*J. Vet. Sci.* (2000), **1**(1), 19–26

JOURNAL OF
Veterinary
Science

# Expression and characterization of the flavoprotein domain of gp91$^{phox}$

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**Truncated forms of gp91$^{phox}$ were expressed in *E. coli* in which the N-terminal hydrophobic transmembrane region was replaced with a portion of the highly soluble bacterial protein thioredoxin (TRX). TRX-gp91$^{phox}$ (306-569), which contains the putative FAD and NADPH binding sites, showed NADPH-dependent NBT (nitroblue tetrazolium) reductase activity, whereas TRX-gp91$^{phox}$ (304-423) and TRX-gp91$^{phox}$ (424-569) were inactive. Activity saturated at about a 1:1 molar ratio of FAD to TRX-gp91$^{phox}$ (306-569), and showed the same Km for NADPH as that for superoxide generating activity by the intact enzyme. Activity was not inhibited by superoxide dismutase, indicating that it was not mediated by superoxide, but was blocked by an inhibitor of the respiratory burst oxidase, diphenylene iodonium (DPI). In the presence of Rac1, the cytosolic regulatory protein p67$^{phox}$ stimulated the NBT reductase activity, but p47$^{phox}$ had no effect. Truncated p67$^{phox}$ containing the activation domain (residues 199-210) stimulated activity approximately 2-fold, whereas forms mutated or lacking this region failed to stimulate the activity. Our data indicate that: 1) TRX-gp91$^{phox}$ (306-569) contains the binding sites for both pyridine and flavin nucleotides; 2) this flavoprotein domain shows NBT reductase activity; and 3) the flavin-binding domain of gp91$^{phox}$ is the target of regulation by the activation domain of p67$^{phox}$.**

**Key words:** gp91$^{phox}$; FAD and NADPH binding sites; NBT reductase activity.

## Introduction

Neutrophils and macrophages produce superoxide ($O_2^-$) and secondary reactive oxygen species ($H_2O_2$, HOCl) that participate in killing of phagocytized microorganisms [1, 7, 44, 5]. Superoxide generation is catalyzed by a multicomponent enzyme, the respiratory burst oxidase or NADPH oxidase. The catalytic moiety is a plasma membrane associated flavocytochrome $b_{558}$ which is composed of two subunits gp91$^{phox}$ and p22$^{phox}$ [29, 35, 45, 40, 21]. The flavocytochrome is inactive in resting cells, but upon cell stimulation, the flavocytochrome is activated by assembly with the cytosolic regulatory proteins p47$^{phox}$ [6, 11, 19, 50] and Rac (Rac2 and/or Rac1) [36, 20, 13].

The large subunit of the flavocytochrome, gp91$^{phox}$ has a highly hydrophobic N-terminus which is predicted to contain 5~6 transmembrane helices [41, 12, 49]. The flavocytochrome contains two hemes [31, 8], which reside solely in gp91$^{phox}$ [54] as well as a single FAD [31, 23,1 4]. Various models [8, 54] suggest that the heme groups both reside within the hydrophobic N-terminal half of the molecule, and specific histidines within this region have been suggested as heme ligands. The relatively hydrophilic C-terminal half of gp91$^{phox}$ is homologous to several flavoprotein dehydrogenases, particularly in putative FAD and NADPH binding sequences [39, 43, 47] (Fig. 1), and is therefore predicted to form an independently folding flavoprotein domain. Direct binding of native FAD and FAD analogs to flavocytochrome $b_{558}$ has been demonstrated by several groups [31, 14, 39]. Localization of the FAD binding region is predicted from studies using plasma membranes from a chronic granulomatous disease patient with a point mutation at His-338, which showed low FAD content in plasma membrane and failed to produce superoxide [53]. The location of the NADPH binding site is not well established. Although gp91$^{phox}$ contains regions homologous to known NADPH binding sites (Fig. 1), direct binding of NADPH or $NADP^+$ has not been demonstrated. Different affinity labeling analogs of NADPH show binding to either gp91$^{phox}$ [38, 15] or p67$^{phox}$ [46]. The latter result has led to the suggestion that p67$^{phox}$ contains the binding site for pyridine nucleotide (or a portion thereof) and that activation might involve bringing this binding site into juxtaposition with the flavin moiety on the flavocytochrome [46].

Individual role for the cytosolic regulatory proteins in activating the NADPH oxidase have been proposed in recent studies. p47$^{phox}$ functions as a regulated adapter

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protein in a cell-free system; while it is not essential for cell-free NADPH oxidase activity, it increases the affinity of $p67^{phox}$ and Rac1 by about 2 orders of magnitude [16, 24]. Both Rac and $p47^{phox}$ provide binding sites for $p67^{phox}$, and Rac may function similarly to $p47^{phox}$ in binding and anchoring $p67^{phox}$ (i.e., both may be regulated adapter proteins). We have proposed that it is $p67^{phox}$ that is the direct regulator of electron transfers within the flavocytochrome. An "activation domain" localized within amino acid residues 199~210 in $p67^{phox}$ is essential for cell-free NADPH oxidase activity [18], and a point mutation at residue 204 eliminates NADPH oxidase activity without affecting either the binding of $p67^{phox}$ to $p47^{phox}$ or Rac, or the assembly of the mutant $p67^{phox}$ in the NADPH oxidase complex [18]. The target for this activation domain on $p67^{phox}$ is unknown, but we hypothesize that it is localized within the flavoprotein domain of $gp91^{phox}$.

In this study, we have investigated the putative flavoprotein domain of $gp91^{phox}$. The hydrophobic transmembrane heme-containing domain was eliminated and replaced by a highly soluble portion of bacterial thioredoxin. Using TRX-$gp91^{phox}$ (306~569), which is predicted to contain both FAD and NADPH binding sites, the NADPH diaphorase activity was detected and investigated. Our results indicate that this domain contains both the NADPH and FAD bindind sites. In addition, the flavoprotein domain responds to regulation by $p67^{phox}$ and Rac, indicating the presence of interaction regions for one or both of these factors.

## Materials and Methods

**Truncation of $gp91^{phox}$:**
Truncated $gp91^{phox}$ clones were obtained by PCR using $gp91^{phox}$ DNA cloned in the pGEX-2T plasmid as the template. The forward primers (CGT**GGATCC**CCTTTC AAAACCATCGACCTA for 304~423, CGT**GGATCC**GGT ACAAATATTGCAATAAC for 424~569, and CGT**GGATCC** AAAACCATCGAGCTACAGATG for 306-569) were designed to introduce a BamHI site (shown in boldface). The reverse primers (CGT**AAGCTT**TTAGACTGACTTG AGAATGGATGC for 304-423, CGT**AAGCTT**TTAGAAG TTTTCCTTGTTGAAAAT for both 306-569 and 424-569) were designed to introduce a Hind III site (shown in boldface) and a stop codon (underlined). These PCR products were purified with a PCR purification kit (Qiagen), and were digested with BamHI and Hind III. The digested samples were purified by 1% agarose gel electrophoresis, and extracted from agar by gel extraction kit (Qiagen). The purified DNA fragments were ligated into the BamHI and Hind III sites of pET-32a(+) vector, and then transformed into BL21 (DE3). Transformants were selected from LB/ampicillin plates, and plasmids were isolated from 2 ml cultures of transformants as described previously [42]. The plasmids were digested with BamH I and Hind III, and were separated on 1% agarose to confirm the presence of the insert. The clones were sequenced to rule out unexpected mutations and to confirm the truncations.

**Expression and purification of recombinant proteins:**
Recombinant proteins $p47^{phox}$ and wild-type $p67^{phox}$ were expressed in insect cells (sf9 cell) and purified according to Ulinger et al. [51,52]. Rac1 cDNA cloned in pGEX-2T was expressed in DH5a cells as a GST fusion form, and purified by binding to glutathione-Sepharose followed by thrombin cleavage [26]. Truncated and point mutated forms of $p67^{phox}$ -GST fusions were expressed and purified as above except that thrombin was not used and proteins were eluted with 100 mM glutathione [18].

For preparation of TRX-$tgp91^{phox}$ fusion proteins, *E. coli* were grown at 37°C in LB media (1 L) to an $A_{550}$ of 0.4. IPTG (1 mM) was added and cells were shaken at 37° C for 4 hrs. Except for the 424~569 form, truncated forms of $gp91^{phox}$ were initially insoluble. These were solubilized and renatured according to a modification of the method of Gentz et al. [17]. Cells were pelleted by centrifuging at 4,500 × g for 15 min, were resuspended in 6 M guanidine HCl, pH 7.8, 50 mM Tris-HCl, 500 mM NaCl and incubated on ice for 1 hr. Insoluble materail was removed by centifugation at 30,000 × g × 20 min. The supernatant was applied to a nickel chelate affinity resin (ProBond, Invitrogen), which was washed 3 times with several volumes of wash buffer (8 M urea, 500 mM NaCl, 50 mM Tris-HCl, pH 7.8). The protein was eluted with the same buffer containing 500 mM imidazole. Dithiothreitol (DTT) (2 mM) was added and samples were sequentially dialyzed for 5 hrs each against a series of buffers containing 2 mM DTT plus 1 M urea, 0.2 M urea, and then no urea. DTT was removed after the final step by dialysis against 500 mM NaCl, 50 mM Tris-HCl, pH 7.8. Protein concentration was determined according to Bradford [4] and samples were stored at − 80°C before use.

**NBT reductase activity assay:**
NBT reductase activity was determined using a Thermomax Kinetic Microplate reader (Molecular Devices, Menlo Park, CA). Expressed forms of $gp91^{phox}$ were preincubated in most experiments with an equimolar ratio of FAD (or, in the case of the FAD titration, with varying ratios of FAD/protein) for 16 hrs at 4°C before use. The incubation contained 4 μM of FAD-preloaded TRX-$gp91^{phox}$ (306-569), 200 mM arachidonate, combinations of cytosolic regulatory proteins [4.8 mM $p67^{phox}$, 4.2 mM $p47^{phox}$, and/or 5.4 mM Rac1 which had been preloaded with GTPgS (4)], all in a 50 ml volume of assay buffer (100 mM KCl, 3 mM NaCl, 4 mM $MgCl_2$, 1 mM EGTA, and 10 mM PIPES, pH 7.0). DPI was prepared as a 1 mM stock solution in DMSO, and

working solutions were prepared by dilution into assay buffer. An extinction coefficient of 15.1 $mM^{-1}$ $cm^{-1}$ at 264 nm was used to determine its concentration [37]. Three 10 ml aliquots of each reaction mixture were transferred to 96-well assay plates and preincubated for 5 min at 25°C. 240 µl of assay buffer containing 200 mM NADPH and 200 mM NBT was added to each well. NBT reduction was quantified by monitoring absorbance change at 595 nm using an extinction coefficient of 12.6 $mM^{-1}$ $cm^{-1}$ at 595 nm [28].

## Results

**Expression of TRX-fusion forms of truncated gp91$^{phox}$:** The expression strategy was designed based on the idea that the C-terminal half of gp91$^{phox}$ is relatively hydrophilic, and that this domain will fold independently. We initially constructed a series of truncated mutant as the N-terminal GST fusion proteins and $His_6$ fusions. Those were; gp91*phox* (190~569), (228~569), (304~569), (424~569), and (304~423). All constructs except for gp91$^{phox}$ (190~569), which contains a large hydrophobic segment, were expressed at high levels in *E. coli.* However, neither GST nor $His_6$ fusion forms were soluble, and denaturation/renaturation methods (vide infra) failed to generate soluble products.

In contrast, the thioredoxin fused forms of truncated gp91$^{phox}$ [TRX-gp91$^{phox}$ (304~569), TRX-gp91$^{phox}$ (424~569), and TRX-gp91$^{phox}$ (306~569)] were successfully expressed and were readily solubilized using a urea unfolding/refolding method (Fig. 2). TRX-gp91$^{phox}$ (228~569) was also expressed, but it was not possible to solubilize this form. The largest form of the soluble protein, TRX-gp91$^{phox}$ (306~569) is predicted based on sequence homology to contain binding sites for both FAD and NADPH (Fig. 1). The highly soluble TRX domain reportedly improves the solubility of proteins to which it is fused [27], and the vector also encodes a hexa histidine which allows purification under denaturing condition on a

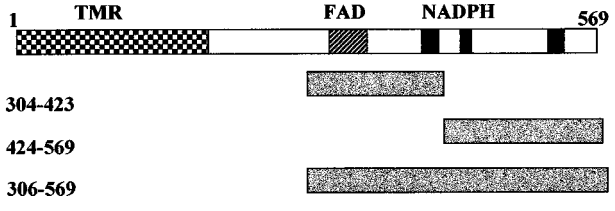


**Fig 1.** Truncations of gp91$^{phox}$. The extremely hydrophobic N-terminus of gp91$^{phox}$ is indicated by the checkered bar, and this region is predicted to contain 5~6 transmembrane regions (TMR) which might have two heme groups. The putative FAD binding region (hatched bar) and NADPH binding regions (filled bars) are indicated. A series of truncated forms indicated by the filled bars were constructed as N-terminal fusion proteins with thioredoxin (TRX).

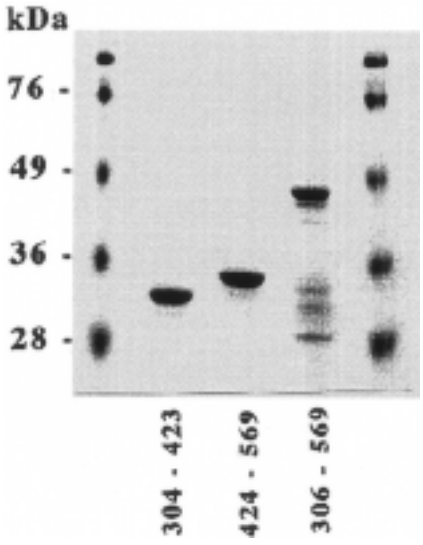


**Fig 2.** SDS-PAGE of expressed TRX-fusion form of truncated gp91$^{phox}$. The TRX-$His_6$-fusion forms of truncated gp91$^{phox}$ were expressed in *E. coli* and were purified by a $Ni^{2+}$-chelating column as described in Materials and Methods. The purified proteins were subjected to SDS-PAGE on a 12% (w/v) polyacrylamide gel, and were visualized by Coomassie-Blue staining. The apparent molecular sizes of TRX fusion forms of gp91$^{phox}$ (304~423), gp91$^{phox}$ (424~569), and gp91$^{phox}$ (306~569) were 32, 34.5, and 48 kDa respectively.

$Ni^{2+}$-chelate affinity matrix. The proteins were purified under denaturing conditions, since TRX-gp91$^{phox}$ (304~423) and TRX-gp91$^{phox}$ (306~569) in particular were poorly soluble and were not retained on a $His_6$ matrix under non-denaturing conditions, despite the fact that they were highly expressed. In contrast, TRX-gp91$^{phox}$ (424~569) was highly expressed and showed good recovery in the presence or absence of 8 M urea. All purified proteins corresponded in size to their predicted molecular weights on SDS-PAGE (Fig. 2).

**Physical properties of expressed proteins:**
Although the flavoprotein domain of gp91$^{phox}$ [TRX-gp91$^{phox}$ (304~423), TRX-gp91$^{phox}$(424~569), and TRX-gp91$^{phox}$(306~569)] were obtained in soluble form that showed no apparent turbidity, they appeared to be an aggregate of 4 or more monomers. TRX-gp91$^{phox}$ (306~569) preincubated with 0.1 mM FAD was chromatographed on a Sephacryl S-300 column equilibrated with 20 mM Tris-HCl buffer, pH 7.4, containing 50 mM NaCl. The proteins eluted at or near the void volume (MW = 200 kDa). On a SDS-PAGE in the absence of DTT, they migrated as large molecular size (apparent size was greater than 106 kDa) smeared band (data not shown). However, when the proteins were treated with SDS sample buffer containing 80 mM DTT, they gave the correct predicted molecular weights of 32, 34.5, and 48 kDa respectively on a 12% SDS-PAGE gel (Fig. 2). The results imply that the

recombinant gp91$^{phox}$ exists in a polymerized state in ordinary buffer even in the presence of detergent unless reducing agent is added, suggesting that there might be intermolecular disulfide bridges. However, DTT interfered with diaphorase assays (below) and was therefore not included.

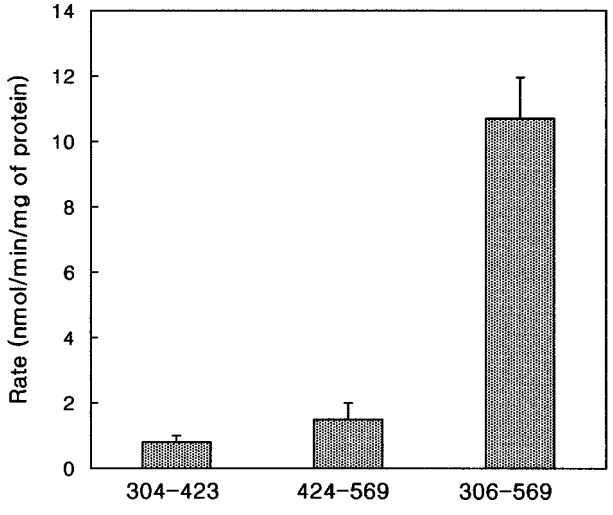


**Fig 3**. NBT reductase activity of TRX-gp91$^{phox}$. Each form of TRX-gp91$^{phox}$ was preincubated with an equimolar amount of FAD for 16 hrs at 4 °C as described under Materials and Methods. NBT reductase activity was measured using 4 mM of each protein and 200 mM arachidonate in a volume of 50 ml. The reaction was initiated by the addition of 10 ml of this mixture to a 240 ml solution cotaining 0.2 mM of NBT in the presence of 0.2 mM NADPH. Error bars show the standard error of the mean (n = 3).

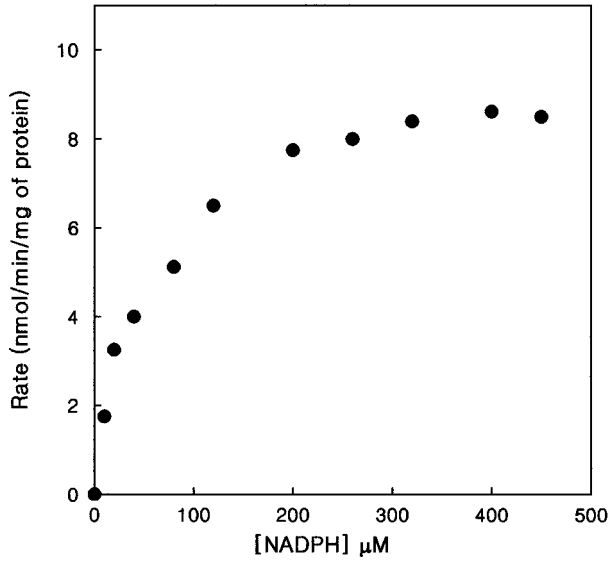


**Fig 4.** NADPH-dependent NBT reductase activity of TRX-gp91$^{phox}$ (306~569). The assay conditions were as described in Fig 3., except that the reaction was initiated by the addition of 10 ml of the activation mixture to a 240 ml solution cotaining 0.2 mM of NBT and the indicated concentrations of NADPH.

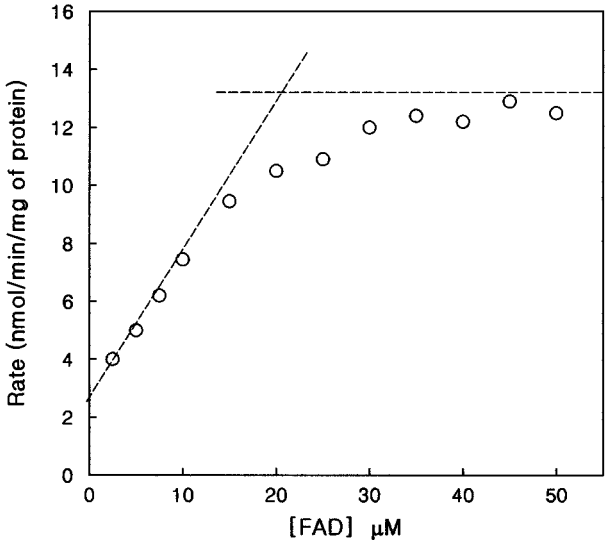


**Fig 5.** FAD-dependent NBT reductase activity of TRX-gp91$^{phox}$ (306~569). TRX-gp91$^{phox}$ (306~569) (20 mM) was preincubated with varying concentrations of FAD for 16 hrs at 4 °C as indicated. NBT reduction of the FAD-reconstituted preparations was measured as described under Materials and Methods using 4 mM TRX-gp91$^{phox}$ (306-569) and 200 mM arachidonate in a 50 ml volume.

**NADPH-dependent NBT reductase activities of TRX-gp91$^{phox}$ (306~569):**

The longest fusion protein, TRX-gp91$^{phox}$ (306-569), showed NADPH-dependent activity (Fig. 3) whereas the shorter forms, TRX-gp91$^{phox}$ (304~423) and TRX-gp91$^{phox}$ (424~569), showed only background NBT reductase activity (Fig. 3). The Km for NADPH in the longest protein was determined to be 45 mM (Fig. 4) in the absence of cytosolic factors. This value is similar to the Km for NADPH (~50 μM) observed in the intact phagocyte NADPH oxidase [15].

**FAD-dependent NBT reductase activities of TRX-gp91$^{phox}$ (306~569):**

Activity was dependent on FAD (Fig. 5), and increased more or less linearly up to a ratio of FAD/protein of approximately 1 : 1, approaching saturation thereafter. Curve fitting revealed an apparent Kd of 740 nM for binding of a single FAD to the protein. The relatively tight binding of FAD and the normal Km for NADPH suggest that the flavoprotein domain of TRX-gp91$^{phox}$ (306~569) achieves a more-or-less native structure following expression and renaturation. The observation of a stoichiometry of FAD binding to protein near 1 : 1 suggests that despite its polymeric state, most of the flavoprotein domain is in an active form.

**Inhibition of NBT reductase activity of TRX-gp91$^{phox}$ (306-569):**

NBT reductase activity of TRX-gp91$^{phox}$ (306~569) was

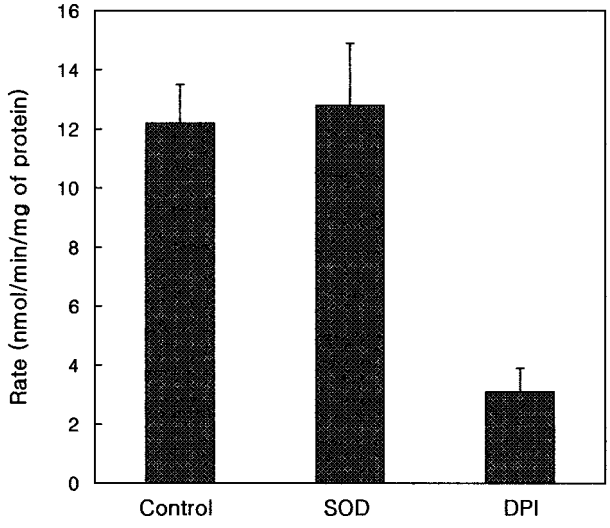


**Fig 6.** Inhibition of NBT reductase activity of TRX-gp91$^{phox}$ (306~569). NBT reduction was measured using 4 mM of FAD-preloaded TRX-gp91$^{phox}$ (306~569) and 200 mM arachidonate in a 50 ml volume in the presence or absence of either 10 units superoxide dismutase (SOD) or 10 mM diphenylene iodonium (DPI). Error bars show the standard error of the mean (n = 3).

not inhibited by superoxide dismutase (Fig. 6) indicating that NBT reduction was not mediated by superoxide. Thus, it seems likely that NBT accepts electrons directly from the reduced FAD. DPI, a known inhibitor of NADPH-dependent superoxide generation by the intact phagocyte oxidase [9], blocked NADPH-dependent NBT reduction (Fig. 6).

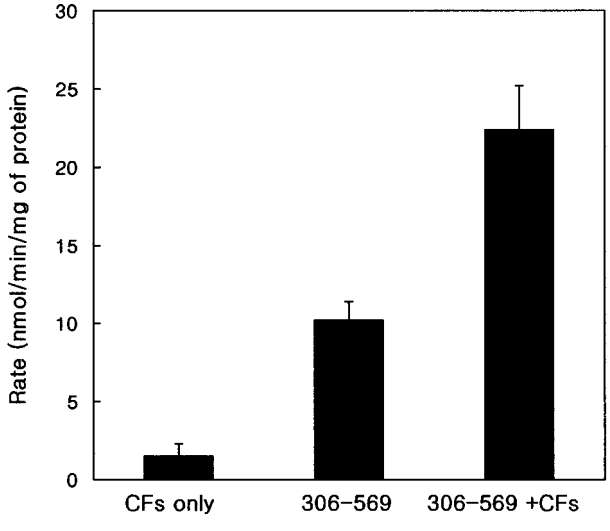


**Fig 7.** Effects of cytosolic factors (CFs) on NBT reductase activity of TRX-gp91$^{phox}$ (306-569). TRX-gp91$^{phox}$ (306-569) (20 mM) was preincubated with 20 mM of FAD for 16 hrs at 4° C before use. NBT reduction was measured using 4 mM of FAD preloaded TRX-gp91$^{phox}$ (306-569) and 200 mM arachidonate in a volume of 50 ml in the presence or absence of the cytosolic factors (4.8 mM p67$^{phox}$, 4.2 mM p47$^{phox}$, 5.4 mM Rac1). Error bars show the standard error of the mean (n=3).

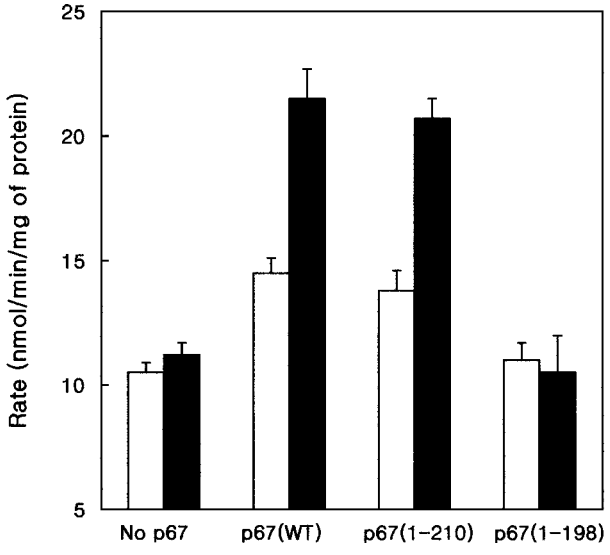


**Fig 8.** Effects of truncation in the activation domain of p67$^{phox}$ on NBT reductase activity of TRX-gp91$^{phox}$ (306-569). The assay conditions were as in Fig 7, except that in the presence (filled bars) or absence (open bars) of Rac1 (5.4 mM). Error bars show the standard error of the mean (n = 3).

**Effects of cytosolic regulatory factors on the NBT reductase activity of TRX-gp91$^{phox}$ (306-569):**

As shown in Figure 7, the NBT reductase activity of TRX-gp91$^{phox}$ (306~569) increased nearly 2-fold in the presence of the cytosolic regulatory proteins p47$^{phox}$, p67$^{phox}$, and Rac1 (GTPgS). Activity was dependent upon TRX-gp91$^{phox}$ (306~569), and cytosolic factors alone showed almost no activity. Thus, one or more cytosolic regulatory proteins can stimulate NBT reductase activity of TRX-gp91$^{phox}$ (306~569). To further explore the requirement for cytosolic factors, the activity was measured in the presence of combinations of cytosolic factors. Elimination of p47$^{phox}$ had no effect on NBT reductase activity (not shown). As shown in Fig. 8, p67$^{phox}$ stimulated the activity in the absence of other cytosolic factors. Rac alone had little or no stimulatory effect, but increased the magnitude of stimulation by p67$^{phox}$ (Fig. 8). The effect of the activation domain of p67$^{phox}$ on NBT reductase activity of the flavoprotein domain was also investigated. p67$^{phox}$ (1-210) which contains the activation domain stimulated activity as well as the wild type p67$^{phox}$. However, p67$^{phox}$ (1-198) which lacks the activation domain was ineffective (Fig. 8).

## Discussion

Based on previous models, the globular portion of the b subunit of cytochrome $b_{558}$ is largely exposed to the solvent, and accessible to NADPH from the cytoplasm [48]. Several flavin-dependent reductases possess a b-stranded barrel structure for FAD binding [30]. The sequence alignment of FAD binding domain of gp91$^{phox}$

and ferredoxin-$NADP^+$ reductase family showed that the amino acid residues 279-400 of $gp91^{phox}$ is homologous to a general FAD binding structure [53]. The HPFT motif (residues 338-341) in this structure is predicted to interact directly with FAD in the flavocytochrome $b_{558}$ model [48], and is conserved in $gp91^{phox}$ in human, porcine, and mouse [55, 3]. The aim of this study was to express a flavoprotein-homology domain of cytochrome $b_{558}$ which lacks the transmembrane heme-binding regions, and to investigate its catalytic properties. $gp91^{phox}$ (306~569) includes most of the predicted b-stranded barrel structure including the HPFT motif and also contains regions which are predicted to form the NADPH binding site (Fig.1). This structure was successfully expressed as a TRX fusion protein and showed low catalytic activity (NBT reductase activities). However, the maximal rate of NBT reduction at saturating FAD concentration was low, about 4 electrons/min/molecule of protein. The low activity may either be an intrinsic property of the flavoprotein domain, or may indicate that the expressed flavoprotein is catalytically inefficient due to its cross-linked nature or absence of an appropriate conformation. In a previous study, the anaeroblic rate of FAD reduction was less than 1% of the aerobic rate [25], and the authors proposed that oxygen induces an conformation which favors flavin reduction. Since there is no heme in TRX-$gp91^{phox}$ (306~569), such a conformational regulation may not be possible. The intact flavocytochrome also catalyzes a low rate of INT reduction [10], but this rate is still approximately 100-200 fold higher than that seen in the present study, suggesting either a less efficient electron transfer in the expressed flavoprotein domain, or additional electron transfer mechanisms in the intact cytochrome. The low activity may make this preparation of limited utility for mechanistic studies, but the model system appears to be adequate for drawing a number of important conclusions.

The diaphorase activity of TRX-$gp91^{phox}$ (306~569) was dependent upon FAD which showed a relatively high binding affinity ($K_d$ = 740 nM). The expressed domain also showed a Km for NADPH of around 50 μM, the same value which is seen for the NADPH-superoxide generating activity of the intact respiratory burst oxidase. These data indicate that the expressed, renatured protein forms a reasonably intact structure, sufficient to bind both NADPH and FAD and to catalyze a diaphorase activity, albeit at a very low rate. These data demonstrate unequivocally that this domain contains binding sites for both an NADPH and an FAD. Although it is possible that $p67^{phox}$ also contains a binding site for NADPH, as was recently proposed [46], these data do not support the idea that such a site is involved in catalysis.

Importantly, these studies also reveal that the flavoprotein domain is the target of regulation by $p67^{phox}$. Previously, we showed that an activation domain in $p67^{phox}$ (residues 199~210) is essential for NADPH-oxidase activation in a cell-free system [18]. Truncated forms of $p67^{phox}$ lacking this region showed no ability to activate the oxidase, despite that fact that these forms bound normally to oxidase components and assembled normally as part of the oxidase complex under cell-free activation conditions. As shown in Fig. 8, the truncation of the activation domain eliminated the ability of $p67^{phox}$ to activate the NBT-reductase activity of the flavoprotein domain. These data indicate that the target of the activation domain of $p67^{phox}$ resides within the flavoprotein domain of $gp91^{phox}$. These data provide a physical explanation for data indicating that the activation domain of $p67^{phox}$ controls the reduction of FAD by NADPH [32]. Interestingly, this study showed that $p67^{phox}$ had little or no effect on NADPH binding, but data were most consitent with regulation of electron/hydride transfer from NADPH to FAD.

These studies failed to provide evidence for an effect of $p47^{phox}$ on regulation at the level of the flavoprotein domain. We have previously shown that $p47^{phox}$ is not essential for NADPH-dependent superoxide generation by the intact respiratory burst oxidase under cell-free conditions [16]. Its role was proposed to be a regulated adapter protein, since its effect was to enhance the affinity of $p67^{phox}$ and Rac by up to 100-fold. Although Rac does not directly activate the flavorprotein domain, a role for Rac is implied by the present studies, since Rac enhances the effect of $p67^{phox}$. Direct binding of Rac to $p67^{phox}$ via the effector region on Rac (residues 26-45) has been demonstrated [33], and Rac is known to bind to the plasma membrane through its C-terminus [26]. A third region on Rac, the insert region (residues 124-135) is essential for optimal activity and is involved in protein-protein interactions within the assembled oxidase [33], this region has been proposed to bind to the cytochrome, although this has not yet been directly demonstrated. Rac may be synergizing with $p67^{phox}$ in activating diaphorase activity of the flavoprotein domain either by binding to $p67^{phox}$, producing an active conformation, or by binding simultaneously to both $p67^{phox}$ and the flavoprotein domain of $gp91^{phox}$. The present studies do not distinguish between these possibilities, but indicate that Rac somehow synergizes with $p67^{phox}$ to activate the flavoprotein domain of $gp91^{phox}$. The ability of $p67^{phox}$ to activate NBT reductase activity in the absence of Rac, however, indicates that $p67^{phox}$ must be interacting directly with the flavoprotein domain, and that it is likely to be the primary regulatory element in this complex and elegant system.

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