Spontaneous activation of NADPH oxidase in a cell-free system: unexpected multiple effects of magnesium ion concentrations

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

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

The role of magnesium ions in the activation of NADPH oxidase has been investigated using flavocytochrome *b*_{–245} and either neutrophil cytosol or mixtures of recombinant p40^{phox}, p47^{phox}, p67^{phox} and Rac2. Purified flavocytochrome *b*_{–245} is highly active (turnover number 120–150 mol of O₂[–]/s per mol of cytochrome haem) in the absence of Mg²⁺, in marked contrast to neutrophil membranes or detergent-solubilized membranes, which have an absolute requirement for Mg²⁺ for NADPH oxidase activity. It was also found that Mg²⁺ affected the anionic amphiphile requirement for oxidase activation, and this was dependent on whether neutrophil cytosol or mixtures of recombinant cytosolic proteins were used in the assay. Unexpectedly we found that,

using purified flavocytochrome *b*_{–245} and recombinant cytosolic proteins, NADPH oxidase undergoes spontaneous activation in the absence of anionic amphiphiles under Mg²⁺-free conditions. The results suggest that Mg²⁺ ions play an important role in NADPH oxidase function, perhaps stabilizing the 260 kDa complex of cytosolic phox proteins or the regulation of a guanine nucleotide-binding protein. We provide evidence that if the latter explanation is correct, the identity of the guanine nucleotide-binding protein is unlikely to be Rap1a.

Key words: flavocytochrome *b*, p47^{phox}, p67^{phox}, Rac2, phagocyte oxidase.

INTRODUCTION

Treatment of neutrophils with a suitable agonist results in the production of a number of reactive oxygen species, such as O₂[–], H₂O₂ and HOCl, which are used by these cells to kill pathogenic organisms as they invade the host. All these reactive species are derived from O₂[–], the product of a tightly regulated, multi-component enzyme system, NADPH oxidase (see [1] for a recent review). In part, the activity of the oxidase is controlled by the compartmentalization of four of the components of the oxidase (p40^{phox}, p47^{phox}, p67^{phox} and Rac2) in the cytosol, separate from the catalytic centre, flavocytochrome *b*_{–245}, which is located in the plasma membrane and specific granule membranes. Of the cytosolic components, p40^{phox}, p47^{phox} and p67^{phox} are largely associated in the form of a 260–290 kDa complex [2,3]. Flavocytochrome *b*_{–245}, comprised of a gp91^{phox}/p22^{phox} heterodimer, contains two haem molecules and a molecule of FAD; the redox centres appear to be contained entirely within the gp91^{phox} subunit [4].

Activation of the oxidase requires the association of the cytosolic components with the membrane-bound flavocytochrome *b*_{–245} [5]. Understanding of the regulation and mechanism of NADPH oxidase has been greatly facilitated by the development of a cell-free activation system in which cytosolic fractions and membrane fractions derived from unstimulated neutrophils are mixed [6,7]. NADPH oxidase activity can then be elicited by the addition of anionic amphiphiles, such as SDS or arachidonic acid. This experimental system has been further refined by the use of detergent-solubilized membranes, highly purified or recombinant flavocytochrome *b*_{–245}, and by the replacement of neutrophil cytosol with mixtures of the recom-

binant cytosolic phox proteins [8–10]. Here we report some unexpected effects of Mg²⁺ ions that we discovered during our kinetic studies of the cell-free system.

EXPERIMENTAL

Isolation of neutrophils and preparation of subcellular fractions

Membranes and cytosolic fractions were prepared from unstimulated human neutrophils as described previously [8] and stored at –80 °C. Where indicated, magnesium was removed from cytosol by dialysis of 10 ml aliquots of cytosol for 16 h against 2 × 5 litres of Mg²⁺-free relaxation buffer containing 2 g of Chelex 100 beads.

Purification, relipidation and refluination of flavocytochrome *b*_{–245}

Flavocytochrome *b*_{–245} was purified using the method described previously (solubilization of salt-washed purified neutrophil membranes in heptyl-β-D-thioglucopyranoside [HTG], treatment of the extract with mixed-bed resins, and heparin column chromatography [11]) with the following modification. After elution from the heparin affinity column, the cytochrome-containing fraction was subjected to FPLC on a CHT-1 ceramic hydroxyapatite column (Bio-Rad, Hercules, CA, U.S.A.) equilibrated with 50 mM K₂HPO₄ buffer (pH 7.4) containing 0.5 mM dithiothreitol, 20 % (v/v) glycerol and 0.5 % HTG. After washing the column with 10 volumes of buffer, flavocytochrome *b*_{–245} was eluted with a 20 ml linear gradient of 50–1000 mM potassium phosphate buffer containing 0.5 mM dithiothreitol, 20 % glycerol and 0.5 % HTG. The peak fractions were

Abbreviations used: HTG, heptyl-β-D-thioglucopyranoside; SOD, superoxide dismutase.

¹ To whom correspondence should be addressed (e-mail scross@scripps.edu).

Table 1 Composition of reaction mixtures used for NADPH oxidase activation

In all reaction types, typical rates of O_2^- production were 10–15 nmol of O_2^- /min per pmol of flavocytochrome b_{-245} (80–125 mol of O_2^- /s per mol of haem). In reaction types D–F, activity was absolutely dependent on the presence of Rac2, p47^{phox} and p67^{phox}. p40^{phox} showed neither synergistic nor inhibitory activity under our experimental conditions.

Reaction type	Source of cytosolic factor	Source of flavocytochrome b_{-245}
A	Neutrophil cytosol	Neutrophil plasma membrane
B	Neutrophil cytosol	Detergent-solubilized membrane
C	Neutrophil cytosol	Purified flavocytochrome b_{-245}
D	Recombinant Rac2, p47 ^{phox} , p67 ^{phox} , p40 ^{phox}	Neutrophil plasma membrane
E	Recombinant Rac2, p47 ^{phox} , p67 ^{phox} , p40 ^{phox}	Detergent-solubilized membrane
F	Recombinant Rac2, p47 ^{phox} , p67 ^{phox} , p40 ^{phox}	Purified flavocytochrome b_{-245}

immediately desalted by passage over a 10 ml gel-filtration column (Bio-Rad Econo-Pac 10G) into buffer B [11] containing 0.5 % HTG. The resulting purified cytochrome was refluorinated and relipidated as described previously [11]. The final product contained 18–22 nmol of haem/mg of protein, based on a molar absorption coefficient of $21.6 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ (cytochrome haem) for the reduced minus oxidized absorbance band at 559 nm [12].

Recombinant phox components

Recombinant p40^{phox}, p47^{phox}, p67^{phox} and Rac2 were expressed in Sf9 cells and purified from cell lysates by ion-exchange and gel-filtration chromatography [13].

Cell-free O_2^- assay

Superoxide dismutase (SOD)-inhibitable rates of O_2^- production were measured in duplicate or triplicate in 96-well microtitre plates as described previously [14]. The final volume of 150 μl of Mg^{2+} -free relaxation buffer [8] contained 100 nM FAD, 10 μM guanosine 5'-[γ -thio]triphosphate, 166 μM NADPH and 100 μM cytochrome c . Each well was paired with a well of identical composition supplemented with 300 units of SOD. Assays using purified membranes (reaction types A and D, see Table 1), detergent-solubilized membranes (reaction types B and E) or purified flavocytochrome b_{-245} (reaction types C and F) employed 0.25–0.5 pmol of cytochrome (0.5–1.0 pmol of haem) per well. Assays using neutrophil cytosol employed 2.5×10^6 cell-equivalents per well. The recombinant cytosolic factors were used in the following quantities per well: p40^{phox}, 10 pmol; p47^{phox}, 10–15 pmol; p67^{phox}, 5 pmol; and Rac2, 2 pmol. Mg^{2+} and SDS were added at the concentrations indicated in the text or Figure legends. Assays were usually started by the addition of NADPH, following a 3 min incubation with SDS. Rates were determined from the maximum rate of cytochrome c reduction in the sample well, minus the rate of cytochrome c reduction in the SOD reference well.

RESULTS

Neutrophil membranes and purified flavocytochrome b_{-245} exhibit different magnesium requirements for maximum NADPH oxidase activity

The Mg^{2+} requirement of the fully soluble cell-free NADPH oxidase system has been described previously [15]. It was shown that using deoxycholate-solubilized neutrophil membranes, the system has an absolute requirement for Mg^{2+} , the concentration

Figure 1 Mg^{2+} requirement of flavocytochrome b_{-245} for oxidase activity during purification

Maximum rates of O_2^- production were determined by measuring the SOD-inhibitable reduction of cytochrome c in a kinetic microtitre-plate reader, as described in the Experimental section, in the presence of dialysed (Mg^{2+} -free) neutrophil cytosol. Rates of O_2^- production are expressed as a percentage of the maximum rate in the presence of 5 mM Mg^{2+} (6–12 nmol of O_2^- /pmol of flavocytochrome b_{-245}) in the presence of 100 μM SDS. The sources of flavocytochrome b_{-245} were neutrophil membranes (■); neutrophil membranes + 1 % HTG (▲); salt-washed neutrophil membranes solubilized in 1 % HTG (◆); detergent extract after mixed-bed resin treatment (●); detergent extract after heparin chromatography (□); and purified cytochrome after hydroxyapatite chromatography (▼). Error bars represent S.E.M.

dependence being approximately hyperbolic with a K_m of approximately 1 mM. As shown in Figure 1, we found a similar Mg^{2+} requirement for both neutrophil membranes and HTG-solubilized membranes; i.e. there was an absolute requirement for Mg^{2+} for oxidase activity that reached a plateau at approximately 3 mM Mg^{2+} . In marked contrast, the purified cytochrome did not have a requirement for Mg^{2+} and, in fact, Mg^{2+} was somewhat inhibitory, with the maximum rate of superoxide production being obtained in the absence of Mg^{2+} . Addition of EDTA (up to 2 mM) to Mg^{2+} -free assays did not cause significant loss of activity (results not shown). These experiments employed neutrophil cytosol that had been extensively dialysed to remove Mg^{2+} as described in the Experimental section. Similar results were obtained using a mixture of recombinant p40^{phox}, p47^{phox}, p67^{phox} and Rac2 ('recombinant cytosol') in place of normal cytosol.

The different magnesium requirements for membranes and purified cytochrome are not related to the presence of Rap1a

Rap1a (Krev1) is closely associated with flavocytochrome b_{-245} and it has been suggested that Rap1a is involved in the regulation of NADPH oxidase activity [16–18]. Since Mg^{2+} is known to affect the GTPase and/or guanine nucleotide-exchange properties of GTP-binding proteins, we hypothesized that the dramatic change in Mg^{2+} requirement between the crude membranes and highly purified flavocytochrome b_{-245} preparations might be the result of the removal of Rap1a during purification. We therefore examined samples from each stage of the purification for the presence of Rap1a by immunoblot, using a commercial anti-Rap1a antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, U.S.A.). The results (Figure 2, upper panel) show that Rap1a is selectively removed during heparin affinity chromatography, and the final cytochrome preparation has undetectable levels of Rap1a. We compared the results of this experiment with

Figure 2 Loss of Rap1a during flavocytochrome *b*₂₄₅ purification does not correlate with NADPH oxidase Mg²⁺-concentration dependence

(Upper panel) Protein immunoblots showing the presence of Rap1a in fractions during flavocytochrome *b*₂₄₅ purification. Samples containing 10 pmol of flavocytochrome *b*₂₄₅ from: neutrophil plasma membranes (lane 1); neutrophil membranes + 1% HTG (lane 2); salt-washed neutrophil membranes solubilized in 1% HTG (lane 3); detergent extract after mixed-bed resin treatment (lane 4); detergent extract after heparin chromatography (lane 5); and purified cytochrome after hydroxyapatite chromatography (lane 6) were subjected to SDS/PAGE on 12.5% acrylamide gels. After electrophoretic transfer of the proteins to PVDF, the blots were probed with a rabbit polyclonal antibody to Rap1a (Santa Cruz Biotechnology Inc.). Goat anti-rabbit antibodies were used to detect the primary antibody and the blots were developed using the Amersham enhanced chemiluminescence (ECL) system (Amersham, Arlington Heights, IL, U.S.A.). (Lower panel) Comparison of NADPH oxidase activity and Rap1a content of fractions from flavocytochrome *b*₂₄₅ purification. O₂⁻-generating activity was measured in the absence of Mg²⁺ as described in the Experimental section using dialysed neutrophil cytosol. Rap1a content was estimated from image analysis of the immunoblot shown in the upper panel using an Alphamager 2000 analyser (Alpha Innotech Inc., San Leandro, CA, U.S.A.). Error bars represent S.E.M. Cyt., flavocytochrome *b*.

the change in Mg²⁺ dependence of the oxidase activity that occurs at each stage during flavocytochrome *b*₂₄₅ purification (Figure 2, lower panel). In contrast to the sudden loss of Rap1a, the change in Mg²⁺ dependence of oxidase activity occurs in a stepwise manner during the purification process, and does not correlate with the removal of Rap1a (Figure 2, lower panel). Addition of Rap1a in the form of flavocytochrome *b*₂₄₅-deficient neutrophil membranes (from a gp91^{phox}-deficient chronic granulomatous disease patient with normal levels of Rap1a [19]) to mixtures of purified flavocytochrome *b*₂₄₅ did not restore the Mg²⁺-requirement (results not shown).

Neutrophil cytosol and 'recombinant cytosol' exhibit different SDS requirements for activity, depending on the Mg²⁺ concentration

We next examined the SDS requirement for NADPH oxidase activation at different Mg²⁺ concentrations (Figure 3, upper panel, reaction type C). At the Mg²⁺ concentrations normally used in the cell-free system (5–7 mM), the SDS optimum is around 110 μM SDS using either purified flavocytochrome *b*₂₄₅ (reaction type C) or HTG-solubilized membranes (reaction type

Figure 3 A comparison of the effects of Mg²⁺ concentration on the SDS requirement for NADPH oxidase activation using neutrophil cytosol or recombinant cytosol

Cell-free activation of NADPH oxidase activity was performed as described in the Experimental section using 0.3 pmol of purified flavocytochrome *b*₂₄₅ per reaction. (Upper panel) Activation of oxidase activity using neutrophil cytosol in the absence (■) or presence (▲) of 5 mM Mg²⁺. (Lower panel) Activation of oxidase activity using recombinant cytosol in the absence (■) or presence of 2.5 mM (◆), 5 mM (▲) or 7.5 mM (▼) Mg²⁺. Error bars represent S.E.M.

B) in combination with cytosol. This compares to 80–100 μM SDS for neutrophil membranes (reaction type A) or 40 μM for deoxycholate-solubilized membranes [14] and the differences presumably reflect the interactions of the different detergents with SDS. Removal of Mg²⁺ resulted in a modest increase in the optimum for SDS to 140 μM. In contrast, recombinant cytosol had an SDS optimum of around 100 μM regardless of Mg²⁺ concentration (reaction type F). In both systems, the activity was higher in the absence of Mg²⁺ (Figure 3).

Recombinant cytosol exhibits SDS-independent activity in the absence of magnesium

Unexpectedly, it was found that, in the absence of Mg²⁺, purified flavocytochrome *b*₂₄₅ and recombinant cytosol were capable of generating O₂⁻ at a significant rate in the complete absence of SDS (Figure 3, lower panel, and Figure 4, reaction type F). These

Figure 4 Time course of O_2^- production by purified flavocytochrome b_{-245} and recombinant cytosol (reaction type F) in the presence and absence of Mg^{2+} and SDS

NADPH oxidase activity was determined as described in the Experimental section in the absence (traces A and C) or presence (traces B and D) of 5 mM Mg^{2+} , and the absence (C and D) or presence (A and B) of 120 μ M SDS. Each trace is representative of at least 4 experiments.

rates were 20–25% of the maximum rates obtained at optimal SDS and Mg^{2+} concentrations. The higher rates obtained in the presence of SDS had an optimum around 100 μ M SDS. Neutrophil cytosol did not support SDS-independent activity, regardless of Mg^{2+} concentration.

DISCUSSION

The results presented above demonstrate that Mg^{2+} exerts at least two apparently unrelated effects on the components of NADPH oxidase in the cell-free system; one effect is on the membrane components and the other on the cytosolic components. First, there is a very different requirement for Mg^{2+} by flavocytochrome b_{-245} in membranes or detergent extracts (reaction types A, B, D and E) as opposed to highly purified cytochrome (reaction types C and F). The gradual loss of the Mg^{2+} requirement during purification is likely to be due to the loss of some protein or lipid during the procedure. Possibly, Mg^{2+} is required to suppress an inhibitory effect of this component. An attractive candidate is a guanine nucleotide-binding protein, such as Rap1a, that might normally inhibit flavocytochrome b_{-245} in the GTP (or GDP)-bound form, but not in the complementary form or in the absence of bound nucleotide. Mg^{2+} would be required for the binding of guanine nucleotides and/or the conversion of one form to the other. However, it is very clear that the loss of Mg^{2+} requirement and the loss of Rap1a from the cytochrome do not correlate and thus Rap1a cannot play this role. It is possible however, that another, as-yet-unidentified, protein performs this function. It is also evident that flavocytochrome b_{-245} does not have a Mg^{2+} requirement for the binding of FAD or NADPH nucleotides since it is fully active in the complete absence of Mg^{2+} ions.

The second effect of Mg^{2+} is that affecting the cytosol. The SDS dependence of neutrophil cytosol (reaction types A–C) differs from the SDS dependence of recombinant cytosol (reaction types D–F) according to the Mg^{2+} concentration. Lowering the Mg^{2+} concentration raises the concentration of SDS required to activate NADPH oxidase optimally when neutrophil cytosol is used, whereas lower concentrations of SDS are required for

maximum activity when Mg^{2+} concentrations are reduced in recombinant cytosol. Differences in SDS requirement for the two different systems are probably due in part to the lower concentration of protein present in the recombinant system. Non-specific binding of SDS to unrelated proteins present in neutrophil cytosol might decrease the effective SDS concentration. More importantly, in neutrophil cytosol, the $p40^{phox}$, $p47^{phox}$ and $p67^{phox}$ are thought to exist as a high-molecular-mass complex. Activation of the oxidase may be achieved, in part, by changes in the composition or intermolecular interactions between the components of the complex. In the cell-free system, there is evidence that this is brought about by SDS disrupting interactions between the components [20–22]. In recombinant cytosol the components are added individually and (initially, at least) cannot be in the form of a heteromeric complex. Thus, there would be no SDS requirement to separate the components of the oxidase. We hypothesize that Mg^{2+} ions may be involved in stabilizing the complex of cytosolic factors and that higher concentrations of SDS may be required to disrupt these interactions when Mg^{2+} ions are present.

The optimum SDS requirement for the system may represent a compromise between the SDS requirement of the cytosol for activation, and an SDS requirement of flavocytochrome b_{-245} for activity. The SDS requirement of the latter is likely to be largely a reflection of the type and amount of lipid and detergent present, depending on the source of the cytochrome (membranes, detergent extract or purified flavocytochrome b_{-245}).

The most unexpected feature of this work is the observation that in the absence of Mg^{2+} , recombinant cytosol exhibits significant activity in the absence of an amphipathic activator. This phenomenon was only observed when highly purified material was the source of flavocytochrome b_{-245} ; it did not occur when ordinary cytosol was used in place of the recombinant proteins, and it did not occur in the presence of Mg^{2+} . This effect does not appear to be a simple consequence of changes in membrane fluidity during flavocytochrome b_{-245} purification, as it was unaffected by temperature (21 °C or 37 °C) and appeared specific for Mg^{2+} ions (Mn^{2+} and Ca^{2+} did not prevent the SDS-independent activation; results not shown). These observations are consistent with both a role for Mg^{2+} in stabilizing the 260 kDa cytosolic complex in an inactive state where it does not interact with flavocytochrome b_{-245} and/or the presence of a Mg^{2+} -dependent G-protein that inhibits NADPH oxidase activation. In the first case, we hypothesize that destabilization (and consequent activation) of the cytosolic complex is promoted by SDS or loss of Mg^{2+} . Thus, less SDS is needed to optimally destabilize the complex in the absence of Mg^{2+} ions, or when recombinant (non-complexed) proteins are used in place of normal cytosol. In the second case, we hypothesize that a guanine nucleotide-dependent protein (distinct from Rap1a) may be associated with flavocytochrome b_{-245} in neutrophil membranes. Normally this protein would function to inhibit NADPH oxidase activity unless Mg^{2+} -dependent nucleotide exchange (or hydrolysis) occurs. The inhibitory function of this protein is lost as it is separated during purification of flavocytochrome b_{-245} and, with it, the Mg^{2+} requirement for oxidase activation.

This work was supported by National Institutes of Health grant AI24838.

REFERENCES

- DeLeo, F. R. and Quinn, M. T. (1996) *J. Leukocyte Biol.* **60**, 677–691
- Park, J.-W., El Benna, J., Scott, K. E., Christensen, B. L., Chanock, S. J. and Babior, B. M. (1994) *Biochemistry* **33**, 2907–2911
- Someya, A., Nagaoka, I. and Yamashita, T. (1993) *FEBS Lett.* **330**, 215–218

- 4 Yu, L., Quinn, M. T., Cross, A. R. and Dinayer, M. C. (1998) *Proc. Natl. Acad. Sci. U.S.A.* **95**, 7993–7998
- 5 Clark, R. A., Volpp, B. D., Leidal, K. G. and Nauseef, W. M. (1990) *J. Clin. Invest.* **85**, 714–721
- 6 Bromberg, Y. and Pick, E. (1984) *Cell. Immunol.* **88**, 213–221
- 7 Heyneman, R. A. and Vercauteren, R. E. (1984) *J. Leukocyte Biol.* **36**, 751–759
- 8 Curnutte, J. T., Kuver, R. and Babior, B. M. (1987) *J. Biol. Chem.* **262**, 6450–6452
- 9 Rotrosen, D., Yeung, C. L. and Katkin, J. P. (1993) *J. Biol. Chem.* **268**, 14256–14260
- 10 Abo, A., Boyhan, A., West, I., Thrasher, A. J. and Segal, A. W. (1992) *J. Biol. Chem.* **267**, 16767–16770
- 11 Cross, A. R. and Curnutte, J. T. (1995) *J. Biol. Chem.* **270**, 6543–6548
- 12 Cross, A. R., Jones, O. T. G., Harper, A. M. and Segal, A. W. (1981) *Biochem. J.* **194**, 599–606
- 13 Leto, T. L., Garrett, M. C., Fujii, H. and Nunoi, H. (1991) *J. Biol. Chem.* **266**, 19812–19818
- 14 Cross, A. R., Yarchover, J. L. and Curnutte, J. T. (1994) *J. Biol. Chem.* **269**, 21448–21454
- 15 Babior, B. M., Kuver, R. and Curnutte, J. T. (1988) *J. Biol. Chem.* **263**, 1713–1718
- 16 Bokoch, G. M., Quilliam, L. A., Bohl, B. P., Jesaitis, A. J. and Quinn, M. T. (1991) *Science* **254**, 1794–1796
- 17 Maly, F.-E., Quilliam, L. A., Dorseuil, O., Der, C. J. and Bokoch, G. M. (1994) *J. Biol. Chem.* **269**, 18743–18746
- 18 Gabig, T. G., Crean, C. D., Mantel, P. L. and Rosli, R. (1995) *Blood* **85**, 804–811
- 19 Quinn, M. T., Curnutte, J. T., Parkos, C. A., Mullen, M. L., Scott, P. J., Erickson, R. W. and Jesaitis, A. J. (1992) *Blood* **79**, 2438–2445
- 20 Sumimoto, H., Kage, Y., Nunoi, H., Sasaki, H., Nose, T., Fukumaki, Y., Ohno, S., Minakami, S. and Takeshige, K. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 5345–5349
- 21 Swain, S. D., Helgeson, S. L., Davis, A. R., Nelson, L. K. and Quinn, M. T. (1997) *J. Biol. Chem.* **272**, 29502–29510
- 22 Hata, K., Ito, T., Takeshige, K. and Sumimoto, H. (1998) *J. Biol. Chem.* **273**, 4232–4236

Received 7 October 1998/26 October 1998; accepted 25 November 1998