flavocytochrome *b*₋₂₄₅ molecule and induce a conformational change to activate it. The p67^{phox} can then dissociate, but leaving the flavocytochrome *b*₋₂₄₅ molecule in the activated state. The p67^{phox} molecule is then able to continue several of these cycles of association, activation and dissociation, before the activated (but p67^{phox}-free) flavocytochrome *b*₋₂₄₅ molecules have time to collapse back to the inactive state. In the experiment illustrated, as the ratio of p67^{phox} is increased, an increasing proportion of flavocytochrome *b*₋₂₄₅ molecules are continuously associated with p67^{phox}, until all of the flavocytochrome *b*₋₂₄₅ present is essentially captured in the activated state. An alternative explanation is that one molecule

Figure 3 Concentration-dependence of NADPH oxidase activity on Rac2

NADPH oxidase activity was determined in the cell-free system as described in the Experimental section. (A) The reaction mixtures contained either 0.15 pmol (■), 0.3 pmol (▲) or 0.6 pmol (▼) of flavocytochrome *b*₋₂₄₅ and an excess of p47^{phox} and p67^{phox}. Each data point is representative of one experiment of three performed in duplicate. The curves are the best fits to a one-site binding (hyperbola) equation calculated using GraphPad prism. The error bars represent the S.E.M. (B) shows a detail of the curve from (A) for the data obtained using 0.3 pmol of flavocytochrome *b*₋₂₄₅. The broken line represents the theoretical maximum binding relationship of 0.3 pmol of Rac2 to 0.3 pmol of flavocytochrome *b*₋₂₄₅. See the text for details.

of p67^{phox} can simultaneously interact with several (at least five) flavocytochrome *b*₋₂₄₅ molecules and cause a conformational change in each that results in activation. This seems highly unlikely, since each flavocytochrome *b*₋₂₄₅ molecule would be in contact with different parts of the p67^{phox} molecule, which does not display the type of multiply repeated amino acid sequences one would expect would be required.

Concentration-dependence of NADPH oxidase activity on Rac2

The concentration-dependence of cell-free NADPH oxidase activity on Rac2 is shown in Figure 3. Recombinant Rac2 was titrated into mixtures of the other *phox* components and the maximum rate of O₂⁻ was determined as described in the Experimental section. As in the case of p67^{phox} above, Rac2 associates and activates oxidase activity with high affinity. Half-maximal activities were obtained with 0.075 pmol of Rac2

(0.15 pmol flavocytochrome b_{-245}), 0.13 pmol of Rac2 (0.3 pmol of flavocytochrome b_{-245}) and 0.19 pmol of Rac2 (0.6 pmol of flavocytochrome b_{-245}). Analysis of the curves at low ratios of Rac2 to flavocytochrome b_{-245} (Figure 3B) is more ambiguous than in the case of the p67^{phox} titrations (Figure 2B). (The curve for 0.3 pmol flavocytochrome b_{-245} is used as illustration). At low ratios of Rac2 to flavocytochrome b_{-245} the curve falls close to, but appears to somewhat exceed, the theoretical line for complete ('infinite' affinity) binding and activation. It is therefore not possible to state with certainty that Rac2 also activates the oxidase complex catalytically, but it appears probable. Thus it appears likely that Rac2 can activate the oxidase through a catalytic mechanism similar to that proposed for p67^{phox} above, but somewhat less effectively.

Although subject to the limitations of the cell-free system (removal of possible modulatory proteins, lack of a requirement for phosphorylation), in summary the data presented here appear to confirm the proposal of Lambeth, Pick and their colleagues [24–26] that the role of p47^{phox} is to provide high-affinity binding site(s) for the association of p67^{phox} and Rac2 with flavocytochrome b_{-245} . Unexpectedly, we demonstrate that the activation process induced by the latter factors seems to be a catalytic mechanism rather than the formation of a stable stoichiometric complex. These results also suggest a mechanism that explains the observations of Koshkin and Pick [27–30] describing NADPH oxidase activity that is supported by flavocytochrome b_{-245} in the absence of any of the cytosolic cofactors. The hypothesis presented above, that flavocytochrome b_{-245} can remain in the activated state for a significant period of time after undergoing a conformational change induced by p67^{phox} and Rac2, suggests that a dynamic equilibrium exists between these two states. Under our conditions the inactive conformation of flavocytochrome b_{-245} is favoured to the exclusion of the active conformation in the absence of the cytosolic *phox* proteins. Interactions of p67^{phox} and Rac2 with flavocytochrome b_{-245} drive the favoured conformation to the active state. We have recently provided evidence that this process is relatively slow [20]. It is possible that, because of methodological differences between our laboratories in the detergents used to solubilize, methods used to purify, or lipids used to reconstitute flavocytochrome b_{-245} , that the flavocytochrome b_{-245} preparations of Koshkin and co-workers are in an environment which is more favourable to the active conformation of flavocytochrome b_{-245} in the absence of the cytosolic *phox* proteins. Consequently they are able to observe significant NADPH oxidase activity in the absence of p47^{phox}, p67^{phox} and Rac.

We hypothesize that the conformational changes in flavocytochrome b_{-245} required for the transition from the inactive to the active state may involve the displacement of residues that normally block the NADPH-binding site [31], a change in spin state of the haem(s) [32] or a combination of both. We have been unable to determine whether the 'inactive conformer' exists *in situ* or whether it is generated during flavocytochrome b_{-245} purification by performing the p67^{phox} titration experiments using neutrophil membranes in place of purified cytochrome. The reason is that, despite extensive manipulation of the experimental conditions, we have only been able to observe very low NADPH oxidase activities using 'recombinant cytosol' in place of neutrophil cytosol when the source of flavocytochrome b_{-245} is neutrophil membranes. This is in marked contrast with the activities observed using solubilized neutrophil membranes (using several different detergents), partially purified, or highly purified flavocytochrome b_{-245} . In these cases, the use of the recombinant *phox* proteins results in activities that meet or exceed the activities seen with neutrophil cytosol. We hypothesize

that neutrophil membranes may contain a factor (possibly Rap1a) that is inhibitory to oxidase activation, and the presence of a second cytosolic factor is necessary to overcome this inhibition. The second factor is missing from 'recombinant cytosol', while the first is removed when neutrophil membranes are treated with detergent. In support of this hypothesis, we have found that the Mg²⁺ requirement for oxidase activity disappears during flavocytochrome b_{-245} purification [18], suggesting that the cytosolic factor may be Mg²⁺-dependent. Secondly, addition of a 'spike' of neutrophil cytosol (insufficient to generate significant oxidase activity) to the cell-free system consisting of 'recombinant cytosol' and neutrophil plasma membranes results in vigorous oxidase activity. This activity approximates to that found using a saturating amount of neutrophil cytosol. Interestingly, the time required to reach maximum activity appears to be significantly longer, suggesting the presence of a rate-limiting factor in the 'spike' of neutrophil cytosol. This hypothesis is under active investigation in our laboratory.

The findings presented above reinforce the conclusion that all the catalytic machinery required for the reactions catalysed by NADPH oxidase (from NADPH binding to oxygen reduction) must be contained within flavocytochrome b_{-245} , and the cytosolic factors p47^{phox}, p67^{phox} and Rac2 do not take part in either the redox reactions or binding of substrates as has been previously proposed.

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