The p67phox Activation Domain Regulates Electron Flow from NADPH to flavin in flavocytochrome b558*

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The p67phox activation domain in p67phox (residues within 199–210) is essential for cytochrome b558-dependent activation of NADPH superoxide (O2) generation in a cell-free system (Han, C.-H., Freeman, J. L. R., Lee, T., Motalebi, S. A., and Lambeth, J. D. (1998) J. Biol. Chem. 273, 16663–16668). To determine the steady state reduction flavin in the presence of highly absorbing hemes, 8-nor-8-S-thioacetamido-FAD (“thioacetamido-FAD”) was reconstituted into the flavocytochrome, and the fluorescence of its oxidized form was monitored. Thioacetamido-FAD-reconstituted cytochrome showed lower activity (7% versus 100%) and increased steady state flavin reduction (28 versus 5%) compared with the enzyme reconstituted with native FAD. Omission of p67phox was critical for regulating flavin reduction, since mutations in this region that decreased O2 generation also decreased the steady state reduction of flavin. Thus, the activation domain on p67phox regulates the reductive half-reaction for FAD. This reaction is comprised of the binding of NADPH followed by hydride transfer. The calculated kinetic deuterium isotope effects on V/Km values permitted calculation of the Kf for NADPH. (R)-NADPD but not (S)-NADPD showed kinetic deuterium isotope effects on V and VK of about 1.9 and 1.5, respectively, demonstrating stereospecificity for the R hydride transfer. The calculated Kf for NADPH was 40 μM in the presence of wild type p67phox and was 55 μM using the weakly activating p67phox(V205A). Thus, the activation domain of p67phox regulates the reduction of FAD but has only a small effect on NADPH binding, consistent with a dominant effect on hydride/electron transfer from NADPH to FAD.

The neutrophil respiratory burst oxidase is a multicomponent enzyme that catalyzes the transfer of electrons from NADPH to O2 to form superoxide (O2·−). The essential membrane-associated component is flavocytochrome b558 (1) which is composed of the glycoprotein gp91phox (2) and a 22-kDa protein, p22phox (3). Cytochrome b558 contains nonidentical hemes and FAD in a molar ratio of 2:1, and its large subunit, gp91phox, has candidate consensus binding sequences for pyridine nucleotide and flavin (4–8). Both hemes were recently shown to reside entirely in gp91phox (9). Thus, gp91phox is the catalytic moiety that transfers electrons from NADPH to O2, and the other components can be considered to be regulatory. Based on chemical precedent and structural models of the enzyme (10, 11), the pathway for electron flow within flavocytochrome b558 has been proposed in Scheme I.

NADPH → FAD → heme A → heme B → O2

SCHEME I

When the cytochrome is purified from neutrophil plasma membranes, the flavin dissociates and activity is lost, but activity and FAD binding can be restored by incubating the enzyme with phospholipids and FAD (4, 6, 7).

The flavocytochrome shows no activity in the absence of the cytosolic regulatory proteins p47phox, p67phox, and Rac, a small GTP-binding protein (12–16). Upon cell activation by exposure to bacteria or chemical activators, these proteins assemble on the plasma membrane in a 1:1:1 ratio with flavocytochrome b558 (17–19). In resting cells, p47phox and p67phox are in a large cytosolic complex (20) along with one or more additional proteins and may translocate en bloc to the membrane. Rac translocates independently of the other cytosolic components (21–24) but can bind directly p67phox (25–28). Details of many of the high affinity protein-protein interactions among cytosolic and some membrane components have been described (29) and involve SH3 domains on p47phox and p67phox and target proline-rich sequences in partner proteins.

A key unanswered question has to do with the mechanism by which assembly of the cytosolic proteins with the cytochrome regulates activity. A cell-free model system consisting of the purified flavocytochrome and recombinant cytosolic factor has proven useful (30), and recent evidence points to unique specialized functions for each of the cytosolic regulatory proteins. p47phox functions as a “regulated adaptor protein,” helping to provide binding sites for the other cytosolic factors (31, 32). Although it is not essential for cell-free activity, it enhances the affinity of p67phox and Rac by 2 orders of magnitude. Site-directed mutagenesis supports a model in which Rac has multivalent interactions, binding simultaneously to p67phox, membrane, and flavocytochrome through distinct regions on Rac (33–35). Data are consistent with a model in which Rac, like p47phox, functions as an adaptor protein, participating in the binding of another essential component(s).

Recent data point to p67phox as the essential factor that activates electron transfer within the flavocytochrome. We recently identified an “activation domain” within p67phox that is essential for NADPH oxidase activity (36). Truncation mutants identified this region within residues 199–210, and a single point mutation at residue 204 completely eliminated NADPH oxidase activity without affecting specific interactions of p67phox with p47phox or Rac, or the assembly of the mutant p67mut within the NADPH oxidase complex. We propose that

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the activation domain on p67phox directly activates a particular step in the electron transfer pathway depicted above in Scheme I. The present studies were undertaken to identify the step that is regulated by the activation domain on p67phox. We provide evidence that the activation domain on p67phox regulates the reduction of FAD by NADPH but does not affect the binding of NADPH itself, consistent with the regulation of the NADPH → FAD hydride/electron transfer reaction.

**EXPERIMENTAL PROCEDURES**

**Materials**—NADPH, NADPH analogs, FAD, arachidonic acid, cytochrome c (type IV, horse heart), thrombin, glutathione, n-octyl glucoside, cholic acid, GTP, S, protease inhibitor mixture, phosphatidylinositol, phosphatidylserine, phosphatidylcholine, and sphingomyelin were purchased from Sigma. Hesper (6.2% hetastarch in 0.9% NaCl) was obtained from American Hospital Supply Corp. Lymphocyte separation medium (6.2% Ficoll and 9.4% sodium diatrizoate) was purchased from Organon Tekniker. Glutathione-Sepharose, heparin-Sepharose CL-6B, DEAE-Sepharose CL-6B, CM-Sepharose CL-6B, ω-aminooxy-agarose, and pGEX-2T vector were purchased from Amersham Pharmacia Biotech. Affi-Gel 10 was purchased from Bio-Rad. Oligonucleotide primers were synthesized and purified by the Emory Microchemical Facility.

**Preparation of 8- Nor-8-Thioacetamido-FAD**—The starting material, 8-chlororiboflavin was generously provided by Dr. Dale E. Edmondson, Emory University. This was converted to 8-chloro-FAD using partially purified FAD synthetase from *Brevibacterium ammoniagenes* (7). The appropriate 8-mercaptopo-FAD was prepared just before use by reaction of 8-chloro-FAD, consumed at pH 8.0, with 5 mM Na2S. Excess unreacted sodium sulfide was removed by P-2 column (2 × 70 cm) chromatography. The 8-mercapto-FAD was reacted with excess iodoacetamide for 24 h at 25°C in the dark, and unreacted iodoacetamide was removed by P-2 column chromatography. The resulting analog showed an absorption spectrum identical with that previously published for 8-nor-8-thioacetamido-FAD (37), and its concentration was determined fluorimetrically. Fluorescence spectra were recorded with a Hitachi model F-3000 spectrophotometer and Hitachi model F-3000 spectrophotometer, respectively.

**Preparation of Deuterated NADPH**—Deuterated pyridine nucleotides were prepared as in Refs. 38–41 with some modifications. The cyano form of NADPH was prepared and was then converted into deuterated NADPH by slow diffusion of D2O into a solution of NADPH in 5 ml of D2O was added to 280 μg of dry NADPH, 0.2 ml of 1 M KOD (in D2O) was added, and the solution was incubated for 2 h in the dark. D2O (35 ml) containing 11 mmol of KH2PO4 was added, and HCN was removed by gentle bubbling with N2 in order to convert cyano NADPH into monodeuterated NADPH. The concentration was determined spectrophotometrically using an extinction coefficient of 18.8 cm−1 mmol−1 at 260 nm. To prepare the R (A) monodeuterated n-NADPH, a 2-fold molar excess of glucose 6-phosphate was added to 25 ml of n-NADPH along with 50 μg of glucose-6-phosphate dehydrogenase (*Leuconostoc mesenteroides*), and reduction was followed spectrophotometrically at 340 nm while maintaining the pH at 7.8–8.0 with 1 M KOH. To prepare the S (B) form of n-NADPH, a 4-fold molar excess of isocitrate and 5 mg of isocitrate dehydrogenase was added to 25 ml of n-NADPH, and the reduction was monitored and the pH was maintained as above. n-NADPH was purified by fast protein liquid chromatography using a BioScale Q-20 anion exchange column (42) using a linear gradient from 0 to 1 M LiCl, pH 7.8; NADPH elutes at 160 mM LiCl. Fractions showing an Amax/A254 ratio of <2.3 were pooled and lyophilized, and LiCl was removed by washing the dried powder with methyl alcohol. The sample was dried under vacuum and stored at −20°C until needed. Samples were analyzed by mass spectrometry to confirm isotopic purity.

**Preparation of Plasma Membrane, Flavocytochrome b558 and Recombinant Cytochrome Proteins**—Human neutrophils were isolated from peripheral blood of healthy donors, and plasma membranes were prepared as described (43, 44). Purification of flavocytochrome b558 from solubilized plasma membranes and reconstitution of flavin-depleted cytochrome b558 with either native FAD or 8-thioacetamido-FAD (heme/ flavin = 2:1, mol ratio) in the presence of both phospholipids (PC/PE/PS/SM/cholesterol = 4:2:1:3:3, w/w; lipids/ protein = 50:1, w/w) and n-octyl glucoside (40 mM) was performed as described (7). Recombinant p47phox and wild type p67phox were expressed in Sf9 insect cells and purified according to Refs. 17 and 45. Rac1 was expressed in DH 5α cells as a glutathione S-transferase fusion protein and was purified by binding to glutathione-Sepharose followed by thrombin cleavage (46). Truncated and point-mutated versions of p67phox developed previously (27) were expressed in *Escherichia coli* and purified by glutathione-Sepharose affinity chromatography with elution using glutathione (27). Samples were dialyzed to remove free glutathione. Protein concentrations were determined according to Bradford (47).

**Assay of Superoxide Generation—**NADPH oxidase activity was assayed spectrophotometrically using superoxide dismutase-inhibitable cytochrome c reduction (44), in a Thermomax Kinetic Microplate reader (Molecular Devices, Menlo Park, CA). Rac was preloaded with 5-fold molar excess of GTP·S for 15 min at room temperature in the absence of MgCl2. For standard assay conditions, the cell-free reaction mixtures included purified cytochrome b558 (275 nm) reconstituted with either native FAD or thioacetamido-FAD, 850 nm p47phox, 900 nm p67phox, 950 nm Rac1 preloaded with GTP·S, and 200–240 μM arachidonate in a total volume of 50 μl. Four 10-μl aliquots of each reaction mixture were transferred to 96-well microassay plates and preincubated for 5 min at 25°C. For each well, 240 μl of solution containing 0.2 mM NADPH and 80 μM cytochrome c in buffer A (20 mM Tris-HCl, pH 7.4, 50 mM NaCl, 4 mM MgCl2, and 1.25 mM EDTA) was added to initiate the reaction. Cytochrome c reduction was quantified by monitoring the absorbance increase at 550 nm using an extinction coefficient of 21.1 M−1 cm−1.

**Calculation of Kinetic Data—**Reported Michaelis-Menten kinetic parameters were determined using a non-linear least squares fit of the data, programmed in Sigma Plot.

**Spectrophotometric and Fluorometric Assays**—Heme content was determined by reduced minus oxidized difference spectroscopy at 424–440 nm using an extinction coefficient of 161 M−1 cm−1 (48). The flavin content of FAD analog-reconstituted cytochrome b558 was estimated fluorimetrically. Fluorescence spectra were recorded with a Hitachi model F-3000 spectrophotometer. Fluorescence changes at 525 nm induced by NADPH-FAD analog oxidoreduction during cell-free NADPH oxidase activation occurred slowly for about 5 min, and the total fluorescence change due to the complete reduction of the FAD analog was measured by adding a few crystals of sodium dithionite. To calculate the percent reduction of the FAD analog at steady state, the fluorescence change at 525 nm attributable to NADPH oxidation was subtracted from that due to oxidoreduction of NADPH and the FAD analog. The time course of heme reduction was derived from the absorbance changes at 558 minus 540 nm, using an extinction coefficient of 21.6 M−1 cm−1 (48).

**RESULTS**

**Reconstitution of Activity with 8-Thioacetamido-FAD**—During detergent solubilization and purification, the flavocytochrome b558 loses flavin so that in its purified form it lacks FAD and has no detectable activity. Activity can be restored using either native FAD (4, 6) or 8-substituted FAD analogs (7).

Reconstitution of activity with flavin requires phospholipids, and we previously found that a mixture of PC, PE, PI, SM and cholesterol (27:10:5:6:0, w/w) and has no detectable activity. Activity can be restored using either native FAD (4, 6) or 8-substituted FAD analogs (7).

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NADPH was assessed fluorophotometrically. Free 8-mercaptoprostanone shows very weak fluorescence (7), but reaction of the 8-mercaptoprostanone with iodoacetamide results in the production of the fluorescent 8-mercaptoprostanone derivative which, unlike native FAD, retains fluorescence when bound to flavocytochrome \( b_{558} \) (49). As shown in Fig. 2, the flavin analog displays a fluorescence emission maximum at 520 nm, and reduction with dithionite resulted in loss of fluorescence. Cytochrome \( b_{558} \) was incorporated into phospholipid/terminal glucoside mixed micelles to provide the required phospholipid while minimizing turbidity. The excitation maximum of 8-thioacetamido-FAD bound to the flavocytochrome is 475 nm, 25 nm red-shifted compared with that of native FAD. This longer wavelength excitation maximum minimizes overlap with the NADPH fluorescence excitation spectrum.

Steady state fluorescence of the thioacetamido-FAD reconstituted into flavocytochrome \( b_{558} \) was analyzed in the presence of the cytosolic regulatory factors \( p67^{\text{phox}} \), \( p47^{\text{phox}} \), and Rac1-GTPyS as well as the activator sodium dodecyl sulfate. The reduction state of heme was also monitored using the increased absorbance at 558 nm minus the absorbance at the isosbestic point 540 nm. Addition of NADPH (0.10 mM) resulted in the partial reduction of the flavin analog (Fig. 3, panel A). The fluorescence decreased approximately linearly for 5 min, after which there was a much more gradual further bleaching of fluorescence. Superoxide generation under these conditions was approximately linear for more than 10 min, indicating that the leveling off in fluorescence change was not due to a loss in activity of the enzyme. Based on the turnover rate of the enzyme with this flavin analog and the predicted oxidation of NADPH (based on the known turnover number), this second phase of slow fluorescence change is due to the oxidation of NADPH, which shows modest fluorescence at this wavelength. Complete reduction of flavin was achieved by adding a few crystals of sodium dithionite to generate a maximal fluorescence change (arrows in Fig. 3). The steady state reduction levels were calculated based on the percent fluorescence bleaching achieved at 5 min, the approximate intersection of the rapid and slow phases, correcting for the decrease in fluorescence contributed by NADPH oxidation. Based on this calculation, the fraction reduction of flavin after steady state has been achieved is 28 ± 3% (Table 1). In contrast to flavin reduction, addition of NADPH produced <2% steady state reduction of heme based on absorbance changes at 558 nm minus 540 nm (Table 1).

**Effect of Cytosolic Regulatory Proteins on the Reduction of Flavin and Heme**—The steady state percent reduction of the FAD analog and heme was determined as above in the complete system or in the absence of either \( p47^{\text{phox}} \) or \( p67^{\text{phox}} \) (Table 1). When \( p47^{\text{phox}} \) was omitted, there was still significant reduction of flavin (21% compared with 28%). However, when \( p67^{\text{phox}} \) was omitted (Table 1 and Fig. 3, panel B), the flavin was almost completely oxidized. The steady state reduction of flavin correlated with the rate of \( O_2^\bullet^- \) generation under the same conditions (Table 1), indicating a functional relationship between flavin reduction and \( O_2^\bullet^- \) generation. In contrast, heme was essentially completely oxidized regardless of the presence of the cytosolic regulatory proteins (Table 1).

**Role of the Activation Domain in \( p67^{\text{phox}} \) in Flavin Reduction**—Previous studies (36) imply that an activation domain within residues 199–210 on \( p67^{\text{phox}} \) activates electron transferers within the flavocytochrome. The truncated \( p67^{\text{phox}} \text{-}(1–198) \) failed to support detectable \( O_2^\bullet^- \) generation (Fig. 4, panel A) and resulted in a very low steady state reduction of 8-thioacetamido-FAD (Fig. 4, panel B). Two forms of \( p67^{\text{phox}} \) mutated within the activation domain, \( p67^{\text{phox}}(V204A) \) and \( p67^{\text{phox}}(V205A) \), were evaluated for their effects on activity and steady state reduction of flavin (Fig. 3, panels C and D). The V204A mutation shows essentially no activity, and the V205A form shows low activity. These mutant forms of \( p67^{\text{phox}} \) supported very low steady state reduction of FAD (Fig. 4, panel B). The activation domain is not involved in the interaction with Rac1 or \( p47^{\text{phox}} \), and \( p67^{\text{phox}} \) mutated in this region assembles normally within the NADPH oxidase complex (36). Thus, mutation of the activation domain suppresses the reduction of flavin by NADPH in flavocytochrome \( b_{558} \).

**Kinetic Deuterium Isotope Effects on NADPH Oxidase Activity**—One attractive hypothesis is that the activation domain on \( p67^{\text{phox}} \) regulates either the binding of NADPH itself or another pre-isotopic step (e.g., a conformational change that might juxtapose the pyridine nucleotide and FAD so as to facilitate electron transfer). The \( K_m \) for a substrate (in this case NADPH) is a complex kinetic term that can differ from the actual binding constant due to the contribution of kinetic terms for post-binding steps. However, a recent treatment of kinetic deuterium isotope effects by Kliman and Matthews (50) led to a simple expression (Equation 1) that allows the direct deter-
The affinity of NADPH for the enzyme was relatively unaffected by flavin reduction, but the plasma membrane preparation, as can be seen, the ratio of NADPH to NADPH oxidase activity is 2.3, and the plasma membrane preparation, but this is insufficient to account for the 3- to 4-fold decrease in V_max seen with this mutation. Thus, the mutation in the activation domain of p67phox does not have a major effect on the affinity of the enzyme for NADPH.

Superoxide Generating Activity Using Pyridine Nucleotide Analogs—The ability to observe a kinetic deuterium isotope effect does not support the idea that NADPH is a “sticky substrate,” defined by Cleland (51), as one that reacts to give products as fast or faster than it dissociates from the enzyme. To test further whether product dissociation could be rate-determining, a series of analogs of NADPH were used as electron-donating substrates, and kinetic parameters were determined as in Fig. 5. Results are summarized in Table III. As shown, for NADPH, deamino-NADPH, and NADH, the V_max values are 320 ± 50, 210 ± 30, and 21 ± 2, respectively. The effect of cytosolic components on NADPH oxidase activity and on steady state reduction of flavin and heme was measured in the presence of phospholipids as described under “Experimental Procedures.” NADPH-dependent superoxide generation was monitored in the presence or absence of p47phox and p67phox. Under the same conditions using the complete system, native FAD gave a turnover of 4.88 μmol O2/min/nmol heme. The extent of flavin analog reduction (Ef) during turnover is expressed as a percentage of the total fluorescence decrease at 525 nm obtained when dithionite was added, using the following expression,

\[ \text{Ef} = \frac{\Delta F_{\text{total}} - \Delta F_{\text{dithionite}}}{\Delta F_{\text{total}}} \times 100 \]  

where \( \Delta F \) is the fluorescence change observed 5 min after addition of 100 μM NADPH, and \( \Delta F_{\text{total}} \) is the total fluorescence change measured after addition of dithionite. The correction factor 0.26 is due to the estimated contribution to the fluorescence change due to NADPH oxidation, which was determined in a separate experiment from the decreased absorbance at 340 nm corrected for changes at this wavelengt due to flavin reduction. The steady state heme reduction level was determined as a percentage of the total dithionite-reducible heme using the reduced minus oxidized absorbance at 558 minus 540 nm.
was essentially identical, whereas the $K_m$ varied over a 34-fold range. Thus, for two substrates that bound more weakly than NADPH based on $K_m$ values, the $V_{max}$ was not increased as would be predicted if NADPH were behaving as a sticky substrate.

**DISCUSSION**

A model has been proposed that attempts to explain individual roles for cytosolic proteins during the protein assembly associated with activation of the respiratory burst (see Introduction). According to this model, it is $p67^{phox}$ that directly regulates the rate-limiting transfer of electrons within the gp91$^{phox}$ subunit through its activation domain within the 198–210 region. In the present study, we have investigated the influence of this region on regulating the rate of specific catalytic steps involved in transferring electrons from NADPH to $O_2$. The reductive half-reaction (Reaction 1) and reoxidative half-reaction (Reaction 2) with respect to FAD within gp91$^{phox}$ are summarized as follows.

\[
\text{NADPH} + E\cdot \text{FAD} \rightarrow \text{NADP}^+ + E\cdot \text{FADH}_2
\]

**REACTION 1**

\[
E\cdot \text{FADH}_2 + 2 \text{heme}_{\text{red}} \rightarrow E\cdot \text{FAD} + 2 \text{heme}_{\text{ox}}
\]

**REACTION 2**

We first used steady state kinetics to investigate whether the activation domain in $p67^{phox}$ stimulates the reductive half-reaction (hypothetical activator 1, above) or the reoxidative half-reaction (hypothetical activator 2, above). If the former were the case then the $p67^{phox}$ should increase the steady state reduction level of FAD, and mutations should lead to a more oxidized state. The opposite should be true if $p67^{phox}$ were functioning like activator 2, above. In addition, the heme should become more reduced. Thus, monitoring the steady state reduction of flavin and heme during turnover will distin-

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2 The $K_m$ value for NADH in our study was approximately 5–6-fold higher than some other published $K_m$ values. The reason for this is not clear at this time, although our data seem clear on this point. Differences may have resulted from differences in preparation or purity of cytochrome $b_{558}$ and/or cytosolic factors, or other assay-specific differences such as activator concentration, presence of GTP$\gamma$S, etc.
guish between these two models.

Two technical problems limit the accuracy and feasibility of monitoring the reduction of native FAD. The extinction coefficient for reduction of FAD is low compared with that of heme, and absorbance changes occur in areas of overlap with the heme. In addition, an earlier study using a high turnover preparation of purified flavocytochrome b558 showed that the flavin remains mostly oxidized during turnover (52), which makes it difficult to see perturbations of the reduction state. These problems were both overcome by the use of a fluorescent FAD analog, 8-thioacetamido-FAD. In addition to providing a robust fluorescence signal that was not affected by the heme, the FAD analog supported a slower turnover. By using flavocytochrome b558 reconstituted with 8-thioacetamido-FAD, the steady state reduction of FAD was 28%, compared with reported values of under 10% in the studies of Koshkin et al. (52).

These features make it straightforward to determine the effect of wild type and mutated forms of p67phox on the steady state reduction of the flavin analog.

This approach provides clear data indicating that the flavin reductive half-reaction is regulated by p67phox, and more specifically, by the activation domain in p67phox. In the absence of p67phox, the flavin is nearly fully oxidized, whereas in the presence of p67phox, the flavin becomes partially reduced. p47phox had little effect on the reduction state of either flavin or heme. The latter was essentially fully oxidized during all turnover conditions, consistent with previous reports that indicate that reduced heme is reoxidized extremely rapidly by molecular oxygen (48, 52). Our data are in agreement with those of Cross and Curnette (53) concerning the role of p67phox in regulating the reduction of flavin but are not consistent with their suggestion of a role for p47phox in regulating heme reduction.

The flavin reductive half-reaction can be further subdivided into the following steps, shown in the upper part of Scheme II. The first step is the binding of NADPH to the enzyme and is described by the on and off rate constants k1 and k2. A second step, described by k3 and its reverse, k4, is included for completeness and describes for example a hypothetical conformational or other non-chemical rearrangements that occur prior to flavin reduction itself. The final step (k5), the hydride transfer from NADPH to FAD, is the isotopically sensitive step.

The present studies have utilized deuterium kinetic isotope effects to investigate this reaction. As described above, using Equation 1, it is possible to derive the actual Kd for NADPH binding to the enzyme from the Km, plus the deuterium isotope effects on K and V/K. As described by Klinman and Matthews (50), Kd in this case is defined as the dissociation constant of substrate from all preisotopic complexes. In a case in which binding is the only preisotopic step (i.e. no conformational or other change prior to hydride transfer), then

\[ K_d = \frac{k_5 k_4}{k_1 (k_3 + k_4)} \]  

We observed a small effect (on the order of 20%) of the p67phox V205A mutation in the Kd for NADPH (Table II). Although seen in three experiments, the effect were not of sufficient magnitude to account for the much larger effect of this mutation on the Vmax. Thus, the Kd is affected only minimally, so it is unlikely that any of the preisotopic steps described by k1, k2, k3, or k4 is significantly regulated by p67phox. A possible exception is that k2 and k3 are both increased by p67phox, leaving the Kd unaffected. However, if k1 were rate-limiting, then increasing k1 to the point where it was no longer rate-limiting (or was less rate-limiting) should unmask a larger isotope effect on Vmax. Thus, these data are most consistent with the regulation by p67phox of the flavin reduction step, i.e. activation of k5 or inhibition of k8. It was also possible that product dissociation from the prior catalytic cycle might be the step that is regulated by p67phox. In the limiting case where product dissociation is

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the single slow step, this should suppress the isotope rate effect. The ability to observe a significant isotope effect in V and $V/K$ therefore argues against this interpretation. In addition, if product dissociation were rate-limiting, then the products of more weakly binding analogs of NADPH should dissociate more readily from their binding sites, increasing the $V_{\text{max}}$. This is not the case (Table III); the $V_{\text{max}}$ is nearly the same for three analogs of NADPH whose $K_m$ values vary 40-fold. These data indicate that product release is not rate-limiting nor is it regulated by p67phox.

Thus, the activation domain of p67phox regulates the reduction of the flavin by NADPH. There is little effect on the affinity of NADPH for forms of the enzyme prior to the hydride transfer step, and data are therefore most consistent with regulation by p67phox of hydride/electron transfer from NADPH to flavin.

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