Architecture of the p40-p47-p67phox complex in the resting state of the NADPH oxidase: a central role for p67phox

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Running Title: Protein-protein interactions in NADPH oxidase assembly

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1Abbreviations: phox, phagocyte oxidase; ITC, isothermal titration calorimetry; SH3, Src homology 3; PX, PhoX domain; TPR, tetratricopeptide repeat; PC, phox and Cdc (also known as OPR motif); PB1, phox and Bem; DLS, dynamic light scattering
SUMMARY

The phagocyte NADPH oxidase is a multi-protein enzyme whose subunits are partitioned between the cytosol and plasma membrane in resting cells. Upon exposure to appropriate stimuli multiple phosphorylation events in the cytosolic components take place which induce rearrangements in a number of protein-protein interactions, ultimately leading to translocation of the cytoplasmic complex to the membrane. To understand the molecular mechanisms which underlie the assembly and activation process we have carried out a detailed study of the protein-protein interactions that occur in the p40-p47-p67phox complex of the resting oxidase. Here we show that this complex contains one copy of each protein which assemble to form a heterotrimeric complex. The apparent high molecular weight of this complex, as observed by gel filtration studies, is due to an extended, non-globular shape rather than to the presence of multiple copies of any of the proteins. Isothermal titration calorimetry measurements of the interactions between the individual components of this complex demonstrate that p67phox is the primary binding partner of p47phox in the resting state. These findings, in combination with earlier reports, allow us to propose a model for the architecture of the resting complex in which p67phox acts as the bridging molecule which connects p40phox and p47phox.
INTRODUCTION

The phagocytic NADPH oxidase is a multi-protein enzyme that catalyses the reduction of molecular oxygen to superoxide in response to invasion of the body with bacterial, fungal and viral pathogens. Superoxide anions are precursors of a variety of reactive oxygen species that are used for killing of the micro-organisms (reviewed in (1-4)). The importance of the NADPH oxidase in host defence is exemplified by the inherited disorder chronic granulomatous disease (CGD) in which patients suffer from recurring infections due to a defect in oxidase activity. The NADPH oxidase consists of six subunits. Four of these, \( p40^{\text{phox}} \), \( p47^{\text{phox}} \), \( p67^{\text{phox}} \) and the small GTPase Rac are cytosolic in unstimulated cells, while \( p22^{\text{phox}} \) and \( \text{gp91}^{\text{phox}} \) form a heterodimeric, membrane-bound flavocytochrome, also known as cytochrome \( b_{558} \). In resting cells, \( p40^{\text{phox}} \), \( p47^{\text{phox}} \) and \( p67^{\text{phox}} \) exist as a tight cytosolic complex of undefined stoichiometry that can be purified by gel filtration chromatography with an apparent molecular weight of 250-300 kDa (5-8). Activation of the NADPH oxidase is initiated by phosphorylation, which is believed to induce conformational changes that subsequently lead to rearrangements in intra- and intermolecular interactions in the \( p40-p47-p67^{\text{phox}} \) complex (9-14). These events culminate in translocation of this complex to the membrane and association with both Rac-GTP and cytochrome \( b_{558} \) to form the active enzyme.

\( P40^{\text{phox}}, P47^{\text{phox}} \) and \( p67^{\text{phox}} \) are multidomain proteins that contain SH3 protein-protein interaction domains (Figure 1). Additionally, \( p40^{\text{phox}} \) and \( p47^{\text{phox}} \) each contain a PX domain, which has recently been shown to bind to phosphatidylinositols and seems to act as a membrane targeting module (15-18). Most PX domains identified so far also contain a Pro-X-X-Pro motif which is the consensus target sequence for SH3 domain binding modules (19). This suggests that PX domains are bifunctional with the potential to coordinate membrane localisation as well as protein assembly during signal transduction events. In support of this
idea it has been shown that the isolated PX domain of p47phox binds to the second SH3 domain of p47phox with an equilibrium dissociation constant (Kₐ) of approximately 50 µM (20).

P47phox plays a central role in the activation process due to its ability to bind to the cytoplasmic region of p22phox, an interaction that is necessary for oxidase activity (21-23). P47phox contains a PX domain, tandem SH3 domains and a polybasic region followed by a proline-rich sequence within its C-terminal region (Fig. 1). Studies by various groups suggest that p47phox exists in an auto-inhibited conformation in the resting state. In this model, the tandem SH3 domains are masked due to an intramolecular interaction with a C-terminal segment while the PxxP motif present in the PX domain simultaneously interacts with the second SH3 domain (24,25). In vivo phosphorylation of multiple serine residues within the C-terminus of p47phox liberates the N-terminal SH3 domain and allows it to interact with a proline-rich region in p22phox. This, in turn, initiates translocation of the cytoplasmic complex to the membrane and activation of the oxidase (10,12,21,24-28).

P67phox is complexed with p47phox in the cytosol of resting neutrophils and this interaction is absolutely required for translocation of p67phox to the membrane. Evidence for this is provided by the observation that neither p67phox nor p40phox are able to translocate in CGD neutrophils lacking p47phox (29-31). Once at the membrane, p67phox interacts with Rac and possibly cytochrome b₅₅₈ to support catalysis by a mechanism that is not fully understood (32-37). On the other hand, no clear function has yet been attributed to p40phox and it can apparently act as an activator or inhibitor depending on the experimental system. Furthermore there are suggestions that it might stabilise p67phox and thus act as a general modulator of NADPH oxidase activity (38-40).

Extensive efforts have been made to identify domains involved in the multiple protein-protein interactions and conformational changes that take place during NADPH oxidase
activation. Nevertheless, the precise nature of the protein-protein interactions that occur during the different stages of the activation process and how these are affected by phosphorylation is still a matter of debate. In particular the C-terminal proline-rich region in p47phox has been variously suggested to bind to SH3 domains present in p67phox and p40phox, as well as to the tandem SH3 domains present within p47phox itself (21-23,25,41-45). Furthermore there are conflicting reports concerning the architecture of the p40-p47-p67phox complex in resting cells. In one scenario, p40phox acts as an adaptor that holds p47phox and p67phox together while an alternative model assumes that p67phox acts as a bridging molecule between p40phox and p47phox (1,29,39,41,44,46,47). A likely explanation for the detection of multiple binding partners for a particular region is that many previous studies have made use of isolated domains. It is possible that such an approach may exclude additional regions that contribute to specificity and/or affinity. In addition, many of the techniques used do not allow a precise quantitative assessment of the affinity of any particular interaction. For these reasons, the relative significance of one interaction with respect to another becomes difficult to assess. Nevertheless, it is possible that all interactions detected so far do take place at various stages of the activation process but occur in a sequential not a simultaneous manner.

Here we describe the thermodynamic and hydrodynamic characterisation of protein-protein complexes of the cytosolic components of the NADPH oxidase. Using a combination of isothermal titration calorimetry, analytical ultracentrifugation and gel filtration chromatography we demonstrate that p40phox, p47phox and p67phox form a trimeric protein complex with a 1:1:1 stoichiometry. Furthermore we show unequivocally that p67phox is the adaptor that links p40phox and p47phox in the resting complex, providing a solid framework upon which the activation process can be further investigated.
EXPERIMENTAL PROCEDURES

Protein Purification - The DNA sequences encoding full-length p67phox(1-526), p67phox(234-526) and p67phox(450-526) “p67-SH3B” were cloned into pGEX-4T1 (Amersham Biosciences) and fusion proteins were overexpressed in E.coli BL21. The cells were lysed in a buffer containing 50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 2 mM EDTA, 4 mM dithiothreitol, 4 mM benzamidine and 1 mM PMSF and the protein was purified on glutathione-Sepharose 4B beads (Amersham Biosciences). The GST-tag was removed using human thrombin (Calbiochem). Further purification was carried out on a Source Q anion-exchange column (Amersham Biosciences) equilibrated in 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA and 4 mM dithiothreitol. Proteins were eluted with a 50 mM to 1 M NaCl gradient in the same buffer. Fractions containing pure protein were pooled and concentrated by ultrafiltration to around 20 mg/ml.

DNA sequences encoding full-length p47phox(1-390), p47phox(1-295), p47phox(1-354) and p47phox(155-390) were cloned into pGEX-6P1 and fusion proteins were overexpressed in E.coli BL21. Cells were lysed in 50 mM Tris-HCl, pH 7.0, 300 mM NaCl, 2 mM EDTA, 4 mM dithiothreitol, 4 mM benzamidine and 1 mM PMSF. The protein was purified on glutathione-Sepharose 4B beads and the GST-tag was removed by incubation with PreScission Protease (Amersham Biosciences). Further purification was carried out on a Source S column equilibrated in 50 mM HEPES, pH 8.0, 50 mM NaCl, 1mM EDTA and 2 mM dithiothreitol, and proteins were eluted with a 50 mM-500 mM NaCl gradient in the same buffer. Fractions containing pure protein were pooled and concentrated by ultrafiltration to around 50 mg/ml.

p40phox cloned into pET-23d (Novagen) was a kind gift of Frans Wientjes, University College, London. The protein was overexpressed in E.coli BL21(DE3) and purified on NiNTA beads (Qiagen) equilibrated with 50 mM NaH2PO4, pH 8.0, 300 mM NaCl, 5mM
mercaptoethanol and 20 mM imidazole. P40phox was eluted with a 20 mM-1M imidazole gradient. Fractions containing pure protein were pooled, dialyzed against 2 l of 50 mM HEPES, pH 8.0, 50 mM NaCl and 2 mM DTT and concentrated by ultrafiltration to ~10 mg/ml.

All sequences were confirmed by nucleotide sequencing and electrospray mass spectrometry of the purified proteins. Protein concentrations were determined spectroscopically using calculated absorption coefficients.

Complexes of p67-p40phox, p67-p47phox and p67-p47-p40phox were obtained by mixing the proteins in 1:1 stoichiometries and purification by gel filtration on a Superdex 200 column (Amersham Biosciences) using a buffer containing 50 mM HEPES, pH 7.0, 100 mM NaCl and 2 mM DTT for p47-p67phox and p40-p47-p67phox and pH 8.0 for p40-p67phox. Fractions containing the complexes were concentrated by ultrafiltration to ~10 mg/ml.

Dynamic light scattering - Dynamic light scattering (DLS) measurements were performed on a DynaPro-801 dynamic light-scattering instrument (Protein Solution). Samples were filtered through 0.02 μm filters and experiments were performed at sample concentrations of 0.5 to 2 mg/ml in the same buffer used for analytical ultracentrifugation measurements. Data were analysed by autocorrelation and the resulting autocorrelation function was then evaluated using single exponential cumulant analysis. From this the translational diffusion coefficient was determined which can subsequently be used to derive the hydrodynamic radius and thereby the molecular weight using an empirically derived relationship between the radius and molecular weights for globular proteins. All calculations were carried out using the software package supplied with the machine.

Analytical ultracentrifugation - The protein partial specific volume, solvent density and viscosity were calculated using the SEDNTERP program (John Philo). Sedimentation equilibrium and velocity studies were carried out using a Beckman Optima XLA analytical
ultracentrifuge. Prior to centrifugation protein samples were dialysed exhaustively against a buffer containing 50 mM HEPES, 100 mM NaCl, 1 mM EDTA and 2 mM β-mercaptoethanol adjusted to the pH used for purification. Equilibrium experiments were carried out on 110 µl samples in an An-60ti rotor using six-channel centerpieces with protein absorbances of 0.8 OD, 0.5 OD and 0.3 OD at λ=280 nm and three different rotor speeds (p67phox at 12000, 15000 and 18000 rpm; p40phox and p47phox at 10000, 13000 and 18000 rpm; p40-p47phox, p40-p67phox, p47-p67phox at 7000, 8500 and 12500 rpm; p40-p47-p67phox at 6000, 8000 and 11000 rpm). Radial scans of the absorbance at 280 nm (pathlength 1.2 cm) were measured at 0.001 cm intervals, at 15°C and twenty-fold averaged. Scans indicating that equilibrium had been reached were used for analysis (typically around 20 hours). Multiple data sets were analysed by non-linear least-squares procedures provided in the Beckman Optima XL-A/XL-1 data analysis software, version 4.1.

Sedimentation velocity studies were carried out at protein concentrations between 0.2 and 1 mg/ml and sedimentation boundaries were monitored at λ=280 nm. The data were analysed using the program SVEDBERG which directly fits sedimentation velocity profiles to give the sedimentation (s) and diffusion coefficient (D) (48). Values obtained in this way were then extrapolated to zero concentration and corrected to standard conditions to yield s20,w and D20,w. Hydrodynamic shape parameters including frictional (ff/fo) and axial ratios (a/b) were calculated with the program Sednterp using the v-bar method.

Isothermal titration calorimetry - Isothermal calorimetric titrations were performed with a Microcal omega VP-ITC (MicroCal Inc., Northampton, USA). All proteins were dialysed against ITC-buffer (25 mM HEPES, 50 mM NaCl, 1 mM EDTA, 2 mM DTT; at the same pH used for purification), and experiments were performed at 15°C. Typically solutions of 10-20 µM of proteins or complexes in the cell were titrated by injection of a total of 290 µl of 100-200 µM of ligands (49). Heats of dilution of ligand into buffer were determined in
control experiments and subtracted from the raw data of the binding experiment prior to data analysis. Data were fitted by least-squares procedures using the evaluation software, Microcal Origin version 5.0 provided by the manufacturer. The data were averaged over two to five ITC experiments. Titrations which followed a two-site binding isotherm were additionally carried out in the presence of 2 mM TCEP ((tris(2-carboxyl)phosphine) in order to ensure that the second binding site detected was not an artifact caused by the use of DTT.

**Analytical gel filtration** - Analytical gel filtration chromatography was carried out on a Superdex 200 HR 10/30 column (Amersham Biosciences) equilibrated with 50 mM HEPES, pH 7.0, 100 mM NaCl and 2 mM MgCl₂ at a flow rate of 0.4 ml/min.

**RESULTS**

*Analytical ultracentrifugation studies*

The proteins used in this study have been overexpressed in *E.coli* in a soluble form. They are monodisperse, as judged by dynamic light scattering, and can be highly concentrated apart from p40phox, which shows a tendency to aggregate at higher concentrations. Similar constructs have been shown in previous studies to retain their biological activity as judged by cell-free NADPH oxidase reconstitution assays, indicating that the proteins are correctly folded upon expression in *E.coli* (9,12,27,33,37). Prior to studying complex formation between the cytosolic phox components, we investigated potential self-association of the individual proteins in sedimentation equilibrium experiments. Analysis of these data can be used to derive the true molecular weight, without making any assumptions about the form and shape of the molecule(s) under investigation. Nine data sets at three different speeds and three concentrations were collected for each protein. Global fitting of these data sets to a single species resulted in a random distribution of residuals indicating that all three proteins exist as monomers in solution (Fig. 2A). The molecular
weights determined for p40\textsuperscript{phox}, p47\textsuperscript{phox} and p67\textsuperscript{phox} are 41036 Da, 43892 Da, and 61621 Da, respectively, in good agreement with the formula molecular weights and those determined by electrospray mass spectrometry (39966 Da, 44963 Da, 60015 Da) (Table 1). P67\textsuperscript{phox} has previously been described as forming dimers based on gel filtration chromatography and small angle neutron scattering studies (45). To investigate if this discrepancy with our data might be due to a non-globular shape of the protein, we carried out sedimentation velocity studies to derive an estimate for the asymmetry of p67\textsuperscript{phox} (Table 2). The velocity data yielded a sedimentation coefficient \(s_{20,w} = 3.2 \times 10^{-13} \text{s}\) and diffusion coefficient \(d_{20,w} = 4.7 \times 10^{-7} \text{cm}^2\text{s}^{-1}\) which are lower than would be expected for a globular protein of 60 kDa (Table 2). The frictional ratio \(f/f_0\), which is an indication of the asymmetry of a protein, can be calculated from these data and has been found to be near 1.2 for globular proteins. The value calculated for p67\textsuperscript{phox}, however, is fairly high \(f/f_0 = 1.65\), indicating that the shape of p67\textsuperscript{phox} significantly deviates from that of a globular protein. Modeling these results as a prolate ellipsoid yields an axial ration \(a/b\) of 12.1, suggesting that p67\textsuperscript{phox} adopts an elongated shape in solution (Table 2). This conclusion is further corroborated by dynamic light scattering studies which showed p67\textsuperscript{phox} to be monodisperse (polydispersity indices 0.1-0.2) and yielded a translational diffusion coefficient of \(4.39 \times 10^{-7} \text{cm}^2\text{s}^{-1}\) which was independent of the salt concentration from 50 to 500 mM NaCl. This value would be consistent with a molecular weight of 120 kDa in the case of a protein that holds a standard globular shape and hydration, close to that of a p67\textsuperscript{phox} dimer. Taken together, these hydrodynamic studies suggest that p67\textsuperscript{phox} adopts an extended and potentially flexible structure thus explaining its apparent dimeric behaviour on gel filtration.

*Formation of dimeric complexes between the cytosolic components of the NADPH oxidase*
The p40-p67phox complex - Isothermal titration calorimetry (ITC) allows direct measurement of the equilibrium binding constant $K_a$ ($K_d=1/K_a$) and the enthalpy of complex formation ($\Delta H$) without the need for producing fusion proteins which can be attached to a solid surface, or the introduction of radioactive or spectroscopic labels. Titration of p40phox into p67phox was exothermic ($\Delta H = -5.4$ kcal/mole) and resulted in the formation of a very tight complex with a dissociation constant of 10 nM and a stoichiometry of 1:1 (Fig. 3A and Table 3). This affinity is in reasonable agreement with that previously obtained by surface plasmon resonance studies ($K_d = 43$ nM, (47)).

The p47-p67phox complex – The binding isotherm for the titration of p67phox with p47phox showed systematic deviations from a single-site binding model indicating that more than one binding event was taking place. Increasing the time between injections had no effect on this apparent biphasic behaviour. A model assuming two independent binding sites resulted in a good fit and yielded dissociation constants of $K_{d1} = 20$ nM and $K_{d2} = 150$ nM and reaction enthalpies of $\Delta H_1 = -8.9$ kcal/mole and $\Delta H_2 = -4.6$ kcal/mole, respectively (Fig. 3B and Table 3). Only the high affinity binding site was fully occupied while the second binding site exhibited a stoichiometry of 1:0.25 (±0.1). The occurrence of a second binding site was rather surprising especially considering the low occupancy of this site. To investigate if p67phox might bind more than one molecule of p47phox we carried out gel filtration analysis of complexes between p47phox and p67phox at varying stoichiometries. Figure 4 shows that a 1:1 mixture elutes as a single species with a retention time that is shorter than that of the individual proteins. In contrast, mixtures containing a molar excess of p47phox or p67phox elute as two species where the second species corresponds to the excess protein (data not shown). Since the ITC data indicated that the second binding site is only between 15 and 30 % occupied we tested if an excess of as little as 10, 20 or 30 % of p47phox might be incorporated into a p47-p67phox complex. As is shown in Fig.4 even these small amounts resulted in the
appearance of an additional p47phox peak, clearly indicating that p47phox and p67phox exist in a stoichiometric 1:1 heterodimeric complex. This conclusion is further corroborated by sedimentation equilibrium studies of a 1:1 complex of p47-p67phox. Fitting of these data to a single species model resulted in a good fit, with residuals which were randomly distributed about the zero value, indicating that the complex is a monodisperse species. The molecular weight calculated from this fit was 105160 Da, which is in very good agreement with its theoretically calculated molecular weight of 104978 Da (Fig. 2B and Table 1). Taken together these observations suggest to us, that the second binding event that we have detected in the p47-p67phox complex is not due to the binding of an additional molecule of p47phox but must be an intrinsic feature of a stoichiometric p47-p67phox complex (see Discussion).

The p40-p47phox complex - P40phox has been proposed to be the adaptor which links p47phox to p67phox in the cytoplasmic complex (41,44,45). However, titration of p40phox with p47phox did not result in any significant heat change suggesting that the two proteins either do not or only weakly interact, that the heat capacity of complex formation $\Delta C_p$ is such that $\Delta H$ is very small at the experimental temperature and/or that the interaction is mainly entropy driven. In order to discriminate between these possible explanations, further studies were carried out using a variety of techniques. In surface plasmon resonance experiments using the BIAcore system p40phox was directly coupled to a Ni-NTA chip via its C-terminal hexahistidine tag and binding of p47phox was monitored. Although there was clear net binding of p47phox to p40phox the data were not of sufficient quality to be able to determine an equilibrium constant from kinetic ‘on’ and ‘off’ rates or equilibrium binding analysis (data not shown). This poor data quality is likely due to the fact that the interaction of the hexahistidine tag of p40phox with the Ni-NTA chip does not seem to be sufficiently tight, resulting in the protein being partially washed off the chip during the experiment. Sedimentation equilibrium runs of a 1:1 mixture of p40phox and p47phox and fitting of the data
to a single species model yielded a molecular weight of 70842 Da providing further evidence that the two proteins interact in solution. However, analysis of these data assuming a monomer-dimer equilibrium is complicated by the fact that the two proteins have different extinction coefficients at 280 nm (ε=39400 M$^{-1}$cm$^{-1}$ for p40phox and ε=57750 M$^{-1}$cm$^{-1}$ for p47phox), which makes it impossible to correctly convert the association constant which is calculated in absorbance units into molar concentrations. Nevertheless, as the molecular weights of the two proteins are very close and there is only a 1.4-fold difference in extinction coefficients, we assumed a mean value of ε$_{280}$=48575 M$^{-1}$cm$^{-1}$ per monomer to estimate a lower limit of 4 μM for the dissociation constant. This value is in very good agreement with a previous estimation for the affinity of this complex which has been made based on small angle neutron scattering studies (45).

**Formation of the trimeric p40-p47-p67phox complex**

To investigate whether the occurrence of a two-site binding isotherm upon interaction of p67phox with p47phox is a particular feature of this complex or whether it also occurs if p67phox is already complexed with p40phox, we titrated a purified p40-p67phox complex with p47phox. Data from this titration could, again, only be fitted to a two-site binding model with dissociation constants of 14 nM and 128 nM and reaction enthalpies of –7.5 kcal/mole and –4.6 kcal/mole, respectively (Fig. 3C). As observed before, the second binding site exhibited a stoichiometry of 1:0.25(±0.1). The similarity between results from this titration and that of p67phox with p47phox suggests that complex formation between p47phox and p67phox takes place independently of the presence of p40phox. In addition it confirms that p40phox and p47phox do not share a common binding site on p67phox. As observed for the p47-p67phox complex, a 1:1:1 mixture of p40phox, p47phox and p67phox elutes as a single species on gel filtration and addition of an excess of any of the components results in the appearance of an additional peak (data
not shown). Sedimentation equilibrium analysis of a 1:1:1 complex showed that it behaves as a single, monodisperse species with a molecular weight of 144546 Da in good agreement with its formula molecular weight of 144944 Da (Fig. 2C). DLS measurements of this complex confirmed its monodispersity (polydispersity index 0.3), however, a diffusion coefficient of $3.44 \times 10^{-7}$ cm$^2$s$^{-1}$ determined by single exponential cumulant analysis which would be consistent with a molecular weight of 240 kDa in the case of a globular protein indicates that the shape of this complex deviates significantly from that of a globular protein. This observation is substantiated by sedimentation velocity studies from which a frictional ratio $f/f_0 = 2.02$ and an axial ratio $a/b = 20.6$ were calculated (Table 2). These values are comparatively high for a protein but can be rationalised by the fact that all three components of this complex are multidomain proteins made up of individual domains that are connected by linkers which are likely to be rather flexible. Thus resulting in a heterotrimeric complex which adopts a highly elongated shape due to the asymmetry of one of its components ($p67^{\text{phox}}$) and due to its flexible organisation.

**Protein-protein interactions in the p47-$p67^{\text{phox}}$ complex**

To further characterise the structural features of complex formation between p47$^{\text{phox}}$ and p67$^{\text{phox}}$ and to understand the reason for the occurrence of a biphasic binding isotherm, we have produced various N- and C-terminally truncated p47$^{\text{phox}}$ and p67$^{\text{phox}}$ fragments and assessed their behaviour in ITC titrations in comparison to the full-length proteins.

The proline rich region in the C-terminus of p47$^{\text{phox}}$ has invariably been suggested to be the target for the SH3 domain in p40$^{\text{phox}}$ as well as C-terminal SH3 domain in p67$^{\text{phox}}$. To investigate if the tight, stoichiometric binding we detect in titrations of p67$^{\text{phox}}$ with p47$^{\text{phox}}$ is due to this interaction we measured complex formation between the isolated C-terminal SH3 domain of p67$^{\text{phox}}$ and full-length p47$^{\text{phox}}$ by ITC. This titration follows a single site binding
isotherm with a 1:1 stoichiometry as would be expected for complex formation between an SH3 domain and its proline rich target. Binding occurred with an affinity of 21 nM and a reaction enthalpy of $-7.4 \text{ kcal/mole}$, indicating that this interaction indeed constitutes the high affinity binding site detected in titrations using the full-length proteins. This conclusion is further substantiated by the fact that removal of the proline rich region (p47\text{phox} 1-354) completely abrogated complex formation. Interestingly, further C-terminal truncation of p47\text{phox} (fragment 1-295) to remove the polybasic region, which is thought to be responsible for the intramolecular masking of the tandem SH3 domains, restored binding and resulted in the formation of a 1:1 complex with a dissociation constant of 6 \text{ \mu M}. This interaction results from a favourable enthalpy change which is of similar size to that observed for the interaction between full-length p47\text{phox} and p67\text{phox}. However, in contrast to that interaction the entropy change exhibited here is unfavourable resulting in a 300-fold lower affinity (Table 3).

To investigate if the N-terminal region in p67\text{phox} including the TPR domain and the proline rich region around amino acids 219-234 might contribute to the apparent biphasic behaviour of p47-p67\text{phox} complex formation we deleted this region (p67\text{phox} 234-end) and measured binding to full-length p47\text{phox} by ITC. This construct behaved almost identically to full-length p67\text{phox}. The titration had to be fitted to a two-site binding model and exhibited a tight, stoichiometric binding site with a K_d of 8 nM and $\Delta \text{H}$ of $-8.8 \text{ kcal/mole}$ while the second binding event again showed an apparent stoichiometry of 1:0.25(±0.1). Removal of the N-terminal portion of p47\text{phox}, including the PX-domain (p47\text{phox} 155-end) had a similar effect, resulting in a two-site binding isotherm with a K_d of 12 nM and reaction enthalpy of $-9.8 \text{ kcal/mole}$ for the first site and a low stoichiometry occupancy for the second. The similarity of these titrations with those of full-length p47\text{phox} and p67\text{phox} but also p67-SH3_B suggests to us that tight, stoichiometric complex formation between p67-SH3_B and the
The NADPH oxidase is a multi-protein enzyme whose activity is regulated by the reversible formation of multiple protein-protein interactions between the cytosolic and membrane components as well as between the cytosolic proteins themselves. Many of these interactions involve SH3 domains and their target proline-rich sequences, which are present in p22phox, p40phox, p47phox and p67phox. Activation of cells by appropriate stimuli leads to phosphorylation of p47phox, which in turn induces intra- and intermolecular rearrangements within a number of these protein-protein interactions. This study was aimed at carrying out a detailed quantitative characterisation of the protein-protein interactions which occur in the resting state of the NADPH oxidase in order to establish a basis upon which the activation process can be investigated.

P40phox, p47phox and p67phox exist as a tight complex in the cytosol of resting neutrophils from which it can be purified by gel filtration with an apparent molecular weight of 250-300 kDa, twice that expected for a complex containing one copy of each of the proteins. Sedimentation equilibrium studies presented here show that the individual proteins exist as monomers, which associate to form a monodisperse, 1:1:1 complex. Addition of an excess of any of the individual proteins to this complex does not result in additional binding. The shape of this complex significantly deviates from that of a globular molecule as evidenced by dynamic light scattering as well as sedimentation velocity studies from which a fractional coefficient of 2.02 was derived. Thus the large molecular weight observed by gel filtration is not due to the existence of multiple copies of any of the proteins in this complex but is rather due to an extended shape and possibly flexible organisation of the heterotrimer.
Extensive studies have been carried out to characterise the domains which mediate the protein-protein interactions within the p40-p47-p67phox complex but in spite of this wealth of data it is still unclear at which stage during the activation process a particular interaction occurs. Our data show that p40phox and p67phox form a very tight complex with a dissociation constant in the low nanomolar range. This interaction has been previously mapped to a region between the two SH3 domains in p67phox, which contains the recently identified BP1 domain and its target sequence, the PC motif, which resides in the C-terminal part of p40phox (50,51). P40phox has often been described as the primary binding partner for p47phox in the resting state due to an interaction of its SH3 domain with the Pro-rich region in the C-terminus of p47phox. Consequently many models for NADPH oxidase architecture assume that p40phox is the link between p47phox and p67phox. However, this Pro-rich region in p47phox has also been described to bind to p67phox and possibly, in an intramolecular fashion, to its own tandem SH3 domains. Since a single, short proline-rich motif can only bind to a single SH3 domain at any one time we carried out ITC studies in order to determine the affinities of the individual oxidase components for one another. We show that the affinity of p40phox for p47phox is comparatively low, in the micromolar range, in accordance with neutron scattering data which estimated the affinity to be around 4.0 µM (45). In contrast, p47phox binds to p67phox with nanomolar affinity. To our surprise this titration did not follow a single site binding isotherm but could only be fitted assuming two non-identical sites. In spite of this behaviour, sedimentation equilibrium analyses and gel filtration chromatography of p47phox and p67phox at various ratios showed convincingly that p47phox and p67phox form a 1:1 complex. This clearly indicates that the second binding event detected by ITC does not reflect an additional binding site. Interestingly, binding of p47phox to a pre-formed complex of p40-p67phox exhibited a similar behaviour. The agreement, within experimental error, between the K_d’s and ΔH’s for the stochiometric binding site determined in these two experiments implies that the presence of
p40phox has no influence on complex formation between p47phox and p67phox in the resting state. By carrying out ITC titrations using a C-terminally truncated p47phox as well as the isolated C-terminal SH3 domain of p67phox we could show that the tight interaction that we detected between full-length p47phox and p67phox is due to binding of the C-terminal Pro-X-X-Pro motif in p47phox to p67-SH3B. Taken together, these results clearly demonstrate that p40phox can not be the link between p47phox and p67phox in the resting state as the affinity of p67phox for the Pro-rich region in p47phox is about 1000-fold higher than that of p40phox.

Our ITC data for complex formation between full-length p47phox and p67phox indicated that a second process is taking place in addition to interaction of the two proteins via their respective C-termini. This process has no influence on the C-terminal SH3 domain-proline rich region interaction. ITC titrations using N-terminally truncated versions of either protein indicated that neither the PX-domain of p47phox nor the TPR domain of p67phox is responsible for this process. At present we are not able to explain the reason for this second event and can only conclude that it seems to require the presence of the tandem SH3 domains of p47phox that are masked by an intramolecular interaction with the subsequent polybasic region (24,25) as well as the C-terminal half of p67phox. Further studies are currently underway to explore this phenomenon.

Based on the data presented here combined with data from other reports we suggest the following model for the NADPH oxidase architecture in the resting state. P67phox is the bridge which connects p40phox and p47phox. No direct interaction takes place in this complex between p40phox and p47phox. Furthermore, association of p40phox with p67phox appears not to induce a conformational change in the p47phox-binding site of p67phox as binding of the latter to the C-terminal Pro-rich region of p47phox follows a similar behaviour in the absence or presence of p40phox. P47phox exists in an auto-inhibited conformation in this complex in which its SH3 domains are masked by intramolecular interactions with a polybasic region in the C-
terminal portion of the protein and possibly with the PX domain. Upon activation phosphorylation induces a conformational change in p47$^{\text{phox}}$ which unmasks the tandem SH3 domains, thereby allowing binding to p22$^{\text{phox}}$ and translocation to the membrane. Babior et al. (26) have shown that phosphorylation of Ser-359 or Ser-370 is absolutely required for translocation as well as oxidase activity. Interestingly both of these serines are adjacent to the poly-proline motif which is the target of the C-terminal SH3 domain of p67$^{\text{phox}}$, suggesting that phosphorylation might interfere with this interaction and may potentially induce the release of p67$^{\text{phox}}$ rendering this sequence accessible for p40$^{\text{phox}}$. Our data show that a second binding site exists between p47$^{\text{phox}}$ and p67$^{\text{phox}}$ that is only accessible once the tandem SH3 domains of p47$^{\text{phox}}$ have been unmasked. This site might become the primary interface between the two proteins if phosphorylation is capable of disrupting the ‘end-to-end’ p47-p67$^{\text{phox}}$ interaction. Further studies are now needed to describe in molecular detail the rearrangements which take place during the activation and assembly process.

ACKNOWLEDGMENTS

We thank Michael B. Yaffe (MIT, Harvard) for clones and plasmids and Frans Wientjes (UCL, London) for p40$^{\text{phox}}$ in pET23. We are grateful to Steve Howell and Lesley Haire (NIMR) for mass spectrometry, John Eccleston for initial help with analytical ultracentrifugation and Steve Smerdon, John Eccleston and Ian Taylor for helpful discussions and critical reading of the manuscript.

REFERENCES

1. Babior, B. M. (1999) Blood 93(5), 1464-76
2. Clark, R. A. (1999) *J Infect Dis* **179**(Suppl 2), S309-17.

3. Nauseef, W. M. (1999) *Proc Assoc Am Physicians* **111**(5), 373-82.

4. McPhail, L. C. (1994) *J Exp Med* **180**(6), 2011-5.

5. Park, J. W., Benna, J. E., Scott, K. E., Christensen, B. L., Chanock, S. J., and Babior, B. M. (1994) *Biochemistry* **33**(10), 2907-11.

6. Park, J. W., Ma, M., Ruedi, J. M., Smith, R. M., and Babior, B. M. (1992) *J Biol Chem* **267**(24), 17327-32.

7. Wientjes, F. B., Hsuan, J. J., Totty, N. F., and Segal, A. W. (1993) *Biochem J* **296**(Pt 3), 557-61.

8. Someya, A., Nagaoka, I., and Yamashita, T. (1993) *FEBS Lett* **330**(2), 215-8.

9. Shiose, A., and Sumimoto, H. (2000) *J Biol Chem* **275**(18), 13793-801.

10. Huang, J., and Kleinberg, M. E. (1999) *J Biol Chem* **274**(28), 19731-7.

11. Swain, S. D., Helgerson, S. L., Davis, A. R., Nelson, L. K., and Quinn, M. T. (1997) *J Biol Chem* **272**(47), 29502-10.

12. Ago, T., Nunoi, H., Ito, T., and Sumimoto, H. (1999) *J Biol Chem* **274**(47), 33644-53.

13. El Benna, J., Faust, R. P., Johnson, J. L., and Babior, B. M. (1996) *J Biol Chem* **271**(11), 6374-8.

14. Park, J. W., and Babior, B. M. (1997) *Biochemistry* **36**(24), 7474-80.

15. Ellson, C. D., Gobert-Gosse, S., Anderson, K. E., Davidson, K., Erdjument-Bromage, H., Tempst, P., Thuring, J. W., Cooper, M. A., Lim, Z. Y., Holmes, A. B., Gaffney, P. R., Coadwell, J., Chilvers, E. R., Hawkins, P. T., and Stephens, L. R. (2001) *Nat Cell Biol* **3**(7), 679-82.

16. Kanai, F., Liu, H., Field, S. J., Akbary, H., Matsuo, T., Brown, G. E., Cantley, L. C., and Yaffe, M. B. (2001) *Nat Cell Biol* **3**(7), 675-8.
17. Cheever, M. L., Sato, T. K., de Beer, T., Kutateladze, T. G., Emr, S. D., and Overduin, M. (2001) Nat Cell Biol 3(7), 613-8.
18. Xu, Y., Hortsman, H., Seet, L., Wong, S. H., and Hong, W. (2001) Nat Cell Biol 3(7), 658-66.
19. Ponting, C. P. (1996) Protein Sci 5(11), 2353-7
20. Hiroaki, H., Ago, T., Ito, T., Sumimoto, H., and Kohda, D. (2001) Nat Struct Biol 8(6), 526-30.
21. de Mendez, I., Homayounpour, N., and Leto, T. L. (1997) Mol Cell Biol 17(4), 2177-85
22. de Mendez, I., Adams, A. G., Sokolic, R. A., Malech, H. L., and Leto, T. L. (1996) Embo J 15(6), 1211-20
23. Sumimoto, H., Hata, K., Mizuki, K., Ito, T., Kage, Y., Sakaki, Y., Fukumaki, Y., Nakamura, M., and Takeshige, K. (1996) J Biol Chem 271(36), 22152-8
24. Sumimoto, H., Kage, Y., Nuno, H., Sasaki, H., Nose, T., Fukumaki, Y., Ohno, M., Minakami, S., and Takeshige, K. (1994) Proc Natl Acad Sci U S A 91(12), 5345-9
25. Leto, T. L., Adams, A. G., and de Mendez, I. (1994) Proc Natl Acad Sci U S A 91(22), 10650-4
26. Johnson, J. L., Park, J. W., Benna, J. E., Faust, L. P., Inanami, O., and Babior, B. M. (1998) J Biol Chem 273(52), 35147-52
27. Hata, K., Ito, T., Takeshige, K., and Sumimoto, H. (1998) J Biol Chem 273(7), 4232-6
28. Delo, F. R., Nauseef, W. M., Jesaitis, A. J., Burritt, J. B., Clark, R. A., and Quinn, M. T. (1995) J Biol Chem 270(44), 26246-51.
29. Dusi, S., Donini, M., and Rossi, F. (1996) Biochem J 314(Pt 2), 409-12
30. Heyworth, P. G., Curnutte, J. T., Nauseef, W. M., Volpp, B. D., Pearson, D. W., Rosen, H., and Clark, R. A. (1991) J Clin Invest 87(1), 352-6
31. Uhlinger, D. J., Tyagi, S. R., Inge, K. L., and Lambeth, J. D. (1993) J Biol Chem 268(12), 8624-31.
32. Diekmann, D., Abo, A., Johnston, C., Segal, A. W., and Hall, A. (1994) Science 265(5171), 531-3.
33. Han, C. H., Freeman, J. L., Lee, T., Motalebi, S. A., and Lambeth, J. D. (1998) J Biol Chem 273(27), 16663-8.
34. Koga, H., Terasawa, H., Nunoi, H., Takeshige, K., Inagaki, F., and Sumimoto, H. (1999) J Biol Chem 274(35), 25051-60.
35. Koshkin, V., Lotan, O., and Pick, E. (1996) J Biol Chem 271(48), 30326-9.
36. Dang, P. M., Cross, A. R., and Babior, B. M. (2001) Proc Natl Acad Sci U S A 98(6), 3001-5.
37. Diebold, B. A., and Bokoch, G. M. (2001) Nat Immunol 2(3), 211-5.
38. Cross, A. R. (2000) Biochem J 349(Pt 1), 113-7.
39. Sathyamoorthy, M., de Mendez, I., Adams, A. G., and Leto, T. L. (1997) J Biol Chem 272(14), 9141-6.
40. Tsunawaki, S., Kagara, S., Yoshikawa, K., Yoshida, L. S., Kuratsuji, T., and Namiki, H. (1996) J Exp Med 184(3), 893-902.
41. Fuchs, A., Dagher, M. C., Faure, J., and Vignais, P. V. (1996) Biochim Biophys Acta 1312(1), 39-47.
42. Finan, P., Shimizu, Y., Gout, I., Hsuan, J., Truong, O., Butcher, C., Bennett, P., Waterfield, M. D., and Kellie, S. (1994) J Biol Chem 269(19), 13752-5.
43. Ito, T., Nakamura, R., Sumimoto, H., Takeshige, K., and Sakaki, Y. (1996) FEBS Lett 385(3), 229-32.
44. Wilson, L., Butcher, C., Finan, P., and Kellie, S. (1997) Inflamm Res 46(7), 265-71.
45. Grizot, S., Grandvaux, N., Fieschi, F., Faure, J., Massenet, C., Andrieu, J. P., Fuchs, A.,
    Vignais, P. V., Timmins, P. A., Dagher, M. C., and Pebay-Peyroula, E. (2001)
    *Biochemistry* **40**(10), 3127-33.
46. Leusen, J. H., Verhoeven, A. J., and Roos, D. (1996) *J Lab Clin Med* **128**(5), 461-76
47. Wientjes, F. B., Panayotou, G., Reeves, E., and Segal, A. W. (1996) *Biochem J* **317**(Pt 3), 919-24
48. Philo, J. S. (1997) *Biophys J* **72**(1), 435-44.
49. Wiseman, T., Williston, S., Brandts, J. F., and Lin, L. N. (1989) *Anal Biochem* **179**(1),
    131-7
50. Ito, T., Matsui, Y., Ago, T., Ota, K., and Sumimoto, H. (2001) *Embo J* **20**(15), 3938-46.
51. Nakamura, R., Sumimoto, H., Mizuki, K., Hata, K., Ago, T., Kitajima, S.,
    Takeshige, K., Sakaki, Y., and Ito, T. (1998) *Eur J Biochem* **251**(3), 583-9.
FIGURE LEGENDS

FIG. 1. Schematic showing the domain structure of the cytosolic components of the NADPH oxidase. The borders of the individual domains have been taken from the Pfam and the Prosite databases. Shown are the sizes of the full-length and truncated proteins used in this study.

FIG. 2. Determination of solution molecular masses by sedimentation equilibrium. The lower panels show the equilibrium data and the best fits (continuous curves). The upper panel shows the residuals plotted versus radial position. A, Equilibrium distribution of p67phox at a loading concentration of 7.5 µM, rotor speed 18,000 rpm, 15°C. B, Equilibrium distribution of p47-p67phox at a loading concentration of 2.4 µM, rotor speed 12,500 rpm, 15°C. C, Equilibrium distribution of p40-p47-p67phox at a loading concentration of 1.8 µM, rotor speed 11,000 rpm, 15°C.

FIG. 3. Isothermal titration calorimetry measurements of complex formation between phox proteins. A. Titration of 100 µM p67phox into 11 µM p40phox. The integrated heats from which the heat of dilution has been subtracted are shown as well as the fit to a single site binding isotherm which yielded $K_d = 10$ nM and $\Delta H = -5.4$ kcal mol$^{-1}$. Titration of B. 204 µM p47phox into 21 µM p67phox and C. 130 µM p47phox into 13 µM p40-p67phox. The raw data of the titration of p47phox into p67phox are shown in the top panel of B. The integrated heats from which the heat of dilution has been subtracted are shown in the other panels. Data shown in B. and C. could not be fitted to a single site binding model but had to be fitted to a two site binding function. The dotted line in the lower panel in B. shows a fit to a single site binding model, clearly indicating systematic deviations. Analysis of the
data yielded B. \( K_{d1} = 20 \text{ nM}, \Delta H_1 = -8.9 \text{ kal/mole}, N = 1.0 \pm 0.15 \) and \( K_{d2} = 150 \text{ nM}, \Delta H_2 = -4.6 \text{ kal/mole}, N = 0.25 \pm 0.1 \); C. \( K_{d1} = 14 \text{ nM}, \Delta H_1 = -7.5 \text{ kcal/mole}, N = 1.0 \pm 0.1 \) and \( K_{d2} = 128 \text{ nM}, \Delta H_2 = -4.6 \text{ kal/mole}, N = 0.25 \pm 0.1 \).

FIG. 4. Analytical gel filtration of \( \text{p47}^{\text{phox}} \) and \( \text{p67}^{\text{phox}} \) and complexes thereof. Gel filtration was performed in 50 mM Hepes, pH 7.0, 100 mM NaCl and 2 mM MgCl\(_2\) at a flow rate of 0.4 ml/min and protein concentrations of 50 \( \mu \text{M} \). The solid line (-) shows the elution profile of the individual \( \text{p47}^{\text{phox}} \) and \( \text{p67}^{\text{phox}} \) proteins as well as a 1:1 \( \text{p47}/\text{p67}^{\text{phox}} \) complex. The dotted lines show the elution profiles of a 1:1 mixture of \( \text{p47}^{\text{phox}} \) and \( \text{p67}^{\text{phox}} \) plus an excess of (---) 10 \% \( \text{p47}^{\text{phox}} \), (\ldots) 20 \% \( \text{p47}^{\text{phox}} \) and (--) 30 \% \( \text{p47}^{\text{phox}} \). Inset: Fractions eluting at 29.5 min, 33 min and 36.5 min were separated on a 12 \% SDS-polyacrylamide gel and stained with Coomassie brilliant blue.

FIG. 5. Model for the \( \text{p40-p47-p67}^{\text{phox}} \) complex in the resting state of the NADPH oxidase. Domains are indicated by the same symbols as used in FIG.1. \( \text{p47}^{\text{phox}} \) is shown in its autoinhibited form in which the polybasic region and possibly the PX domain interact with the tandem SH3 domains. The sites of protein-protein interactions are indicated by arrows. The positions of Ser-359 and Ser-370 in \( \text{p47}^{\text{phox}} \) are highlighted.
TABLE 1

*Molecular weights determined from sedimentation equilibrium experiments*

All measurements were carried out at three different loading concentrations and three different speeds as described in Experimental Procedures and fitted simultaneously. Standard deviations were calculated from fits to individual data sets. The theoretical molecular weights were calculated from the amino acid sequences, taking into account the amino acids that are left at the N-terminus after cleavage with thrombin or PreScission protease. All listed values of $M_{\text{app}}$ represent values obtained from fits of the data to ideal, single-component models.

| Protein                  | MW (Da)       | Theoretical MW (Da) |
|-------------------------|---------------|---------------------|
| p67phox                 | 61621 ± 2800  | 60015               |
| p47phox                 | 43892 ± 2600  | 44963               |
| p40phox                 | 41036 ± 3700  | 39966               |
| p67phox-p47phox         | 105160 ± 3700 | 104978              |
| p67phox-p40phox         | 107550 ± 10000 | 99981               |
| p67phox-p47phox-p40phox | 144546 ± 3500 | 144944              |
| p47phox-p40phox         | 70842 ± 2500  | 84929               |
TABLE 2

Hydrodynamic shape parameters of p67\textsuperscript{phox} and the p40-p47-p67\textsuperscript{phox} complex

Conditions for sedimentation velocity and dynamic light scattering (DLS) measurements are described in Experimental Procedures. \(S_{20,w}^0\) and \(D_{20,w}^0\) are the sedimentation coefficients and translational diffusion coefficients extrapolated to zero concentration and corrected to standard conditions. \(f/f_0\) is the fractional ratio derived from \(S_{20,w}^0\). \(a/b\) is the axial ratio calculated assuming a prolate ellipsoid model.

|                       | p67\textsuperscript{phox} | p40-p47-p67\textsuperscript{phox} |
|-----------------------|-----------------------------|-----------------------------------|
| \(S_{20,w}^0\) (Svedbergs) | 3.23                        | 4.60                              |
| \(D_{20,w}^0\) (x10\textsuperscript{-7}) cm\textsuperscript{2}s\textsuperscript{-1} | 4.70                        | 3.30                              |
| \(D^0\) (DLS) (x10\textsuperscript{-7}) cm\textsuperscript{2}s\textsuperscript{-1} | 4.39                        | 3.44                              |
| \(f/f_0\)              | 1.65                        | 2.02                              |
| \(a/b\)                | 12.10                       | 20.60                             |
TABLE 3

Thermodynamic parameters of complex formation between the cytosolic components of the NADPH oxidase

All measurements were performed in 25 mM HEPES, 50 mM NaCl, 1 mM EDTA, 2 mM DTT at pH 7.0, 7.5 or 8.0, depending on the pIs of the individual proteins and complexes formed. TΔS was calculated from the measured values of K\text{a} and ΔH. The stoichiometry of complex formation N is 1.0 ± 0.15 for titrations which were fitted to a single site binding model and N\textsubscript{1} = 1.0 ± 0.15 and N\textsubscript{2} = 0.25 ± 0.1 for titrations which had to be fitted to a two site binding model. The errors reflect standard deviations for repeated titrations.

| titration                          | K\textsubscript{a\textsubscript{1}} (x10\textsuperscript{7}M\textsuperscript{-1}) | ΔH\textsubscript{1} (kcal/mole) | TΔS\textsubscript{1} (kcal/mole) | K\textsubscript{a\textsubscript{2}} (x10\textsuperscript{7}M\textsuperscript{-1}) | ΔH\textsubscript{2} (kcal/mole) |
|-----------------------------------|-------------------------------|-------------------------------|-----------------------------|-------------------------------|-------------------------------|
| p47\textsuperscript{phox} + p67\textsuperscript{phox} | 5 ± 3                          | -8.9 ± 0.9                    | 1.2                         | 0.67 ± 0.2                   | -4.6 ± 1                      |
| p67-p40\textsuperscript{phox} + p47\textsuperscript{phox} | 7.3 ± 1                        | -7.5 ± 0.3                    | 2.8                         | 0.78 ± 0.08                  | -4.6 ± 0.2                    |
| p47\textsuperscript{phox} (155-end) + p67\textsuperscript{phox} | 8 ± 1                          | -9.8 ± 1.2                    | 0.6                         | 1.14 ± 0.5                   | -6.6 ± 1.1                    |
| p47\textsuperscript{phox} + p67\textsuperscript{phox} (234-end) | 12.5 ± 3                       | -8.8 ± 0.4                    | 1.9                         | 1.1 ± 0.16                   | -4.8 ± 1.1                    |
| p47\textsuperscript{phox} + p67\textsuperscript{phox} (450-526) | 4.8 ± 1.6                      | -7.4 ± 0.2                    | 2.7                         |                               |                               |
| p67\textsuperscript{phox} + p40\textsuperscript{phox} | 10 ± 2.7                       | -5.4 ± 0.4                    | 5.1                         |                               |                               |
| p47\textsuperscript{phox} (1-295) + p67\textsuperscript{phox} | 0.017 ± 0.006                  | -8.8 ± 0.9                    | -1.9                        |                               |                               |
Figure 1
Figure 2

(A) Absorbance at 280 nm vs. Radius (cm)

(B) Absorbance at 280 nm vs. Radius (cm)

(C) Absorbance at 280 nm vs. Radius (cm)

p67^{phox}

p67^{phox} - p47^{phox}

p67^{phox} - p47^{phox} - p40^{phox}
Figure 3
Figure 4
Figure 5

The diagram illustrates the interaction between p67^phox^ and p40^phox^. The p67^phox^ protein contains TPR, SH3, and PB1 domains, while p40^phox^ contains PX, SH3, and PC domains. The diagram shows the P-rich regions and the interactions at 10 nM and 20 nM concentrations.
Architecture of the p40-p47-p67<sub>phox</sub> complex in the resting state of the NADPH oxidase: a central role for p67<sub>phox</sub>

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*J. Biol. Chem.* published online January 16, 2002

Access the most updated version of this article at doi: [10.1074/jbc.M112065200](http://doi.org/10.1074/jbc.M112065200)

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