Simultaneous Presence of p47<sup>phox</sup> and Flavocytochrome b<sub>245</sub> Are Required for the Activation of NADPH Oxidase by Anionic Amphiphiles

EVIDENCE FOR AN INTERMEDIATE STATE OF OXIDASE ACTIVATION* (Received for publication, February 10, 1999, and in revised form, March 23, 1999)

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We have examined the kinetics of NADPH oxidase activation induced by arachidonic acid or SDS in a cell-free system using mixtures of recombinant Phox proteins and purified flavocytochrome b<sub>245</sub>. Activation of oxidase activity required the simultaneous presence of p47<sup>phox</sup>, flavocytochrome b<sub>245</sub>, and the anionic amphiphile. The activation of electron transfer reactions was much more rapid when iodonitrotetrazolium violet was used as electron acceptor than when oxygen alone was the acceptor. We propose that this difference represents an intermediate activation state of NADPH oxidase in which electron flow can proceed from NADPH to enzyme flavin (and hence to iodonitrotetrazolium violet) but not from flavin to heme (or not between the hemes). A model for NADPH oxidase activation is presented that is consistent with these observations.

NADPH oxidase is a multicomponent electron transport system mainly expressed in phagocytic cells of the immune system. Normally dormant, NADPH oxidase is activated after stimulation of these cells resulting in the vigorous production of superoxide (O<sub>2</sub><sup>-</sup>) at the expense of NADPH (1). The physiologic role of the enzyme is to contribute to microbial killing by the generation of reactive oxygen species derived from O<sub>2</sub><sup>-</sup> that are toxic to microorganisms. Genetic defects in components of NADPH oxidase lead to a severe predisposition to bacterial and fungal infections, a syndrome known as chronic granulomatous disease (2). The NADPH oxidase enzyme complex consists of a membrane-bound flavocytochrome, flavocytochrome b<sub>245</sub> that contains two molecules of heme and one molecule of FAD in a gp91<sup>phox</sup>-p22<sup>phox</sup> heterodimer (3–8). In unstimulated cells, the other components of the complex, p47<sup>phox</sup>, p67<sup>phox</sup>, and Rac2, are present in the cytosol; upon activation, they associate with flavocytochrome b<sub>245</sub> on the membrane to form the active enzyme (9–13). A fourth cytosolic component, p40<sup>phox</sup>, is also implicated in NADPH oxidase activity, but the role it plays is poorly understood at present (14, 15). Understanding of the mechanism and activation of NADPH oxidase has been greatly facilitated by the development of a cell-free system. In the simplest form, NADPH-dependent O<sub>2</sub><sup>-</sup> can be generated by mixing neutrophil membranes (containing flavocytochrome b<sub>245</sub>) and neutrophil cytosol (containing p40<sup>phox</sup>, p47<sup>phox</sup>, p67<sup>phox</sup>, and Rac2) with an anionic amphiphile and NADPH. In both whole cells and the cell-free system, there is a characteristic lag period before the oxidase activity appears after the addition of neutrophils (to whole cells) or amphiphile (to the cell-free system). Previous work has shown that a decrease in the lag period can, only be achieved by simultaneous incubation of neutrophil membranes and cytosol with the anionic amphiphile and not by preincubation of one or the other with anionic amphiphile (16, 17). Recent reports using intrinsic tryptophan fluorescence and circular dichroism suggest that SDS and AA<sub>3</sub> caused conformational changes in p47<sup>phox</sup> at concentrations similar to those required to induce oxidase activity (18, 19). Furthermore, Sumimoto and co-workers (20) reported that C-terminally truncated forms of p47<sup>phox</sup> and p67<sup>phox</sup> exhibit a diminished requirement for SDS, which would be consistent with p47<sup>phox</sup> (or p67<sup>phox</sup>) being a primary target for anionic amphiphiles.

We have previously provided evidence that electron transfer in NADPH oxidase is regulated at several points. First, at the site where electrons enter the oxidase, because in the nonactivated enzyme, FAD does not become reduced in the presence of NADPH (21, 22). Second, there is a kinetic barrier between the flavin center and the heme, because they are not in thermodynamic equilibrium during oxidase turnover (23–26). Finally, rapid electron transfer within the oxidase only occurs in the presence of oxygen, suggesting regulation at the level of heme (21, 23, 27, 28). To further understand the events that occur during oxidase activation, we have taken advantage of a cell-free system consisting of highly purified flavocytochrome b<sub>245</sub> and recombinant p47<sup>phox</sup>, p67<sup>phox</sup>, and Rac2 to perform experiments aimed at dissecting the target(s) of the anionic amphiphile during oxidase activation.

EXPERIMENTAL PROCEDURES

Recombinant Proteins—Oligonucleotide primers corresponding to each of the human NADPH oxidase subunits were used to amplify respective cDNA from a human neutrophil cDNA library (CLONTECH, Palo Alto, CA). Polymerase chain reaction-amplified products were purified by agarose gel electrophoresis and ligated into the baculovirus transfer vector pVL1393 (Pharmingen, San Diego, CA). Constructs were used to transform Escherichia coli and single colonies were picked from plated LB broth cultures under selective pressure of 50 μg/ml carbenicillin. Purified vector clones were sequenced to confirm cDNA sequence integrity. Purified pVL1393 plasmid was used for homologous recombination with BaculoGold baculovirus (Pharmingen) before infec-

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1 The abbreviations used are: AA, arachidonic acid; GTPγS, guanosine 5′-O-(3-thiotriphosphate)tetralithium salt; INT, iodonitrotetrazolium violet; SOD, superoxide dismutase; PIPES, 1,4-piperazineethanesulfonic acid; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.
Anionic Amiphile Activation of NADPH Oxidase

Fig. 1. NADPH oxidase activation is independent of anionic amphiphile at high p67phox and Rac2 concentrations in the absence of p47phox. NADPH oxidase activity was determined as described under “Experimental Procedures.” The reactions were initiated by the addition of 160 μM NADPH after 5 min of preincubation with or without SDS. The shaded bars refer to the standard concentrations of Phox proteins (2.0 nm flavocytochrome b, 100 nm p47phox, 67 nm p67phox, 290 nm Rac2). The open bars refer to a system containing high concentrations of cytosolic phox components (2.0 nm flavocytochrome b, 3.1 μM p47phox, 4.0 μM p67phox, and 175 nm Rac2). For comparison, the high concentration system employed by Lambeth and colleagues (31) used 10 nm flavocytochrome b, 6.0 μM p47phox, 6.0 μM p67phox, and 2.0 μM Rac1, and the system used by Pick and colleagues (32) used 1.75 nm flavocytochrome b, 880 nm p47phox, 2.95 μM p67phox, and 1.0 μM Rac2. The values are the means ± S.E. of two separate experiments performed in duplicate.

Large scale production of recombinant oxidase factors was carried out in Sf9 cells (rp47phox and rpRac2) or Hi5 cells (rp40phox and rp67phox). Briefly, master viral stocks were used at a multiplicity of infection of 5–10, infecting 2 × 10^6 insect cells in 15-20 ml of TMN-FH medium (Pharmingen) in 150-mm diameter culture dishes; 50 of these plates were used for each production run. Cells were allowed to incubate for three days at 28 °C before harvest; all subsequent manipulations and purifications were carried out at 4 °C. Insect cells were obtained by scraping the culture dishes and centrifuging the pooled cultures at 500 × g for 7 min. The cell pellet was resuspended in 200 ml of phosphate-buffered saline and again centrifuged at 500 × g for 7 min. This cell pellet was then resuspended in 50 ml of dissection buffer: 10 mM PIPES, pH 7.3, 100 mM KCl, 3 mM NaCl, 3.5 mM MgCl2, 48 μg/ml [4-2-aminoethoxybenzyl]sulfonyl fluoride, HCl], 1 μg/ml aprotinin, 151 μg/ml benzamidine, 5 μM diisopropylfluorophosphate, 50 μg/ml leupeptin, 0.7 μg/ml pepstatin. Cell suspensions were disrupted by nitrogen cavitation (450 psi, 20 min) and (for the cytosolic factors rp40phox, rp67phox, and the cavitate was subjected to a 10,000 × g centrifugation for 10 min. The resulting supernatant was then collected and centrifuged at 100,000 × g for 1 h; the supernatant from this high speed spin used as starting material for chromatographic purifications. Treatment of the cavitate differed slightly for rpRac2: only the 100,000 × g centrifugation was performed, and the resulting pellet was then solubilized in 50 ml of extraction buffer: 200 ml Tris, pH 7.5, 0.6% (w/v) CHAPS. This extract was centrifuged at 100,000 × g for 1 h, and the supernatant was collected for subsequent chromatography.

rp40phox was purified at pH 6.0 by anion exchange (HiLoad Q-Sepharose fast flow) (Amersham Pharma Biotech), hydrophobic interaction chromatography (HiLoad phenyl-Sepharose high performance) (Amersham Pharma Biotech). Pure rp40phox was frozen to −80 °C at 10 μM in 20 mM Tris, pH 7.5, 0.1 mM dithiothreitol, 0.15 mM phenylmethylsulfonyl fluoride. rp47phox was purified at pH 7.5 by cation exchange (HiLoad SP-Sepharose fast flow), heparin chromatography (1 ml HiTrap heparin) (Amersham Pharma Biotech), hydrophobic interaction chromatography (HiLoad phenyl-Sepharose high performance), and size-exclusion gel chromatography (HiLoad SuperDex 75) (Amersham Pharma Biotech). rp67phox was frozen to −80 °C at 10 μM in 20 mM Tris, pH 7.0, 0.1 mM dithiothreitol, 0.15 mM phenylmethylsulfonyl fluoride.

rpRac2 detergent extract was purified by anion exchange (HiLoad Q-Sepharose fast flow). Fractions containing pure rpRac2 were pooled (∼15 ml) and diluted to 100 ml with nucleotide loading buffer: 20 mM Tris, pH 7.5, 1 mM EDTA, 2 mM dithiothreitol, 100 μM GTPγS. Concentration was performed in a 200-ml stirred cell through a YM10 membrane (Amicon, Beverly, MA) to ∼6 ml, and volume brought back to 100 ml with fresh nucleotide loading buffer. The sample was again centrifuged to ∼6 ml, then brought to 100 μM fresh GTPγS and 5 mM MgCl2. rpRac2 was then frozen to −80 °C at 2 μl.

Each of the recombinant oxidase factors was analyzed by mass spectrometry to confirm correct molecular weight; proteins were also subjected to SDS-polyacrylamide gel electrophoresis and subsequent silver staining to assess homogeneity. Western blotting of SDS-polyacrylamide gel electrophoresis gels and subsequent incubation with anti-peptide (all subunits) and monoclonal (p47 only) antibodies showed that the recombinant proteins were immunoreactive and migrated identically to their counterparts in purified human neutrophil cytosol.

Activation of NADPH Oxidase in the Cell-Free System—Cell-free NADPH oxidase assays were performed at 21 °C in 96-well plates as described previously (29) using a final concentration of 80 μM SDS or 20 μM AA. These concentrations were found to elicit the maximum rate of O2 generation. Reactions were carried out in a final volume of 150 μl, which contained 0.3 pmol of flavocytochrome b, 15 pmol of p47phox, 10 pmol of p67phox, and 4 pmol of Rac2. 10 pmol of p40phox was included where noted. For preincubation assays, each reaction mixture was split into three equal parts. The first contained the proteins to be incubated with anionic amphiphile, the anionic amphiphile (80 μM SDS or 20 μM AA) and cytochrome c. The second contained NADPH and the proteins not to be preincubated with amphiphile. The third portion contained relaxation buffer and either 160 μM SDS or 40 μM AA, to maintain the final amphiphile concentration in the assay at 80 μM SDS or 20 μM AA. GTPγS was always included in the portion containing Rac2, and FAD was always included in the portion containing flavocytochrome b. After 5 min of preincubation, the three portions were mixed together in the microtiter plate, and the reduction of cytochrome c was monitored at 550 nm. The time taken between mixing and the first measurement was approximately 25 s. The “complete” preincubation mixture refers to the reaction mixture that contained all the assay components (including anionic amphiphile) with the exception of NAPDH.

To check that the rates of reduction of cytochrome c reflect the true O2-generating activity of the system and are not a result of a lack of sensitivity at low rates of O2 production, control assays were carried out at a range of cytochrome c concentrations using a sensitive spectrophotometer (Perkin-Elmer Lambda 18) and 1-cm path length cuvettes. A 5-fold increase in cytochrome c concentration gave the same rate as the standard assay over a wide range of oxidase activities and therefore probably accurately reflects the true rate of O2 even at low rates of O2 production (data not shown).

INT reductase assays were performed as described above for the O2 assay, except 100 μM INT was substituted for the cytochrome c and 300
units/ml of SOD were included in the mixture. The rate of reduction of INT was determined from the absorbance change at 490 nm as described previously (30).

Spectrophotometric Assays—In cases where it was advantageous to examine the earliest parts of the time course, assays were performed in semi-micro cuvettes in a Perkin-Elmer Lambda 18 spectrophotometer. Each reaction mixture contained the same concentrations of reactants as the microtiter assay in a final volume of 750 μl.

Measurement of the Lag Time—Three methods were used to estimate the lag times: (a) time to maximum reaction velocity; (b) extending a tangent from the steepest part of the curve to the x axis, and; (c) measuring the onset time (time taken to produce an arbitrary amount of O_2
). Each method gave qualitatively the same results. Of the three, the onset time was found to give the most reproducible results and was used to calculate the data presented here. The onset time of each reaction was taken as the time needed by that mixture to generate the same amount of O_2 as made by the complete reaction mixture during the initial 100 s following the addition of NADPH.

RESULTS

NADPH Oxidase Activity Is Independent of Both p47phox and Anionic Amphiphile at High Concentrations of p67phox and Rac2—Two groups have recently reported that NADPH oxidase can be activated in a cell-free system in the absence of p47phox, under conditions where p67phox and Rac are present at much higher concentrations than are normally employed (31, 32). In both cases, they reported that the activity was also dependent upon the presence of anionic amphiphile (AA or lithium dodecyl sulfate). In our hands however, we found p47phox-independent oxidase activity was completely independent of anionic amphiphiles at concentrations of p67phox and Rac similar to those used by Pick, Lambeth, and co-workers (31, 32) in these previous reports (Fig. 1). This difference may be due to differences in the flavocytochrome b-245 preparation (purity, reconstituted lipid composition, FAD content, detergent, etc.) or that we employed Rac2 in our experiments as opposed to Rac1. More than 96% of Rac in human neutrophils is the Rac2 form (33). Because the absence of p47phox allowed activation of the system without addition of amphipathic stimulus, the data strongly suggest that the primary target for the anionic amphiphiles was p47phox.

Kinetics of O_2
 Production after Preincubation of Mixtures of Phox Components with SDS—As shown previously, preincubation of all phox components together with SDS for 5 min (the complete mixture) before the addition of NADPH completely ablished the lag seen under conditions of no preincubation (Fig. 2, trace b versus trace a). Preincubation of flavocyto-
chrome $b_{-245}$ with SDS alone or preincubation of cytosolic phox components with SDS alone, did not shorten the lag for NADPH oxidase activity below the control reaction without any SDS preincubation. Similarly, preincubation of flavocytochrome $b_{-245}$ with SDS and preincubation of cytosolic components with SDS concomitantly but in separate tubes did not significantly reduce the lag time (Fig. 2, trace c). Preincubation of the recombinant cytosolic components with SDS in the absence of flavocytochrome $b_{-245}$ tended to decrease the final maximum activity, however, consistent with previous studies using neutrophil cytosol (34) (Fig. 2, trace c). The results of preincubation of cytosolic components individually and in combination with flavocytochrome $b_{-245}$ and SDS are shown in Fig. 3. The lag was only significantly reduced when p47$^{phox}$ and flavocytochrome $b_{-245}$ were preincubated together with SDS (bars D, H, and J). Preincubations of p67$^{phox}$ + flavocytochrome $b_{-245}$ + SDS (bar E) or Rac2 + flavocytochrome $b_{-245}$ + SDS (bar F) did not reduce the lag. Somewhat surprisingly, the simultaneous presence of p67$^{phox}$ + p47$^{phox}$ + flavocytochrome $b_{-245}$ + SDS (bar G) in the preincubation mixture did not decrease the lag, despite the fact that preincubation of p47$^{phox}$ + flavocytochrome $b_{-245}$ + SDS (bar D) alone did. This suggests that p67$^{phox}$ can interfere with productive interactions between p47$^{phox}$ and flavocytochrome $b_{-245}$. Interestingly, if Rac2 is also present, the lag is once again reduced (bar J), suggesting that interactions between p67$^{phox}$ and Rac2 may be important during the activation process.

We have recently reported that in the absence of Mg$^{2+}$ there is a significant SDS-independent spontaneous activation of oxidase activity in this system (29). A series of experiments using Mg$^{2+}$-free conditions gave qualitatively the same results as those shown, except in each case there was more O$_2$-generating activity evident at the time of the first reading in the Mg$^{2+}$-free experiments.

Kinetics of O$_2$ Production after Preincubation of Mixtures of phox Components with AA—A similar series of experiments to those described above was performed using AA in place of SDS. In both types of experiment, the maximal velocity is similar regardless of activator, although the onset time is somewhat shorter with AA. As in the case of SDS, preincubation of flavocytochrome $b_{-245}$ with AA (Fig. 4, bar B) or preincubation of cytosolic components with AA (bar C) did not shorten the lag for NADPH oxidase activity below the control reaction without any AA preincubation (bar A). Similarly, preincubation of flavocytochrome $b_{-245}$ with AA or recombinant cytosol with AA concomitantly but separately did not significantly reduce the lag time. As in the case of SDS, preincubation of p47$^{phox}$ + flavocytochrome $b_{-245}$ + AA (Fig. 4, bar D) caused significant reduction in the lag. Unlike the SDS incubations, however, the combination of p47$^{phox}$ + p67$^{phox}$ + flavocytochrome $b_{-245}$ + AA (bar G) reduced the lag maximally, whereas the combination of p47$^{phox}$ + Rac2 + flavocytochrome $b_{-245}$ + AA (bar H) did not reduce the lag at all. This suggests that Rac2 can interfere with productive interactions between p47$^{phox}$ and flavocytochrome $b_{-245}$ when AA is the activator. Because this is not the case for SDS, it indicates there may be significant differences in the way in which SDS and AA cause oxidase activation.

The Effect of p40$^{phox}$ on the Kinetics of NADPH Oxidase Activation—The effect of p40$^{phox}$ was also examined in a series of experiments. In no case did p40$^{phox}$ appear to affect the lag time or maximal velocity of the reaction (data not shown).

Kinetics of INT Reduction after Preincubation of Mixtures of phox Components with SDS—We next examined the kinetics of activation of INT reductase activity. We have previously shown that INT accepts electrons from the flavin center of NADPH oxidase and that there is separate control of electron transfer to INT and to the cytochrome c assay at low rates of O$_2$ formation, as described under “Experimental Procedures.” Most strikingly, there is a very high rate of INT reductase activity in assays where p47$^{phox}$ + flavocytochrome $b_{-245}$ + SDS or p47$^{phox}$ + Rac2 + flavocytochrome $b_{-245}$ + SDS were preincubated, and these activities had virtually no lag (Fig. 5A, traces a and b). The rates in both cases were much higher than the rate obtained when all the components (except NADPH) were preincubated together (trace c). In contrast, preincubations containing flavocytochrome $b_{-245}$ + SDS, p67$^{phox}$ + flavocytochrome $b_{-245}$ + SDS, or p67$^{phox}$ + Rac2 + flavocytochrome $b_{-245}$ + SDS showed the longest lag and the lowest maximal rates (Fig. 5B, traces d, f, and g). Incubation of the cytosolic factors with SDS in the absence of flavocytochrome...
Fig. 5. The effect of the composition of the SDS preincubation mixture on the kinetics of INT reductase activity by NADPH oxidase. INT reduction was followed using a kinetic microtiter plate assay as described under “Experimental Procedures.” In panel A, the preincubation mixtures contained SDS and as follows: flavocytochrome b$_{245}$ + p47$^{phox}$ + Rac2 (a); flavocytochrome b$_{245}$ + p47$^{phox}$ + p67$^{phox}$ (b); no Phox proteins (no preincubation) (d); flavocytochrome b$_{245}$ + p47$^{phox}$ + p67$^{phox}$ + Rac2 (complete preincubation) (e); p47$^{phox}$ + p67$^{phox}$ + Rac2 (f). In panel B, the preincubation mixtures contained SDS and as follows: flavocytochrome b$_{245}$ + Rac2 (a); flavocytochrome b$_{245}$ + p67$^{phox}$ (b); flavocytochrome b$_{245}$ + p67$^{phox}$ + Rac2 (c); flavocytochrome b$_{245}$ (d); complete preincubation but no NADPH addition (e). The traces are the averaged values of two wells of a representative experiment of three performed in duplicate.

Table I

The effect of the preincubation conditions on the rate of INT reduction

| Components preincubated with anionic amphiphile | Rate of INT reduction activated with |  |
|-----------------------------------------------|-------------------------------------|---|
|                               | cytochrome b$_{245}$ | p47$^{phox}$ | p67$^{phox}$ | Rac2 | SDS | AA |
| +                               | 2.30 ± 0.36 | 4.99 ± 0.39 |
| +                               | 2.54 ± 0.41 | 3.49 ± 0.14 |
| +                               | 5.31 ± 0.83 | 4.07 ± 0.27 |
| +                               | 1.09 ± 0.07 | 3.55 ± 0.09 |
| +                               | 5.24 ± 0.99 | 3.41 ± 0.29 |
| +                               | 1.13 ± 0.16 | 4.10 ± 0.30 |
| +                               | 1.90 ± 0.23 | 3.91 ± 0.10 |
| +                               | 1.48 ± 0.19 | 4.07 ± 0.10 |
| +                               | 0.92 ± 0.22 | 3.14 ± 0.04 |
| +                               | 0.56 ± 0.16 | 3.42 ± 0.10 |
| +                               | 0.93 ± 0.33 | 3.12 ± 0.14 |
| +                               | 1.64 ± 0.3  | 3.81 ± 0.12 |
| +                               | 1.52 ± 0.11 | 3.66 ± 0.05 |
| +                               | 1.25 ± 0.38 | 3.99 ± 0.18 |
| +                               | 1.49 ± 0.26 | 4.06 ± 0.15 |
| +                               | 1.19 ± 0.10 | 4.25 ± 0.15 |

b$_{245}$ also gave rise to rather low linear rates (Table I).

Kinetics of INT Reduction after Preincubation of Phox Components with AA—As in the case of SDS activation, the lag was much less pronounced during AA-dependent activation when INT was the electron acceptor. Unlike SDS activation, however, all incubations showed broadly similar initial rates, with the incubation containing all the Phox proteins having the highest rate. Preincubations of cytosolic factors with AA in the absence of flavocytochrome b$_{245}$ again gave somewhat lower rates of INT reductase activity (Table I).
Kinetics of NADPH Oxidation, INT Reduction, and \( \text{O}_2 \) Formation in the Presence and Absence of INT—Further evidence for an intermediate state of activation of NADPH oxidase was provided by experiments following NADPH oxidation in the presence or absence of INT. NADPH oxidation was recorded at 340 nm after the addition of SDS to the cuvette. The results are shown in Fig. 6. As can be seen from the traces, in the presence of INT, the onset of NADPH oxidation is faster than in the absence of the dye, and the rate is fastest during the first 2 min. In the absence of INT, NADPH oxidation follows a similar time course to \( \text{O}_2 \) production, with a long lag before reaching the maximum rate. The complementary experiments are shown in Fig. 7.

**Fig. 6.** The kinetics of NADPH oxidation by NADPH oxidase in the presence or absence of the electron acceptor INT. NADPH oxidation was determined spectrophotometrically as described under “Experimental Procedures.” The reactions were started by the addition of SDS. The mixing time (before the start of the recording) was approximately 10 s. Trace \( a \), NADPH oxidation in the absence of INT; trace \( b \), NADPH oxidation in the presence of 100 \( \mu \text{M} \) INT. The traces are representative of three separate experiments.

**Fig. 7.** The kinetics of INT reduction and \( \text{O}_2 \) formation after the activation of NADPH oxidase by SDS. INT reduction and \( \text{O}_2 \) formation were measured spectrophotometrically as described under “Experimental Procedures.” The reactions were started by the addition of SDS. The mixing time (before the start of the recording) was approximately 10 s. Trace \( a \), \( \text{O}_2 \) production (cytochrome \( c \) reduction); trace \( b \), INT reduction. The traces are representative of three separate experiments.
Fig. 7 following the reduction of cytochrome c by O$_2^-$ (at 550 nm) or the reduction of INT (at 490 nm). The traces parallel the NADPH oxidation rates, with the reduction of INT having a rapid onset and reaching the maximum rate within the first 2 min.

**DISCUSSION**

The principal action of both SDS and AA appears to be to promote an interaction between p47$^{phox}$ and flavocytochrome b$_{245}$. This process requires the simultaneous presence of both flavocytochrome b$_{245}$ and p47$^{phox}$ and results in almost complete elimination of the lag and subsequent high rates of oxidase activity. The secondary effect of SDS appears to be to promote interactions between Rac2 and flavocytochrome b$_{245}$ (or the flavocytochrome b$_{245}$p47$^{phox}$ complex), whereas the secondary effect of AA appears to promote interactions between p67$^{phox}$ and flavocytochrome b$_{245}$.

Taken together, the results presented above support a model in which association of the cytosolic factors with flavocytochrome b$_{245}$ initially results in an intermediate state of activation of NADPH oxidase by a relatively rapid process. In this intermediate state, electron transfer is facilitated from NADPH to INT. The traces parallel the oxidase activity. The secondary effect of SDS appears to be to promote an interaction between Rac2 and flavocytochrome b$_{245}$ (or the flavocytochrome b$_{245}$p47$^{phox}$ complex), whereas the secondary effect of AA appears to promote interactions between p67$^{phox}$ and flavocytochrome b$_{245}$.

A model that describes the observed kinetics is shown in Scheme 1. For simplicity, only the reactions involving flavocytochrome b$_{245}$ are shown. In this model, the anionic amphiphile (SDS or AA) causes a conformational change in p47$^{phox}$ that causes it to associate with flavocytochrome b$_{245}$. It seems that this conformational change only occurs in a productive manner when flavocytochrome b$_{245}$ is also present in the incubation mixture, because preincubation of p47$^{phox}$ with SDS or AA does not decrease the lag if flavocytochrome b$_{245}$ is not present. This complex is represented by p47$^{cyt}$. The binding of p47$^{phox}$ to flavocytochrome b$_{245}$ allows the high affinity binding of p67$^{phox}$ and Rac2 as previously proposed by Lambeth and colleagues (35), resulting in the intermediate state in which electron transfer can proceed from NADPH to INT. The formation of this intermediate state is relatively rapid, and consequently, little lag is evident in the onset of INT reductase activity. A subsequent slow step, possibly involving a conformational change in flavocytochrome b$_{245}$ (cyt $\rightarrow$ cyt$^a$ in Scheme 1), results in the final fully active NADPH oxidase. The relative rapid formation of the intermediate complex relative to the fully activated form would explain why INT reductase activity dominates the initial phase of oxidase activity, whereas O$_2^-$ production predominates the latter phase (Fig. 6).

It will clearly be of interest to study the factors that affect the formation of this intermediate state of oxidase activation and establish whether it is a reversible process.

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