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Biochimica et Biophysica Acta 1657 (2004) 1–22

BIOCHIMICA ET BIOPHYSICA ACTA
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Review

The NADPH oxidase of professional phagocytes—prototype of the NOX electron transport chain systems

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Received 13 May 2003; received in revised form 16 March 2004; accepted 16 March 2004
Available online 5 May 2004

Abstract

The NADPH oxidase is an electron transport chain in ''professional'' phagocytic cells that transfers electrons from NADPH in the cytoplasm, across the wall of the phagocytic vacuole, to form superoxide. The electron transporting flavocytochrome *b* is activated by the integrated function of four cytoplasmic proteins. The antimicrobial function of this system involves pumping K^+ into the vacuole through BK_{Ca} channels, the effect of which is to elevate the vacuolar pH and activate neutral proteases. A number of homologous systems have been discovered in plants and lower animals as well as in man. Their function remains to be established.

Keywords: Free radical; Flavin; Cytochrome; Neutrophil; Microbicidal; Enzyme; NOX; Antioxidant; Ion channel

1. Introduction

The NADPH oxidase is found in ''professional'' phagocytes including neutrophils (PMN), eosinophils, monocytes and macrophages, cells that form the core of the innate immune system, and is required for their efficient function. These cells circulate in the blood and migrate through capillaries into the tissues where they crawl between cells in search of bacteria, fungi and tissue debris, which they engulf and digest (Fig. 1).

This multi-component enzyme complex is of particular interest because it is the prototype of a newly discovered family of electron transport systems that consist of a transmembrane flavocytochrome that interacts with a variety of activating cytosolic proteins, including a small GTP-binding protein. The discovery of this system and the elucidation of its components, organization and regulation have been important in its own right for developing our understanding of innate immune mechanisms. In the last few years the identification of a number of homologous electron transport chains in a variety of tissues in mammals, and in plants, has greatly increased the general involvement of such systems in biology in its widest sense. These NADPH oxidase (NOX) systems transport electrons across membranes—in addition to their essential role in innate immunity, they have been shown to be important for such diverse processes as the development of roots in plants [104], cuticle in *C. elegans* [94] and wings in *Drosophila*.

2. Respiratory burst—history

The ''extra respiration of phagocytosis'' was first described by Baldridge and Gerrard in 1933 [16] when they noticed that neutrophils demonstrated a dramatic increase in oxygen uptake when phagocytosing bacteria. It was assumed for many years that this ''respiratory burst'' was a response of the cell's mitochondria to provide the extra energy required to engulf the particles. It was only when the respiratory burst was shown to be insensitive to classic inhibitors of mitochondrial oxidative metabolism such as cyanide and azide [255] that the unusual nature of this process was realised. It was subsequently found to be essential for the efficient killing of microbes that were adequately engulfed, but not killed, in the absence of oxygen [274].

2.1. CGD—description

Chronic granulomatous disease (CGD) [296] is a rare inherited disorder characterized by the absence of NADPH

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0005-2728/$ - see front matter © 2004 Published by Elsevier B.V.
doi:10.1016/j.bbabio.2004.03.008

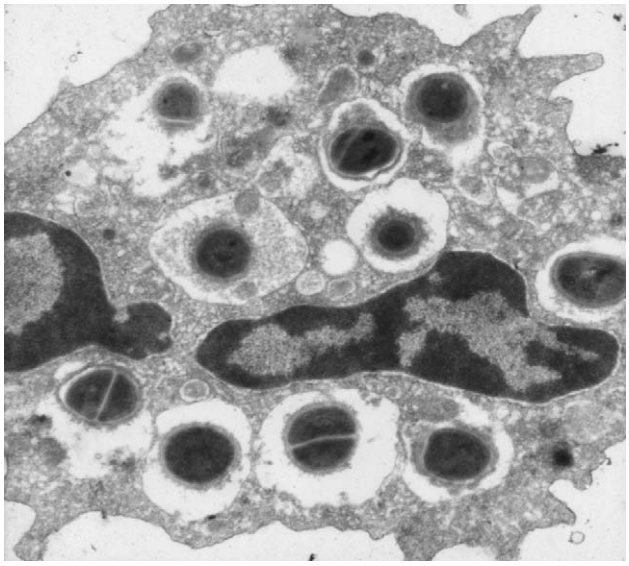

Fig. 1. Electron micrograph of neutrophil. A section through a neutrophil (about 10 μm in diameter) containing 10 *S. aureus* within phagocytic vacuoles taken after about 30 s. after mixing the cells and bacteria. Granules can be seen in the cytoplasm and degranulating into the vacuoles in which granule contents can also be seen.

oxidase (NOX2) activity. It results from mutations in the genes coding for its component proteins, most of which, as well as the functions of the proteins themselves, have been identified through the study of cells from patients with this syndrome.

Phagocytes lacking NADPH oxidase activity are unable to kill bacteria and fungi efficiently, with the predicted consequence that these patients are profoundly immunodeficient, demonstrating frequent, severe, acute and chronic, often fatal, infections.

Treatment consists of prophylactic and therapeutic antibiotics. Bone marrow transplantation can be effective and gene therapy offers potential for the future.

2.2. Identification of the nature of the oxidase

Enormous difficulties were experienced in the identification and purification of this oxidase, causing it to be labeled the ''elusive pimpernel'' [15]. The earliest experiments attempted to purify the oxidase from whole-cell homogenates and crude subcellular fractions, which were incubated with a variety of potential substrates to demonstrate oxidase or diaphorase activity [169]. A variety of activities were detected, but the interpretation of these experiments was complicated by the lack of specificity of the oxidation of many of these substrates and the autocatalytic nature of many of the resulting reactions, accelerated by the presence of metal ions, lipids and reductants in the reaction mixture. Although a number of disparate ''enzymes'' were discovered and described as defective in CGD, none stood the test of time.

Despite the development of a solubilised active oxidase [20,110,292,306] from stimulated cells, complete purification proved impossible because of its instability, particularly its exquisite sensitivity to salts, which prevented most chromatographic separations [19].

2.3. The NADPH oxidase is a transmembrane electron transport chain containing a flavocytochrome b—discovery, rediscovery, controversy and resolution

A great deal of frustration could have been avoided had the observation in the early 1960s of a *b*-type cytochrome in neutrophils been more widely recognized. These descriptions [134] were of the molecule responsible for stable NADI oxidase activity. This reaction, which produces a blue compound, indophenolblue, from dimethyl *para*-phenylen diamine and α-napthol [146], was originally thought to be produced by a specific enzyme, indophenol oxidase, but later shown to be a nonspecific indicator of oxidases. Subsequently it was also shown to be associated with rabbit neutrophils, localising to the granules [278]. In a thorough chromatographic characterisation of this cytochrome, the possibility was even raised that this new cytochrome might have something to do with neutrophil respiration insensitive to cyanide and antimycin A [277]. This linkage was never clearly made, possibly because it was thought the cytochrome might be an unusual cytochrome P420 (an inactivated form of cytochrome P450), and these reports in the Japanese literature were overlooked [169,170].

The *b*-type cytochrome was rediscovered in human neutrophils and shown to be missing in the commonest (X-linked) form of CGD [268,269]. When it was initially purified [133], only a single protein that ran on SDS gels with a molecular mass of about 60–100 kDa (because of its heavy glycosylation [132]) co-purified with the haem. This protein was initially called cytochrome b_{558} and its gene was found by positional cloning and shown to be abnormal in patients with X-CGD [252,294]. Subsequently the cytochrome was shown to be a heterodimer, when a 22-kDa protein co-purified with the haem and the larger protein. Both subunits were shown to be missing in X-CGD [231,257]. The larger and smaller proteins were then called the β and α subunits, respectively, and subsequently gp91^{phox} and p22^{phox}.

Initially, there was strong resistance to the idea that this cytochrome might be part of the oxidase (thought at the time to be a flavoprotein [117]), especially when it was shown to be present in some CGD patients and not others [25]. Ultimately, it has been clearly demonstrated that the catalytic core of the NADPH oxidase is a membrane-bound flavocytochrome, containing two molecules of haem and one molecule of flavin adenine dinucleotide (FAD) [60,89,102,174,272] with an absorbance maximum of the α band near 558 nm.[1] For this reason, it is now usually referred to as flavocytochrome b_{558}.

[1] In fact, the reduced-minus-oxidized absorbance maximum of human flavocytochrome b558 is 559.3 nm at room temperature.

3. Flavocytochrome b_{558}: structure and mechanism

3.1. Structure and energetics

3.1.1. gp91^{phox} (β-subunit, NOX2, CYBB gene)

Flavocytochrome b_{558} is a membrane-bound heterodimeric protein consisting of one molecule of p22^{phox} (α-subunit, the production of the *CYBA* gene) and one molecule of gp91^{phox} (β-subunit, *CYBB* gene) [85,86,148,257,307] (Table 1).

Flavocytochrome b_{558} contains all the catalytic machinery for the oxidation of cytosolic NADPH and the reduction of O_2 to form O_2^-. This enzymatic process requires the participation of three redox co-factors: one molecule of FAD and two molecules of iron protoporphyrin IX (haem *b*) [51,53,219]. The haem groups are nonequivalent [219] and have different redox potentials [60] (Section 3.1.5). All these redox centers, as well as the NADPH binding site, are located on gp91^{phox} [88,240,272,293,326].

gp91^{phox} is a heavily glycosylated (~85–100 kDa) N-linked glycoprotein [132] with a 65-kDa protein core (Fig. 2).

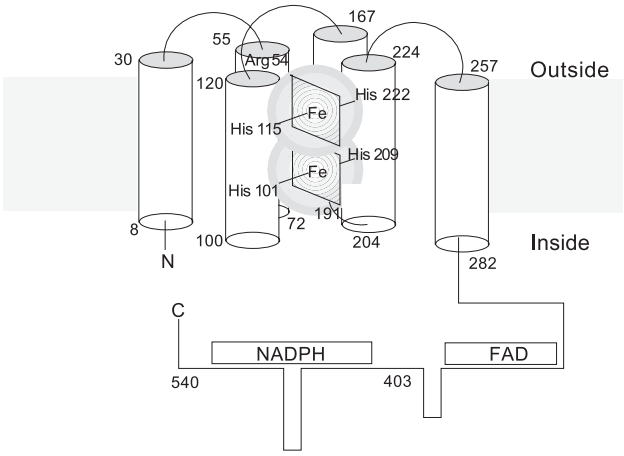

Fig. 2. Model of gp91^{phox} model. The six transmembrane helices and NH_2- and COOH-terminal tails are arranged as indicated. The two non-identical haems are located within the membrane as shown. The haem with $E_{m7} = -265$ mV is toward the outer face of the membrane coordinated between His115 and His222. The inner haem, $E_{m7} = -225$ mV is coordinated by His101 and His209. Arg54, which is hydrogen bonded to the propionate side chain of the outer haem, is indicated. The cytosolic NADPH and FAD-binding regions are shown toward the C-terminus of the protein. In the membrane, gp91^{phox} is tightly associated with the p22^{phox} subunit (not shown).

3.1.2. gp91^{phox} C terminus: NADPH and FAD binding

The hydrophilic C-terminal (282–570) portion of gp91^{phox} contains the FAD and NADPH binding sites that have distant but recognizable homology to the large family of ferredoxin-NADP reductase (FNR) proteins, of which cytochrome P450 reductase, nitric oxide synthase [272,293] and yeast ferric reductase [276] are members. The FAD is non-covalently bound with a K_d of 20–70 nM, a value that is probably partly dependent on the lipid environment [177,179,181,222,230,326]. Consistent with these observations are the existence of a number of naturally occurring CGD mutations within the FAD and NADPH binding motifs that ablate binding of the appropriate nucleotide. As expected, these motifs are also well conserved within the other members of the NADPH oxidase (NOX) family (Fig. 3 and Section 10). The overall homology of the cytosolic domain to the FNR family has allowed modeling studies to be made that suggest the presence of a large insertion that could potentially function as a regulatory domain [293]. In keeping with this notion, there is considerable evidence from the literature that there are multiple direct interactions between both p47^{phox} and

Table 1
Properties of the phagocyte respiratory burst oxidase (phox) components

	gp91^{phox}	p22^{phox}	p47^{phox}	p67^{phox}	p40^{phox}	Rac2
Gene and locus	*CYBB*; Xp21.1	*CYBA*; 16q24	*NCF-1*; 7q11.23	*NCF-2*; 1q25	*NCF-4*; 22q13.1	*Rac2*; 22q13.1
Amino acids	570	195	390	526	339	192
Molecular weight:						
Predicted	65,338 Da	20,959 Da	44,684 Da	59,735 Da	39,039 kDa	21,429 Da
By SDS-PAGE	~90 kDa	22 kDa	47 kDa	67 kDa	40 kDa	22 kDa
Glycosylation	Yes	No	No	No	No	No
p*I*	9.26	10.1	9.58	6.12	7.28	7.87
Phosphorylation	No	Minor	Yes	Minor	Yes	?
Location in PMN						
Resting	Specific granule and plasma membrane		Cytosol	Cytosol	Cytosol	Mainly cytosol
Stimulated	Plasma membrane and phagosome		Membrane	Membrane	Membrane	Membrane
Abundance pmol/10^6 cells (cytosol conc.)	1.0–2.0	1.0–2.0	6.0 (2750 nM)	1.0 (460 nM)	1.0 (460 nM)	2.6 (1200 nM)
Functional domains (see Fig. 2)	C-terminus binds cytosolic components. Haem, FAD and NADPH binding regions	C-terminal proline-rich region	Phosphorylation sites. PX domain, 2 SH3 domains, proline-rich region	Tetratricopeptide repeat, 2 SH3 domains, proline-rich domains	PX and SH3 domains, octicosapeptide repeat	GDP/GTP-binding; insert and effector regions, isoprenylation site

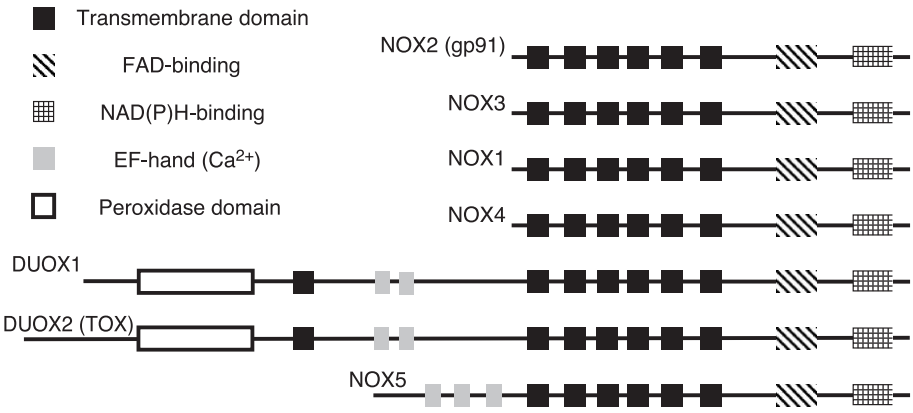

Fig. 3. Structural motifs in the NOX/DUOX family. Structural motifs in the family members are present as indicated. See text for details.

p67^{phox} and gp91^{phox}, particularly in the inserted region thought to overlie the NADPH binding site (450–504) [293] and between p47^{phox} and the extreme N-terminal part of gp91^{phox} [79,191,192,251].

3.1.3. gp91^{phox} N-terminus: haem co-ordination

The hydrophobic N-terminal half of gp91^{phox} contains six membrane-spanning α-helices. Helices III and V each contain two histidine residues appropriately positioned (101:209 and 115:222) to co-ordinate two haem prosthetic groups perpendicular to the plane of the membrane (Fig. 2). These histidine residues are completely conserved among all the NOX family members. Site-directed mutagenesis studies support the proposal that these histidine residues form the axial ligands to the haem groups [21,102]. Resonance Raman [149], visible, CD [322] and EPR [107,108,206,301] spectroscopy are all consistent with low-spin (hexacoordinate) configuration of both haem groups, implying that electron transfer to oxygen occurs via the haem edge rather than through direct coordination to the haem iron. This is in keeping with the known insensitivity of the oxidase to cyanide, azide and carbon monoxide, classic haem "poisons". Interestingly, this outer-sphere process is exactly the mechanism of the reverse reaction between O_2^- and cytochrome *c*, which is the most common method used to detect O_2^- production. Despite this well-documented fact, many researchers argue that O_2 "must" bind directly to the haem iron. It has been suggested that one (or possibly both) haem(s) may undergo a low-spin to high-spin transition during activation of the oxidase which might allow direct oxygen binding to the haem-iron and/or promote proton conductance through gp91^{phox} (Section 8.1) [90,199]. However, this would appear to be contrary to the careful studies of Fujii et al. [107,108] and Isogai et al. [150,151], who have presented extensive evidence that the active form of NADPH oxidase requires the haem to be in the low-spin (hexacoordinate) state. The predicted placing of the haem groups (one toward the inner face and one toward the outer face) is consistent with their function to transport electrons from the NADPH (via FAD) on the inside (cytosol) across the membrane to the interior of the phagocytic vacuole where molecular O_2 is reduced to form O_2^-. In biology, transmembrane electron transport is very often (but not exclusively) by means of di-haem membrane proteins. Two haems are required to span the width of the membrane to allow electrons to transfer at kinetically significant rates. Within proteins, electron transfer rates fall off exponentially with the distance between donor and acceptor, with a ~10-fold decrease in rate for every 1.7-Å separation [225]. Biological membranes are ~25-Å thick and thus at least two redox centers are required. The proposed spacing and orientation of the haems in gp91^{phox} is consistent with other known cytochromes that function to transport electrons across membranes and is very similar to that of the mitochondrial bc_1 complex where members of the pair of histidine axial ligands are also ~100 amino acids apart and axial pairs 14 amino acids apart [321]. p22^{phox} contains an invariant histidine residue (H94) that had been proposed as one of the axial ligands to one of the haem groups. Recent site-directed mutagenesis studies have conclusively shown this not to be the case [22].

The second (120–167) and third (224–257) external loops of gp91^{phox} contain the N-linked glycosylation sites (asparagines 132, 149 and 240) [308]; these are only partly conserved among mammals. During synthesis of flavocytochrome b_{558}, haem insertion into gp91^{phox} is required before heterodimer formation, which in turn precedes glycosylation [77,324]. Although the human neutrophil flavocytochrome b_{558} is heavily glycosylated, this appears to vary among phagocytes and among species [172]. Glycosylation is not a requirement for gp91^{phox} catalytic function [223].

3.1.4. p22^{phox}

p22^{phox} is a 194-amino-acid (~21 kDa) protein with a hydrophobic, membrane-spanning N-terminus (1–132) that crosses the membrane at least two (and possibly four) times. The cytoplasmic hydrophilic C-terminus (133–194) contains a proline-rich domain (151–160) that is known to be important in binding of p47^{phox} to flavocytochrome b_{558} [189,288]. A naturally occurring mutation in this domain

(156Pro → Gln), while giving normal amounts of protein, abolished translocation of $p47^{phox}$ and $p67^{phox}$ to the membrane after neutrophil activation and resulted in CGD [193]. Other regions of $p22^{phox}$ have also been implicated as sites of interaction between $p22^{phox}$ and both $p47^{phox}$ and $p67^{phox}$ [67]. Thus, it seems likely that the only function of $p22^{phox}$ is to provide high-affinity binding sites for the cytosolic NADPH oxidase subunits. It is noteworthy, however, that for full processing and maturation, association of $gp91^{phox}$ and $p22^{phox}$ is required, at least in neutrophils [77].

In neutrophils, the stable expression of each subunit is mutually dependent on the expression of the other. Thus, mutations causing the loss of $p22^{phox}$ expression in CGD lead to the loss of expression of both $p22^{phox}$ and $gp91^{phox}$ subunits and vice versa. This does not apply to all cell types, however, and low level $p22^{phox}$ expression can be detected in many cell types and tissues in the absence of $gp91^{phox}$ [232,233]. Similarly, in cell-lines, $gp91^{phox}$ has been expressed in the absence of $p22^{phox}$. By using such model systems, it has been possible to confirm that all the redox centers are contained within the $gp91^{phox}$ subunit [325].

3.1.5. Energetics of the flavocytochrome b_{558} redox centers

The redox potentials of the flavin redox couples have been measured using EPR spectroscopy by Kakinuma [160] as $E_{m1} = -256$ mV (FAD/FAD$^{\bullet}$) and $E_{m2} = -304$ mV (FAD$^{\bullet}$/$FADH_2$) with the formation of a neutral semiquinone, although this semiquinone does not accumulate to a significant extent during enzyme turnover [95,179]. Thus, the overall midpoint potential of the FAD center is -280 mV (for comparison, the E_m of the NADPH/NADP couple is ~ -317 mV).

The redox potentials of the haems in $gp91^{phox}$ are -225 and -265 mV at pH 7.0. When first determined, these potentials were too close together to be distinguished [56], but the serendipitous discovery of an X-linked CGD patient with an Arg54 → Ser mutation in $gp91^{phox}$ allowed the dissection of redox titration curves into two separate components. In the mutant, the haem center with $E_{m7} = -265$ was shifted to -300 mV making deconvolution of the titration possible [56,60]. Structural comparisons with other membrane cytochromes suggest that invariant arginine residues near the membrane surface form hydrogen bonds with the negatively charged propionyl side chains of the membrane-embedded haem. Arg54 is predicted to be at the outer membrane surface in a position where it could perform this function. Substitution of the positively charged arginine to an uncharged serine would decrease the electron withdrawing nature of this group and thus lower the redox potential of the haem (in this case by 35 mV). Similar effects have been demonstrated in yeast *iso*-1-cytochrome *c* when glutamine (-30 mV) or asparagine (-34 mV) was substituted for the equivalent arginine that forms a hydrogen bond with one of the haem propionate side chains [66]. In combination, these observations allow the assignment of the redox potentials to the individual haem groups in the wild-type enzyme; the inner haem having $E_{m7} = -225$ mV and the outer haem $E_{m7} = -265$ mV.

Although not formally proven, it is now generally believed that NADPH oxidase does not contain any other redox centers or redox-active metal ions, although $p67^{phox}$ has been reported to be capable of (vanishingly) small rates of NADPH oxidase activity at high NADPH concentrations [69].[1] Early reports suggested the involvement of ubiquinone [41,42,111] or pyrroloquinoline quinone (PQQ) [23] in oxidase function, but these appear to have been artifactual [55,196].

3.1.6. Electron transfer mechanism

There are seven separate steps of electron transfer during a single turnover of NADPH oxidase, as illustrated in Fig. 4. The initial step is the transfer of two electrons from NADPH to oxidized FAD. As in many biological processes, FAD (or

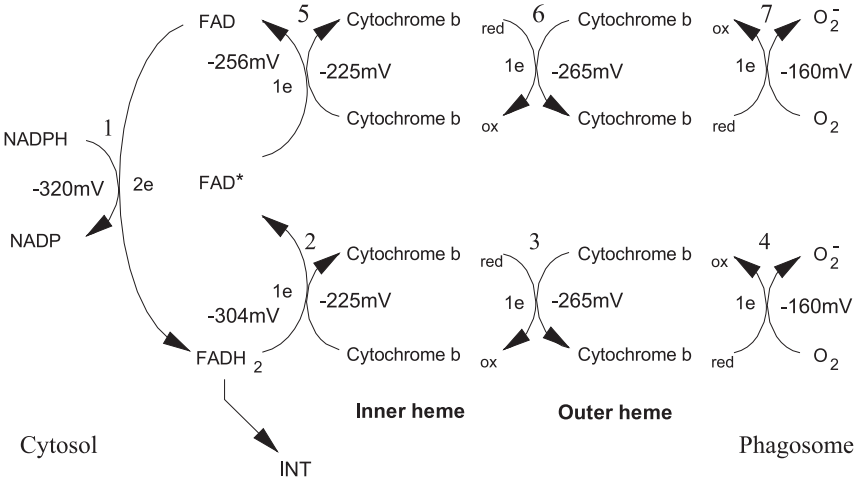

Fig. 4. Electron transfer pathways within flavocytochrome b_{558}. The seven electron transfer steps are numbered. Electron transfer takes place from NADPH in the cytosol, across the membrane to the phagocytic vacuole. See text for details.

other flavins) are used to convert two-electron transfer processes into single electron transfer steps in cases where only one (or an odd number of) electron(s) are required for an enzymatic reaction [309]. The stereochemistry of the hydride transfer has been determined to be from the 4-pro *R* position of NADPH, as is the case with other members of the FNR family, by measurement of the kinetic deuterium isotope effect [218]. The K_m for NADPH has been generally reported to be in the range 25–35 μM for neutrophil plasma membranes and slightly lower for purified flavocytochrome b_{558}. The K_m for NADH is at least 10–20 times higher, thus the oxidase exhibits a marked preference for NADPH. At the prevailing intracellular concentrations of NADH and NADPH (both ~ 70 μM), it undoubtedly utilizes the latter almost exclusively. This initial electron transfer process appears to be the principal rate-limiting step of oxidase turnover. Although it is frequently suggested that the oxidase is regulated by changes in nucleotide-binding (i.e. through alteration of K_m NADPH), there is little experimental evidence that this is the case—consistent with labeling studies, where NADPH analogues appear to label resting or activated flavocytochrome b_{558} with approximately equal efficiency [88,240,272].

The second electron transfer step is from reduced FAD ($FADH_2$, $E_m = -304$ mV) to the inner haem ($E_m = -225$ mV) (Fig. 5), generating the FAD semiquinone ($FAD^{\bullet}$). In the third and fourth steps, the electron is passed from the inner to the outer haem ($E_m = -265$ mV) and thence to oxygen forming superoxide ($E_m = -160$ mV). The reported K_m for oxygen varies quite widely in the literature, but most values are in the range of 5–10 μM. The fifth, sixth and seventh steps recapitulate the third, fourth and fifth, the exception being in the fifth step, where it is the flavin semiquinone ($E_m = -256$ mV) that is the donor to the inner haem, and thus the driving force for electron transfer is less.

Two points are noteworthy: first, little flavin semiquinone is observed during enzyme steady-state enzyme turnover, despite there being measurable amounts of reduced FAD [95,181]. This is consistent with the reactions involving haem being more rapid than the reactions involving FAD. The degree of flavin reduction reported in the literature is also quite variable (< 5–40%) probably due to differences in enzyme preparation, intrinsic enzyme activity and reaction conditions (amphiphile and cytosolic factors) that in turn determine the degree to which flavin reduction is the rate-limiting step [46,51,59,177,179,218]. Second, the third and sixth steps in this electron transfer scheme are energetically unfavorable, since the inner haem has a higher midpoint redox potential than the outer haem (Fig. 5). It is not known if this unexpected arrangement has a functional significance, but it may partially explain that the presence of oxygen is necessary for rapid electron flow of electrons into and through NADPH oxidase, as the absence of a terminal electron acceptor will cause electrons to accumulate on the inner haem. Under anaerobic conditions, the rates of flavin and haem reduction are approximately 1000 times slower than the known rate of electron transfer in the aerobic enzyme [51,177]. The slow rate of anaerobic haem reduction has been used as an argument to "prove" that haem of $gp91^{phox}$ is not involved in catalysis of O_2^-, and that NADPH oxidase is a simple flavoprotein (for example [12]). This argument conveniently ignores the equally slow kinetics of anaerobic flavin reduction! [43,53]. It has never been clear how a flavoprotein with an internal flavin domain (and no other active redox centers) could transfer electrons from the cytosolic face to the outer face of the membrane. Measurement of reoxidation rates of the reduced components, combined with steady-state experiments, confirmed that under aerobic conditions both haem and flavin within NADPH oxidase are kinetically competent to generate O_2^- at the observed rate [59,95]. Thus, oxygen is necessary for rapid electron flow. The ability of the haem to rapidly react with oxygen, the appropriate steady-state levels of reduction of haem and flavin and the ability of oxygen to accelerate

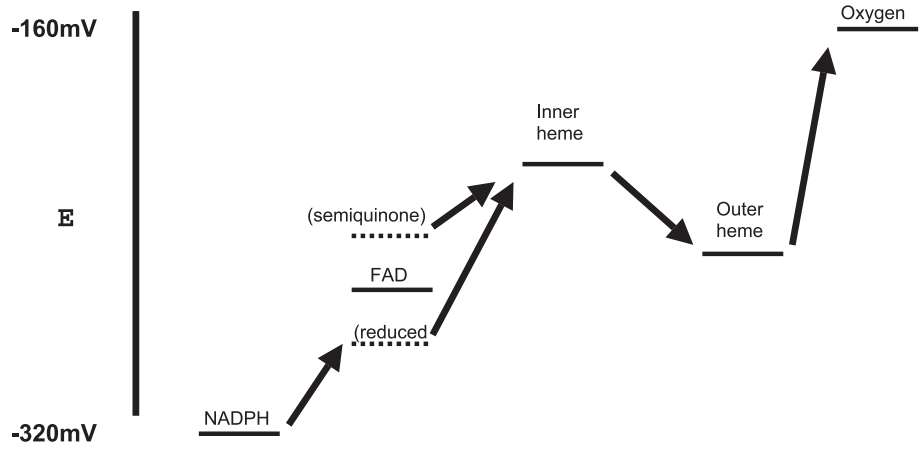

Fig. 5. The energetics of electron flow within flavocytochrome b_{558}. Note that the overall electron transfer from NADPH ($E_m = -317$ mV) to oxygen (E_m $O_2^-/O_2 = -160$ mV) is energetically favourable, transfer from the inner haem to the outer haem is not. In addition, the two electron transfer steps from FAD to haem during the reaction cycle (steps 2 and 5 in Fig. 3) are not equivalent.

electron flow have been confirmed by several laboratories [150,177,179,218,222]. Under optimal in vitro conditions, flavocytochrome b_{558} exhibits a turnover number of around 300/s at 22 °C, with a pseudo first-order haem reoxidation rate of 4.7 ms [48,56,59].

4. Cytosolic phox proteins—discovery, structures and interactions

4.1. Discovery

The demonstration that flavocytochrome was absent in only some CGD patients led to the identification of other forms of the disease [25] that were inherited in an autosomal recessive manner as a result of abnormalities in cytosolic proteins that are also components of the system.

Most patients with autosomal pattern of inheritance of CGD usually possess a normal flavocytochrome b_{558} [260]. However, their cells were unable to transfer electrons to this molecule when stimulated [266], indicating a problem with the activation system or a missing proximal electron transporting molecule. The heterogeneity of this condition was confirmed by complementation conferred by fusion of cells from the subjects with the different patterns of inheritance [128]. A 44–47-kDa protein that was normally heavily phosphorylated upon activation of normal neutrophils was shown to be missing in most (but not all) cases of autosomal recessive CGD [264].

Two technical developments then led to the discovery and characterisation of the components of the oxidase other than flavocytochrome b_{558}. These were as follows.

4.1.1. The development of a "cell-free" assay

Several groups independently developed methods of activating the NADPH oxidase by mixing neutrophil subcellular fractions with NADPH and anionic amphiphiles (30–100 μM arachidonic acid or SDS, although other amphipathic lipids can also fulfill this requirement) [31,32,62,139,202]. A mixture of crude membrane preparations and factors within the cytoplasm was required for activity. The factors missing in the cells from the majority of autosomal recessive CGD patients were in the cytosol and not the membrane fraction that housed the flavocytochrome b_{558} [63,64], and two of these cytosolic factors appeared to be involved [65,237].

4.1.2. The production of antibodies to $p47^{phox}$ and $p67^{phox}$

The involvement of a GTP binding protein in the NADPH oxidase was first suggested by Gabig et al. [109] and Seifert et al. [273] when they showed that guanine nucleotides stimulated the oxidase in a cell-free system. Doussière et al. [91] then demonstrated that GTP promoted the formation of a complex between a cytosolic factor and a membrane protein.

In an attempt to purify this cytosolic protein, cytosol was passed over a column of immobilised GTP, and the eluate, which enhanced activity in the cell-free assay, was used to immunise rabbits. This experiment had a remarkably successful outcome. Of all the proteins in this complex mixture, good antibodies were only produced to two proteins, both from the phox system, and each was missing from a different type of autosomal recessive CGD [304]. Neither of these two proteins was subsequently found to bind GTP.

One protein was identified as the 47-kDa phosphoprotein previously shown to be missing in most cases of autosomal recessive CGD [264], and the other had a molecular mass of about 67 kDa. The antiserum was then used to clone the genes, coding for $p47^{phox}$ [305] and $p67^{phox}$ [190], respectively.

4.1.3. p21rac

After the discovery of $p47^{phox}$ and $p67^{phox}$ [304], it became clear that they were not sufficient to reconstitute the active oxidase when combined with membranes in the cell-free assay [237]. A GTP-dependent complementing factor that restored oxidase activity when mixed with $p47^{phox}$, $p67^{phox}$ and membranes [1] was purified from cytosol and was shown to be rac1 [2] or rac2 [173]. Although rac2 is the predominant form in neutrophils, rac1 can compensate for its deficiency as shown by normal oxidase activity in rac2 deficient cells [126]. Under resting conditions, rac is coupled to another protein, GDP dissociation inhibitor (GDI), which maintains it in the inactive form bound to GDP [236]. The lipid modification on the tail of the rac is housed in a hydrophobic pocket in GDI [163].

4.2. Details of cytosolic phox proteins

4.2.1. $p67^{phox}$ (NOXA2)

$p67^{phox}$ is a 59,735-Da protein[2] (526 amino acids) with a p*I* of 6.12. Like the other cytosolic phox proteins, $p67^{phox}$ is rich in motifs involved in protein/protein interactions (Fig. 6). These include two SH3 domains (central and C-terminal), two proline-rich regions flanking the central SH3 domain, an N-terminal TPR (*t*etratrico*p*eptide *r*epeat) and a PB1 domain C-terminal to the central SH3 domain. The TPR domains are thought to bind rac [84]. PB1 domains are known to interact with octicosapeptide motifs [152] and $p67^{phox}$ binds to $p40^{phox}$ through this domain [211]. As will be described later, this protein is absolutely required to induce electron transport through the flavocytochrome, consequently these proteins have been called the NOXA (*NOX A*ctivator) family. Thus, a homolog of $p67^{phox}$ has just been described [17,116] and termed NOXA1. NOXA1 differs from $p67^{phox}$ in lacking the first SH3 domain and possessing an additional hydrophobic region. It appears to be co-expressed with NOX1 and NOXO1 (below) in the colon epithelium.

[2] p67phox and p47phox have apparent MWs of 67 and 47 kDa by SDS gel electrophoresis.

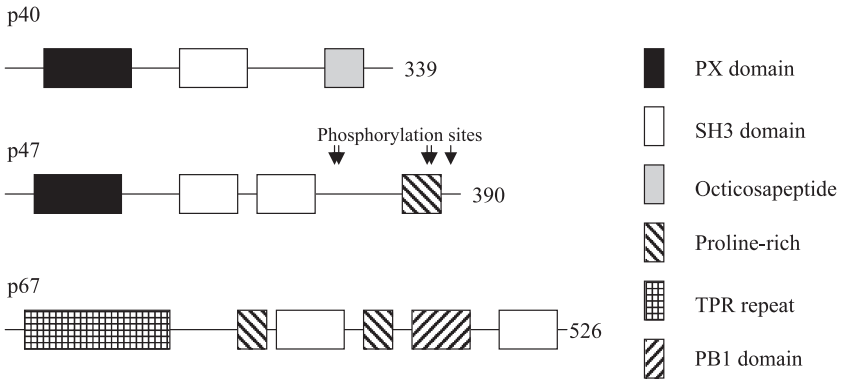

Fig. 6. Regions of p40^{phox}, p47^{phox} and p67^{phox} involved in protein/protein interactions. Serine residues of p47^{phox} that are phosphorylated during oxidase activation are indicated with arrows. See text for details of the structural motifs.

4.2.2. p47^{phox} (NOXO2)

p47^{phox} is a basic protein (p*I* = 9.6) of molecular weight 44,681 Da (390 amino acids) that is heavily phosphorylated during neutrophil activation. p47^{phox} contains a number of well-defined motifs (Fig. 6), including a PX domain (involved in phosphoinositide binding), two SH3 domains (involved in protein/protein interactions) and at least one proline-rich motif (the reciprocal target for SH3 domain interactions). This protein appears to stabilize and organize the oxidase complex and consequently they are called the NOXO (*NOX O*rganizer) proteins. Consequently, the novel homolog of p47^{phox} that has been described very recently [17,116] is termed NOXO1. It differs from p47^{phox} in lacking the auto-inhibitory domain and the pKC phosphorylation sites. NOXO1 is co-expressed with NOX1 in the colon epithelium where it appears to be involved in the regulation of NOX1 activity.

4.2.3. p40^{phox}

p40^{phox} was discovered when it co-purified with p67^{phox} (to which it is tightly bound) [284,314]. It is a protein of 39,039 Da (339 amino acids), strongly homologous with p47^{phox} with an N-terminal PX domain, followed by an SH3 domain. Towards the C-terminus, there is an octicosapeptide repeat (OPR [239], also known as PC domain [211]) (Fig. 6). This domain seems to be involved in the binding of p40^{phox} to p67^{phox}. This protein probably functions as a shuttle partner, transporting p67^{phox}, which does not contain a PX domain, to the membrane of the phagocytic vacuole by binding to phosphatidylinositides (Section 7.1).

5. Molecular genetics of CGD—role of the different oxidase components

Defects in any one of four genes give rise to the known forms of CGD [58,142,248]. *CYBB* (coding for gp91^{phox}, NOX2) is located on the X chromosome and accounts for about 65% of cases, exclusively in males (except in rare female carriers where there is extreme lyonization). The other three genes are all autosomal with *NCF1* (p47^{phox} or NOXO2 protein) *NCF2* (p67^{phox} or NOXA2) and *CYBA* (p22^{phox}) causing about 25%, 5% and 5% of cases, respectively. Defects in the fifth necessary component (rac2) give rise to a spectrum of neutrophil dysfunction, including a CGD-like syndrome [8,317] although oxidase activity can be normal in these cells, depending upon the stimulus used [7].

In most cases, the genetic mutation results in the complete loss of the protein for which it codes. Rarely the mutation affects a regulatory, nonstructural, part of the protein, and in these cases, the protein is expressed but does not function. Under these circumstances, the mutation can be very instructive as to the normal role of that part of the molecule in the active oxidase complex. Analysis of these mutations has provided much of the specific evidence of the domains responsible for protein/protein interactions of the subunits and the binding of the redox centers to flavocytochrome b_{558}.

A small subgroup of CGD patients have what is known as "variant" CGD [194]. In these cases there is partial loss of a protein or its function and often as much as 10% [33,157,247], and up to 30% (Malech, H., personal communication), of normal oxidase activity can be measured.

No instances of CGD have been identified where a lesion of p40^{phox} is causal. This could mean that it only plays a relatively unimportant regulatory role, or that mutations result in a lethal phenotype.

6. Cell biology of oxidase components

6.1. Cell distribution

The NADPH oxidase is primarily found in the professional phagocytic cells. These include neutrophils, monocytes, macrophages (including various members of the macrophage lineage such as Kupffer cells in the liver, alveolar macrophages, microglia in the CNS) and eosinophils. It is not present in the immature promyelocytic HL-60

cell line but accumulates as these cells mature to neutrophils following induction [245].

There have been reports of this oxidase in other cells. These include fibroblasts [203] although this activity seems to be produced by another superoxide generating system [204] possibly a NOX isoform. It was also suggested that it might play an oxygen-sensing role in carotid body cells in which it was thought to have been detected [4]. Oxygen sensing is normal in mice lacking gp91^{phox} [11] and another sensing system seems to be operative in most cells [200].

6.2. Subcellular distribution

The initial description was that the flavocytochrome was distributed between the plasma membrane and specific granules in neutrophils [265,267,271] from which it was incorporated into the wall of the phagocytic vacuole [267].

There has been some disagreement with this initial assignment of location with the suggestion that almost all the flavocytochrome *b* resided in the membrane of the specific granules [24] and that activation involved the fusion of these granules with the phagocytic vacuole. This does not explain the subcellular distribution of the flavocytochrome in monocytes, macrophages, eosinophils [261] or HL-60 cells [245], none of which contain specific granules.

It has also been proposed that the small amount of flavocytochrome *b* associated with the membranes is not found in the plasma membrane but in a distinct organelle called the "secretory vesicle", which is suggested to act as a storage system for plasma membrane associated molecules [35,275]. However, these "organelles" contain relatively large amounts of human serum albumin which can only have arrived there by endocytosis of the plasma into invaginated plasma membrane. There would not seem to be an obvious need to consider these secretory vesicles as unique organelles, and that the association of the flavocytochrome b_{558} with the plasma membrane should suffice.

7. Activation of the NADPH oxidase

7.1. Cytoskeletal interactions, conformational changes and phosphorylation

After a particle is phagocytosed, the oxidase is activated after the vacuole has closed [270], and only in that region of the plasma membrane that makes up the wall of the vacuole [29] (Fig. 7). Targeting of an active oxidase to this part of the membrane appears to be dependent upon the accumulation of phosphatidylinositol phosphates at this site. The characteristic lag of about 20–30 s between the activating stimulus and oxidase activity [259] is probably accounted for by PIP metabolism and translocation of the cytosolic factors, although the cell-free system (which is independent of PI metabolism) also exhibits a lag (Section 8.1).

The vacuolar membrane is the site of activity of PI3 kinases and phosphatases that act on phosphoinositol to generate a variety of phosphoinositol phosphates (PIPs)

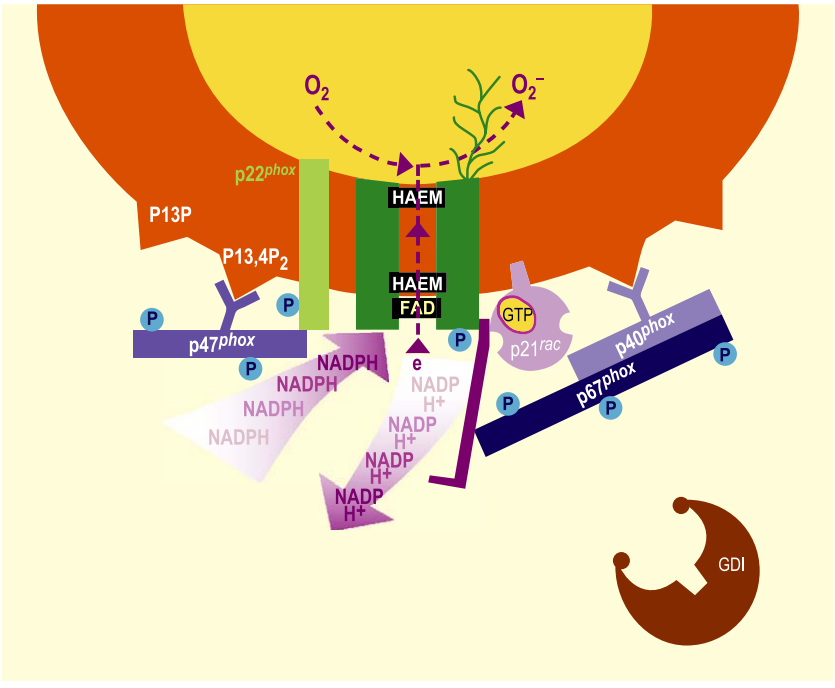

Fig. 7. Schematic representation of the components of the active NADPH oxidase. Cytosolic factors, p47^{phox} and p67^{phox} are phosphorylated and translocate from the cytosol to the membrane where they interact with the flavocytochrome *b* and with p21^{rac} which is normally maintained in the cytosol in its GDP bound state in association with GDI. The interaction of these factors might induce electron transport by inducing a conformational change, providing the substrate NADPH access to its binding site.

[96]. The N-terminal regions of $p40^{phox}$ and $p47^{phox}$ contain homologous stretches of 120–130 amino acids that form a structure called the phox homology or PX domain [239] (Fig. 6) that binds to these PIPs and directs these proteins to this activated membrane [161,328] (reviewed by Wientjes and Segal [316]). There are slight differences in the PIP binding pocket of $p40^{phox}$ and $p47^{phox}$ resulting in different specificities of the associations [161]. $p47^{phox}$ [144] has a greater affinity for PI(3,4)P2 than PI(3)P whereas the binding pocket of $p40^{phox}$ [28] is too cramped for PI(3,4)P2 but binds PI(3)P tightly. $p40^{phox}$ might be responsible for transporting $p67^{phox}$, which does not contain a PX domain, to the membrane.

The cytoskeleton also plays a role in the dynamics of the movement of cytosolic components. Initially a dense meshwork of polymerised cytoskeletal proteins lines the membrane at the base of the phagocytic vacuole and protrudes all around it to produce the pseudopodia around the phagocytic cup which then unite around the particle to close the vacuole. The vacuole then migrates to the interior of the cell, rupturing through the polymerised cytoskeleton as it does so. However, it still remains surrounded by a network of cytoskeletal proteins such as coronin, paxillin and vimentin [125,241]. The influence of the different states of the cytoskeletal proteins on the access of the substrate to the flavocytochrome and of the cytosolic phox proteins and rac to the membrane is unknown. $p47^{phox}$ and $p67^{phox}$ do not exist in simple solution in the cytosol, but are associated with the cytoskeleton, in particular with moesin, coronin and actin [125,216,291,315,320].

Phosphorylation of $gp91^{phox}$ [114], $p22^{phox}$ [114,243], $p67^{phox}$ [93,103] and $p40^{phox}$ [27,285] is seen in association with activation of the oxidase and may only involve a small fraction of these proteins. However, the most heavily phosphorylated of all the components is $p47^{phox}$, which is phosphorylated at multiple serine residues [143]. The most important sites appear to be S379, S303/304 and S359/S370 [98,158] (Fig. 6) and may function in an hierarchical fashion, some being phosphorylated in the cytosol and being required for translocation, while others being phosphorylated at the membrane and being required for activation itself [158] and likely causing a conformational change [76,228,229]. The kinases involved have not been clearly identified although many have been implicated [145,176,184,220,227]. Phosphorylation of specific $p47^{phox}$ residues may also be involved in deactivation of the oxidase [227].

Activation of electron transport depends upon a change in the conformation of the flavocytochrome, possibly providing NADPH access to the active site. Alternatively, electron transfer may be facilitated between the flavin and haem of flavocytochrome b_{558} [47,83]. The former mechanism might be accomplished by displacing the small helix that is predicted in the molecular model to occupy this binding site in the inactive state [293]. This conformational change appears to require contact between the cytosolic proteins and the flavocytochrome b_{558}, and to do that, the phox proteins have to move from the cytosol to the vacuolar membrane. This movement is accomplished by specific changes in the membrane of the vacuole and in the proteins themselves.

7.2. Intermolecular associations of the components of the NADPH oxidase

Our knowledge regarding the assembly of the oxidase is indirect in the absence of structural evidence of the intermolecular interactions. We know that the cytosolic phox proteins transiently associate with phagosomes [6] but that this association is unstable when the flavocytochrome is not present [141,143] (Fig. 7).

$p67^{phox}$ attaches directly to flavocytochrome b_{558}, and in combination with rac in the GTP bound form, is sufficient to induce electron transport [47,70,106,178] although in the absence of $p47^{phox}$, micromolar rather than nanomolar concentrations of $p67^{phox}$ are required [47,178]. Rac2 translocates to the membrane [3] independently of the cytosolic phox proteins [140], and also binds to the flavocytochrome b_{558}. Chimeras of rac and $p67^{phox}$ also elicit superoxide production [118,207]. In the absence of $p47^{phox}$, much greater concentrations of $p67^{phox}$ are required for activity and the complex with the flavocytochrome b_{558} is less stable. $p47^{phox}$ appears to be an adaptor molecule that forms a bridge between $p22^{phox}$ and $p67^{phox}$, as well as binding to cytoplasmic regions of $gp91^{phox}$ [78,178], thereby stabilising the attachment of $p67^{phox}$ to flavocytochrome b_{558}. There is also evidence that $p47^{phox}$ does functionally affect flavocytochrome b_{558} directly [46,47,61]. $p47^{phox}$ has two SH3 domains, the ligand binding domains of which face each other to form a groove in which its C-terminal polybasic region fits [124]. It has been suggested that upon activation this polybasic region is phosphorylated, inducing a release from its autoinhibitory role and making the groove accessible to bind the proline-rich tail in the C-terminal portion of $p22^{phox}$ [124,226,229,280,289]. Arachidonic acid generated through the activity of phospholipase-A2 might be important for activation of the oxidase in the intact cell [68]. There is also good evidence that $p47^{phox}$ directly interacts with cytoplasmic loops of $gp91^{phox}$ [79,251].

The mechanism by which rac promotes activation by $p67^{phox}$, to which it binds [84], remains to be clarified. It is not known what causes the separation of rac from its complex with GDI in the cytosol. It translocates to the membrane independently from $p67^{phox}$ and $p47^{phox}$ [82,118,119,140] and its GDP is probably exchanged for GTP on the membrane through the action of P-Rex1, a 185-kDa guanine-nucleotide exchange factor (GEF) [311], that is activated by PtdIns(3,4,5)P3 and the βγ subunits of heterotrimeric G proteins.

The requirement for $p40^{phox}$ in controlling NADPH oxidase activity is rather obscure. $p40^{phox}$ becomes phosphorylated during neutrophil activation and translocates to the plasma membrane in a similar manner to $p47^{phox}$. Unlike the bulk of $p47^{phox}$, in resting neutrophils $p40^{phox}$ and $p67^{phox}$ are largely associated with the cytoskeleton. Work

from several laboratories has suggested both negative [254,303] and positive [45,97,300] regulatory roles for this protein. The finding that it is responsible for translocating $p47^{phox}$ and $p67^{phox}$ to the vacuolar membrane [183] makes the latter more likely and its principal role may be targeting to PIP-rich regions (Section 7.1).

Although $p40^{phox}$ is not required for activity of the cell-free oxidase system, it can modulate this activity. Using recombinant cytosolic proteins, it has been shown that $p40^{phox}$ increases the affinity of $p47^{phox}$ for flavocytochrome b_{558} [45]. This finding sprang from the observation that "recombinant cytosol" ($rp47^{phox}$, $rp67^{phox}$, rprac2) could only activate oxidase activity weakly when added to crude neutrophil membranes, yet was fully competent in activating purified flavocytochrome b_{558}. This deficiency could be partially overcome by the addition of $rp40^{phox}$ (or a sub-stimulatory "spike" of natural cytosol) to the activation mixture. These experiments also suggest that $p47^{phox}$ is the limiting component in neutrophil cytosol [50]. In the absence of $p47^{phox}$, $p40^{phox}$ could elicit a small amount of oxidase activity ($\sim$ 5%), possibly by increasing the affinity of $p67^{phox}$ and/or Rac for flavocytochrome b_{558} [45]. It was proposed $p40^{phox}$ could increase the affinity of $p47^{phox}$ for flavocytochrome b_{558} by promoting the conformational change in $p47^{phox}$ that occurs during activation; cooperatively increase the binding of $p47^{phox}$ by binding to both $p47^{phox}$ and flavocytochrome b_{558}; or bind to $p67^{phox}$ and/or rac2 in such a way as to increase their affinity for cytochrome-associated $p47^{phox}$.

8. Regulation of electron flow

8.1. Mechanisms and kinetics

In both whole cells and cell-free NADPH oxidase activation systems, there is a characteristic lag before oxidase activity appears after the addition of stimulus (to whole cells) or amphiphile (in cell-free systems). By exploiting this phenomenon and the steady-state levels of reduction of haem and flavin, several laboratories have obtained evidence that the cytosolic factors have individual roles in regulating electron flow within the oxidase [46,47,61,83,179,218]. In principal, this may be achieved by controlling the oxidase at several points: (1) binding of NADPH to the enzyme; (2) electron (hydride) transfer from NADPH to FAD; (3) electron transfer from FAD to the inner haem; (4) electron transfer between the haems; and (5) electron transfer to oxygen. In fact, it appears there is control of electron flow at two distinct points: between NADPH and enzyme FAD, and between enzyme FAD and haem [46,61,83]. This results in a situation in which the oxidase exists in at least three kinetic states: inactive, intermediate and fully active. In the intermediate state, electron transfer can occur from NADPH to the flavin center of flavocytochrome b_{558}, but not to the haem or oxygen (Fig. 8). Electron flow to the flavin can be measured by a dye reductase assay, since the dye iodonitrotetrazolium iodide accepts electrons directly from reduced, enzyme-bound FAD [47,50,61]. Thus, the intermediate state of the oxidase can be analyzed in cell-free assays using highly purified or recombinant proteins. Measurements of the kinetics of activation of oxidase activity using INT or O_2^- formation as outputs showed that there is significant INT reductase activity before the appearance of O_2^-, depending on the incubation conditions. Similarly, in the presence of INT, the onset of NADPH oxidation is much shorter than in its absence. Thus, the formation of the intermediate state is relatively rapid compared to the conversion of the intermediate state to the fully active oxidase [47]. In the fully active enzyme, steady-state measurements of the reduction level of flavin and haem indicate the rate-limiting step becomes electron transfer from NADPH to FAD, and not the binding of NADPH or the later electron transfer steps. It is this first step that appears to be principally regulated by $p67^{phox}$ [179,218]. Bokoch and Diebold [83] have proposed that Rac and $p67^{phox}$ have independent roles in regulating this electron transfer step, but must interact with each other in order to activate the electron transfer steps subsequent to FAD reduction. Differential [105] regulation of the initial electron transfer step (NADPH $\rightarrow$ FAD) from subsequent electron transfer step(s) by the individual cytosolic factors (or amphiphile) may form the mechanistic basis for the intermediate state of oxidase activation.

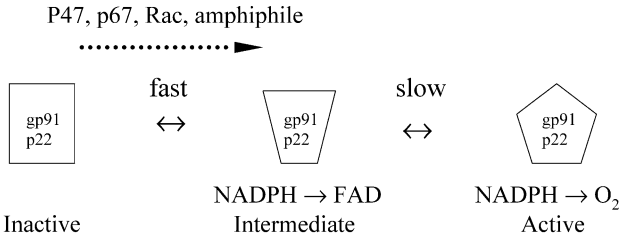

Fig. 8. Equilibrium model for NADPH oxidase activation. This model is a simplified version of the kinetic model developed in reference [47]. In this model, the anionic amphiphile causes a change in conformation in $p47^{phox}$ that allows it to associate with flavocytochrome b_{558}. This association allows the high affinity binding of $p67^{phox}$ and rac resulting in an intermediate activation state where electron transfer can occur from NADPH to FAD. The formation of this intermediate state is relatively rapid. A subsequent slow step, possibly involving a conformational change in flavocytochrome b_{558}, results in the fully active oxidase. The occupancy of each activation state may be influenced by additional factors, such as Mg^{2+} [49].

There is also evidence that the anionic amphiphile may play a direct role in regulating electron transfer (in addition to its effect in causing a conformational change in $p47^{phox}$ [50,280,289]). Two possible mechanisms can be envisaged. First, the amphiphile may assist the cytosolic factors in causing a change in the conformation in

flavocytochrome b_{558} [200]. Second, the amphiphile may induce a change in the axial co-ordination of one of the haems, possibly enabling it to bind O_2 [90,199] (Section 3.1.3).

Importantly, and surprisingly, the activation process is thought to be catalytic and dynamic in nature, rather than through the formation of a fixed stoichiometric complex [48]. It has been generally assumed that the activation of NADPH oxidase involves the formation of a stable complex between flavocytochrome b_{558} and the cytosolic factors in a stoichiometric complex. Further, it is assumed that this association converts flavocytochrome b_{558} (containing all the catalytic machinery) from an inactive to an active state and dissociation converts the flavocytochrome b_{558} back to the resting form. However, simple titration experiments strongly suggest that in the cell-free system at low ratios of $p67^{phox}$ (and to a lesser extent Rac2), these proteins are capable of activating flavocytochrome b_{558} in a catalytic fashion, rather than through the formation of a stoichiometric complex. In these experiments, it was found that one molecule of $p67^{phox}$ was competent to activate a minimum of five flavocytochrome b_{558} molecules, and similar (but less pronounced) effects were seen for Rac2. It was proposed that after undergoing a conformational change induced by $p67^{phox}$ and Rac2, flavocytochrome b_{558} remains in the activated state for a significant period after the cytosolic factors have dissociated.

This suggests that a dynamic equilibrium exists between (at least) the three states of the oxidase (inactive, intermediate and active, described above and in Fig. 8). Under normal conditions the inactive conformation of flavocytochrome b_{558} is exclusively favored over other conformations. Interactions with $p67^{phox}$ and Rac2 drive the favored conformation toward the intermediate and activated states; the conversion process(es) being relatively slow [47].

Support for this concept is provided by the observation that in a specific detergent and lipid environment, purified guinea pig flavocytochrome b_{558} displays NADPH-dependent, O_2^--generating activity independent of anionic amphiphile or cytosolic factors [180,181]. This [181] suggests the role of lipid and cytosolic subunits is to perturb the equilibrium that normally favors an inactive flavocytochrome b_{558} to favor the active form. Perhaps due to methodological differences, the preparations of Koshkin and Pick [180,181] are in an environment that is more favorable to the active conformation of flavocytochrome b_{558} even in the absence of cytosolic phox proteins. Consequently, they observe significant oxidase activity in the absence of $p47^{phox}$, $p67^{phox}$, Rac and amphiphile (although the presence of these molecules enhances activity in their experiments).

The conformational change responsible for these changes in activity may involve displacement of residues that normally block the NADPH binding site [293], changes in the relative orientation of the redox centers to facilitate electron transfer, a change in spin state of the haem(s) [87,90,199] or a combination of these factors.

8.2. Oxidase inhibitors

Despite an intensive search, the identification of a truly specific inhibitor of NADPH oxidase has proved elusive, although more than 120 had been described by 1990 [44] and more than 350 have been described in the current literature (Cross, unpublished). This susceptibility to inhibition is perhaps due to the complex nature of the oxidase and its regulatory pathways. The most widely used inhibitor, diphenylene iodonium [52], has like most inhibitors "lost specificity over time" and is now known to inhibit many flavoproteins, including xanthine oxidase (a source of O_2^-) and nitric oxide synthase. Two inhibitors that may stand the test of time are apocynin [283] and a $gp91^{phox}$-tat fusion peptide [244].

9. Products of the oxidase and indications as to their antimicrobial role

9.1. Reactive oxygen species

Oxygen radicals and their reaction products, collectively referred to as reactive oxygen species (ROS), are produced as a consequence of NADPH oxidase activity (Fig. 9). Since ROS can react with organic molecules, and because the oxidase is required to kill a number of important microbial pathogens, an enormous body of literature has developed that has causally linked the ROS directly to the death of the microbe. Extrapolations from these studies have implicated ROS in a wide range

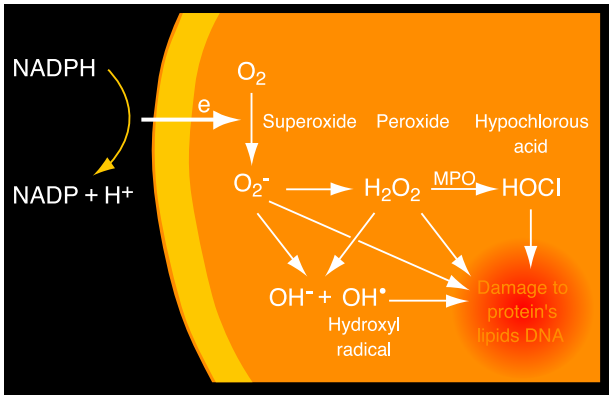

Fig. 9. Proposed toxic products of NADPH oxidase in neutrophils. The oxidase generates superoxide which dismutates to form hydrogen peroxide. The peroxide and superoxide can react to produce hydroxyl radicals in the presence of metal ions. Hydrogen peroxide can also serve as substrate for myeloperoxidase mediated oxidation of halides. Reactive oxygen species and hypohalous acids could produce damaging interactions with cellular constituents.

of pathological processes, and free-radical scavengers have been proposed as panaceas for their prevention and treatment. There were many good reasons for believing the theories relating to the direct toxicity of oxygen radicals and their metabolites. They sound reactive, and therefore toxic! Ionizing radiation, which generates free radicals, is clearly highly toxic to cells, although this is predominantly through damage to DNA. As will be described below, many of these issues need to be readdressed.

9.1.1. O_2^-

Although the superoxide anion radical, O_2^-, had been recognised in chemical systems for many years, the proof of its existence in biology followed the discovery of the enzymatic function of superoxide dismutase by McCord and Fridovich [201]. It was not long before it was shown that neutrophils produce large amounts of O_2^- [14] which then dismutate to produce the previously observed H_2O_2 [153].

Despite concerns relating to its toxicity [99], it was claimed that O_2^- had been shown to kill microbes directly [13,130,250].

9.1.2. $OH^\bullet$

O_2^- and H_2O_2 can combine to generate the very reactive hydroxyl radical ($HO^\bullet$) in the Haber–Weiss reaction. This requires a metal such as iron in the Fenton reaction, $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^- + HO^\bullet$. $HO^\bullet$ has been measured in a broken cell preparation [7] and has been implicated as a microbicidal agent [249]. These radicals are probably not found in intact cells [40] because lactoferrin, which is unsaturated in neutrophil granules, inhibits the generation of $HO^\bullet$ [30] and other free-radical reactions [127] by binding free copper and iron.

9.1.3. H_2O_2±*myeloperoxidase*

H_2O_2, which is used as a topical antiseptic [208], is produced by neutrophils and has been thought to be capable of killing microbes within them [39,195]. In combination with myeloperoxidase (MPO), a very abundant protein in neutrophils (which gives pus its characteristic green colour), it was thought that this enzyme catalyses the H_2O_2-dependent oxidation of halides that can react with and kill microbes. Experiments with the myeloperoxidase–H_2O_2–halide system demonstrated that this enzyme can kill bacteria in the test tube [130,131,167,168,170], and myeloperoxidase mediated halogenation has been the accepted basis of microbial killing for several decades.

Supportive evidence was provided by the finding that catalase negative organisms rarely infect patients with CGD [112]. The explanation was that these bacteria generated enough H_2O_2 to catalyse their own MPO-mediated halogenation within the vacuole of the neutrophil [147,238]. In vitro mutagenesis was used to generate strains of *Staphylococcus aureus* containing varying levels of catalase. Their virulence in mice was found to be inversely proportional to their catalase content [198]. Recently, however, doubts have been cast upon this theory. Catalase-deficient *A. nidulans* [36] and *S. aureus* [205] were shown to be as virulent as the catalase-positive varieties in a mouse model of CGD.

9.1.4. *Ozone*

More recently, it has been found that antibodies can catalyse the formation of ozone [312], and this has also been suggested as a possible mechanism by which bacteria might be killed and inflammation induced.

Experiments with $^{15}O_2$ showed [258] that almost all the O_2 consumed goes to form water, and little if any is incorporated into the organic matter of the cell or microbe.

9.2. *A new approach to the relationship between NADPH oxidase and microbial killing*

Although the proposal that ROS would be toxic to living cells is intuitively attractive, it was never adequately tested under the conditions pertaining in the phagocytic vacuole. The opportunity was provided by the development of gene targeting which allowed a mouse model to be constructed which was lacking the major neutrophil proteases, elastase and cathepsin G [241,297]. The essential nature of these proteases in the killing process was demonstrated by the fact that this mouse was very susceptible to infection by the same bacteria and fungi that infect CGD patients, and that its neutrophils failed to kill these organisms in vitro. The different organisms showed different susceptibilities to the different proteases. Most importantly, these cells failed to kill the microbes despite an entirely normal respiratory burst and microbial induced iodination. This meant that two separate components of neutrophil biology are required, a normal respiratory burst and a normal complement of granule proteases, and the challenge was to understand the interaction between the two.

The first step to this understanding was to know the conditions under which the products of the oxidase and the granule proteins interact within the vacuole. From knowledge of the O_2^- generated in response to the uptake of a bacterium and the volume of the vacuole as measured on electron micrographs, the amount of O_2^- pumped into the vacuoles can be calculated. It turns out to be enormous—in the region of 4 mol/l. This gargantuan concentration of O_2^- generation is required for effective killing. Some rare patients with CGD have been described in whom the molecular defect is incomplete and some oxidase activity can be detected [33,142,247]. These ‘‘variant’’ patients often demonstrate 10% (and exceptionally up to 30%, Malech, H., personal communication) of normal levels of activity. Thus, these patients are producing at least 400 mM O_2^- but can be as severely affected clinically as those with complete defects. These levels of substrate should be more than adequate for enzymic reactions, and yet they are not

sufficient for efficient killing, suggesting a physico-chemical alteration by the oxidase rather than the production of an enzyme substrate. In this regard, it should be noted that a high concentration of protein, approximately 500 mg/ml, and associated sulphated proteoglycans [175] are released from the granules into the vacuole.

An important additional consideration is that of the pH. Initially the vacuolar pH was thought to become acidic by analogy with that found after several hours in secondary lysosomes of macrophages [221]. In fact, the oxidase initially causes the pH in the vacuole of neutrophils to rise to about 7.8–8, after which it slowly falls [263] for the reasons described in the following sections.

9.3. Compensation of the charge across the vacuolar membrane

The oxidase is electrogenic, transferring electrons, unaccompanied by protons, across the vacuolar membrane [72,135,162,256]. The vacuolar volume is about 0.2 μ^3 with a membrane surface area of about 1.65 μ^2. O_2^- of 0.8–2.0 fmol is produced in each vacuole and thus about $5–10 \times 10^8$ electrons pass across each square micron of membrane. The charge on one electron is 1.6×10^{-19} C, so $3–7 \times 10^8$ charges in 1 μ^2 would produce 4.6×10^{-3} to 1.2×10^{-2} C/cm^2, which, taking the capacitance of the membrane at about 1 μF/cm^2 [234], would depolarize the membrane potential by 4600–11,700 V! Therefore, the charge must be compensated, if electron transport is not to cease almost immediately. It was demonstrated recently that depolarization of the membrane to +190 mV shuts down NADPH oxidase activity completely [75]. This finding confirms the expectation that electron extrusion will be opposed by voltage, and that charge compensation is absolutely required for continuous NADPH oxidase function.

9.3.1. Charge compensation by protons

One of the problems of studying charge compensation across the phagocytic vacuole is inaccessibility. Thus, many studies are conducted in which the cell/s are stimulated with soluble activators of the oxidase, such as PMA, under which circumstances a number of different but related processes are taking place at the plasma membrane. In addition to electron transport [256], there is movement of the compensating ions, and also the movement of protons and other ions to correct for the fall in pH produced by the oxidase, and these two are not necessarily the same.

In addition to the protons remaining in the cytoplasm as a result of charge separation when the electrons are transported from NADPH across the wall of the phagocytic vacuole, additional protons are produced in the cytosol by the hexose monophosphate shunt, which generates NADPH [26], as well as during the production of energy by glycolysis. This proton generation by an active oxidase, estimated to be about 150 mmol/l [213], causes an initial slight fall in cytosolic pH which rapidly returns to normal.

Three mechanisms appear to be associated with the extrusion of these H^+s which are extruded in roughly equimolar quantities with the O_2^- generated [290,302]. The predominant one is a Na^+/H^+ antiport [122,282], and its inhibition by the removal of extracellular Na^+ or blockage with amiloride causes acidification of the cytosol upon stimulation of the cells. In addition, both Zn^{2+} and Cd^{2+} blockable proton channels [136,212] and vacuolar (V)-type H^+ pumps, inhibited by bafilomycins [213], are also present.

It is generally agreed that the charge induced by electron translocation (I_e) through the NADPH oxidase is compensated by proton efflux [75,135,137]. The identity of this channel is currently very contentious. On the one hand, there is a school of thought that proposes that the protons pass through voltage-gated proton channels that are distinct from any NADPH oxidase component [73,74,210] whereas the opposing view is that they pass through the flavocytochrome *b* of the oxidase, gp91phox, itself [138,199,214,299].

One of the hallmarks of the assumption that I_e is largely compensated by proton fluxes is that both Zn^{2+} and Cd^{2+}, known proton channel blockers [74,136,295], were also thought to inhibit O_2^- production [75,137]. The discrepancy between the low μM concentrations of these cations that block proton channels and the mM concentrations needed to inhibit cytochrome *c* reduction, was recently explained by the voltage dependence of I_e whereby Zn^{2+} and Cd^{2+} shift the threshold voltage for activating voltage-gated proton channels into the steeply voltage-dependent region of I_e, thereby attenuating O_2^- production [75].

However, Zn^{2+} and Cd^{2+} do not in fact inhibit the NADPH oxidase, as they have no effect on oxygen consumption but interfere with the superoxide detection system by accelerating the dismutation of superoxide to hydrogen peroxide, thereby bypassing the reduction of cytochrome *c*. In a system in which xanthine–xanthine oxidase generated O_2^-, 3 mM concentrations of these elements induced the dismutation of O_2^- to H_2O_2 at a rate indistinguishable from that catalysed by superoxide dismutase (1 μg/ml). Zn^{2+}, at concentrations three orders of magnitude greater than those causing almost complete blockage to proton channels, was also without effect on the currents measured in electrophysiological studies performed on neutrophils and on eosinophils, or on PMA induced ^{86}Rb efflux from these cells [5].

9.3.2. Charge compensation by K^+

The changes in the vacuolar pH (Fig. 10) hold the key to understanding the nature of the compensating ions [80,156]. This pH is elevated from that of the extracellular medium to 7.8–8.0 [263] despite the release into the vacuole of 500 mg/ml granule protein contents [241] that are maintained at pH 5.0 in the granule by a proton pump [286]. These granule contents are strong buffers and it required about 400 μmol of KOH per gram of granule protein to elevate the pH from 5.0 to 8.0 [241]. Because the vacuole becomes alkalinised despite the entry of these acidic granule contents, the compensating charge cannot be solely in the form of H^+

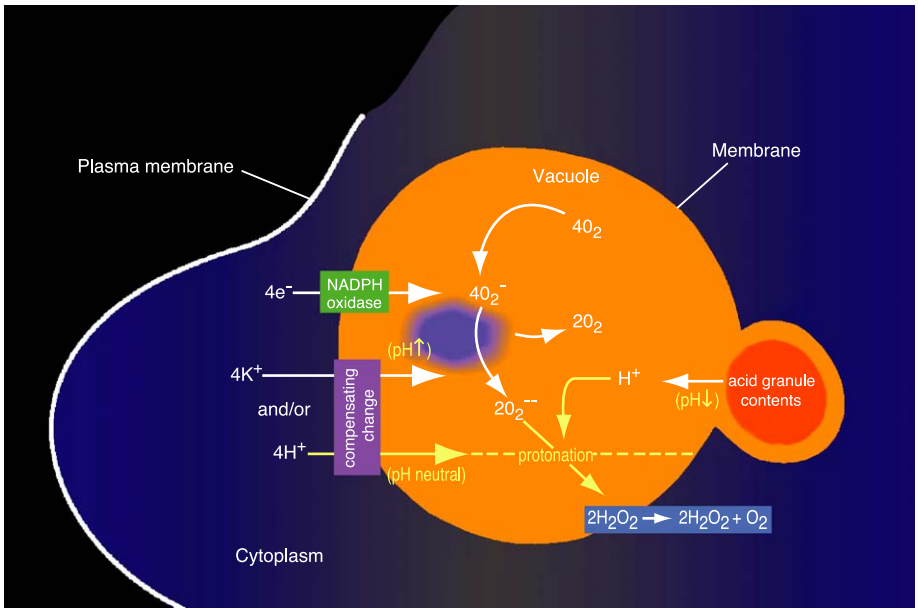

Fig. 10. Schematic representation of ion fluxes and influence on pH of phagocytic vacuole. The interior of the cytoplasmic granules is maintained at a pH of about 5.0 by V-ATPases [123]. The degranulation of these contents into the vacuole causes it to become acidic. The superoxide and its dismutation product, peroxide, become protonated, consuming protons in the vacuole and driving the pH up. The passage of electrons across the vacuolar membrane to produce O_2^- generates a charge across the membrane that must be compensated by the passage of other ions for electron transport to continue. The nature of the ions compensating this charge has a profound effect on the pH in the vacuole. The movement of protons prevents the consumption of those within the vacuole and the pH is not elevated whereas other ions such as Cl^- from, or K^+ to, the vacuole cause it to rise.

from the cytoplasm, because such H^+ flux is ultimately pH neutral (see below).

The only ion other than H^+ that is present in the cytoplasm at high enough concentrations to compensate the charge by passing into the vacuole is K^+. It accumulates in the vacuole at concentrations of up to about 600 mM as a consequence of oxidase activity [241]. Transport of K^+ ions is markedly diminished when the pH rises above 8.0. Thus, charge compensation by K^+ provides an important self-regulating mechanism for elevating the vacuolar pH to between 7.8 and 8.

K^+ flux only accounts for about 6% of the compensating charge [241] and since the proton channel does not appear to compensate all the rest of the charge because its inhibition does not block the NADPH oxidase, some other major ion flux must be involved.

9.3.2.1. The K^+ enters the phagocytic vacuole through the large conductance Ca^{2+}-activated K^+ channel. It is known that neutrophils have a high intrinsic permeability to K^+ ions and contain a number of different types of K^+ conductances. These include inwardly rectifying K^+ channels [197], voltage-gated K^+ channels and Ca^{2+}-activated K^+ channel [182] and ATP-sensitive K^+ [281] channels. K^+ enters the phagocytic vacuole through the large conductance Ca^{2+}-activated K^+ channel [5]. Iberiotoxin (IBTX) and paxilline (PAX), both highly selective and potent inhibitors of the BK_{Ca} channel [113,253,310], prevented the alkalinisation of the vacuole. The IC_{50} values for this effect were in the region of 10 nM for IBTX and PAX, consistent with their IC_{50} for channel block [38,154]. In addition, the BK_{Ca} channel opener, NS1619 [186], significantly augmented the rise in pH to supra-normal levels. A variety of blockers and openers of other K^+ channels were without effect.

$^{86}Rb^+$ release from activated neutrophils after stimulation with PMA was also induced by the BK_{Ca} channel opener NS1619, and even further enhanced by the combination of this opener and PMA. PMA-induced efflux and that caused by NS1619 were both completely abrogated by IBTX and PAX.

BK_{Ca} channels are classically opened by the combination of membrane depolarisation and elevated $[Ca^{2+}]_c$ [159]. The same was found to hold true for this channel in neutrophils and eosinophils. Neither depolarising the membrane nor elevating the cytosolic $[Ca^{2+}]$ was sufficient to fully open the K^+ channel, whereas the combination of these caused as much channel opening as did stimulation with PMA. Although PMA stimulation is well known to depolarise the neutrophil plasma membrane [155], it is generally thought not to elevate $[Ca^{2+}]_c$. One mechanism by which this might occur could be through a drop in pH just beneath the plasma membrane as a consequence of charge separation induced by the oxidase. Corresponding elevations in $[Ca^{2+}]_c$ and falls in pH were seen just beneath the plasma membrane in activated cells [5].

9.3.2.2. The movement of K^+ into the vacuole activates elastase and cathepsin G. The contents of the cytoplasmic azurophil granules are not freely in solution. They are almost exclusively highly cationic proteins that are strongly

bound to the highly negatively charged proteoglycans, heparin and chondroitin sulfate [175], in which state they are inactive. They are activated in the vacuole both by the elevation in pH described above, and by the hypertonic K^+ that breaks the charged interaction between the enzymes and the matrix, releasing them in a soluble form [241]. For these hypertonic conditions to develop, water must be prevented from entering the vacuole in response to the osmotic attraction of the salts. This is achieved by encasing the vacuole in a meshwork of cytoskeletal proteins, including paxillin and vinculin.

The importance of the accumulation of K^+ in the vacuole was shown when this was abrogated either with the K^+ ionophore valinomycin [241] or by blocking the BKCa channel with the specific inhibitors iberiotoxin or paxillin [5]. In both cases, microbial killing and digestion was almost completely prevented. The fact that the microbes were not killed despite the generation of normal quantities of ROS and normal levels of iodination proved that these were not directly involved in the microbicidal process, whereas inhibition of killing by removal of the proteases cathepsin G and elastase proved that these enzymes were.

9.4. Myeloperoxidase

Myeloperoxidase (MPO) is a di-haem protein comprised of two identical heterodimers. Each heterodimer is formed from the post-translational modification of a single polypeptide precursor. The two symmetric halves are linked by disulfide linkage between the two heavy chains. The covalently bound haem has a unique structure and exhibits unusual spectral properties that are responsible for its green colour [100]. MPO constitutes about 5% of the total neutrophil protein and is present in the cytoplasmic granules at very high concentrations. It makes up about 25% of the granule protein to achieve concentrations of about 100 mg/ml (1 mM) in the vacuole.

Experiments with the myeloperoxidase–H_2O_2–halide system demonstrated that this enzyme can kill bacteria in the test tube [130,131,167,168,170]. However, these experiments were conducted under unphysiological conditions, with relatively low concentrations of MPO (50 μg/ml), at low pH (5.0) and, most important of all, in the absence of the high levels of proteins found in the vacuole. When bacteria were exposed to 100 mM H_2O_2 or 1 mM HOCl in the presence of 25 mg/ml granule proteins (technically much more manageable than the experimentally determined 500 mg/ml), killing was almost abolished [242]. Neutrophils clearly iodinate and chlorinate proteins when bacteria are phagocytosed, and this halogenation is dependent upon an active NADPH oxidase and myeloperoxidase [171]. However, it is not the bacterial protein that is being iodinated [262] and chlorinated [37] but the proteins of the enveloping neutrophil, particularly those from the cytoplasmic granules. This would be a highly inefficient system if its primary purpose was to halogenate bacterial proteins.

A few patients were discovered whose neutrophils lacked MPO, who were also thought to be immunodeficient [187] and recently an MPO "knock-out" mouse was shown to be susceptible to yeast but not bacterial infection [9]. However, the advent of automated differential leukocyte counting machines, where the identification of neutrophils was dependent upon a peroxidase stain, revealed that about 1 in 2000 of the general population is MPO deficient without any undue predisposition to infection [215], and the neutrophils of birds do not contain MPO [235].

One possible function of this enzyme is to protect the digestive enzymes from oxidative denaturation by removing H_2O_2 from the phagocytic vacuole, rather than the traditional antimicrobial function ascribed to it.

MPO has catalase activity [165] but this only functions efficiently if the compound II that accumulates is reduced back to the native enzyme. This can be achieved by the high concentrations of O_2^- in the vacuole with which MPO forms an adduct to produce compound III [318].

It is also possible that MPO has a dual function, as a catalase under the conditions pertaining in the vacuole, but in a microbicidal capacity outside the cell where levels of enzyme and substrate would be much lower, and the pH is also generally low at sites of infection and inflammation.

10. The NOX family

Recently, six human homologs of $gp91^{phox}$ have been identified (Table 2).

10.1. Nomenclature of phox proteins

The phox family of proteins ($p22^{phox}$, $gp91^{phox}$, $p47^{phox}$, $p67^{phox}$ and $p40^{phox}$) is named for *ph*agocyte *ox*idase. Their corresponding genes are CYBA and CYBB [for *cy*tochrome *b* subunits α ($p22^{phox}$) and β ($gp91^{phox}$)]; *NCF1* ($p47^{phox}$), *NCF2* ($p67^{phox}$) and *NCF4* ($p40^{phox}$) (for *N*eutrophil *C*ytosolic *F*actor). NCF3 turned out to be rac2. The NOX (*N*ADPH *OX*idase) and DUOX (*DU*al *OX*idase) nomenclature was agreed by interested parties at the Gordon Research Conference on Phagocytes in June 2001. The NOXO1 and NOXA1 (for NOX Organizer 1 and NOX Activator 1) terms were adopted by researchers attending the Banbury Center Conference on NADPH oxidases in November 2002. These names have been approved by the HUGO Nomenclature Committee.

10.2. General features

Evolutionary relationships revealed three main subgroupings of the NOXs. The gp91phox subfamily consisting of Nox1, gp91phox, Nox3 and Nox4 have molecular masses of about 65 kDa. The Duox molecules comprise the second

Table 2
Members of the NOX/DUOX family of NAD(P)H oxidases and related cytosolic factors

Isoform (synonyms)	Locus	Tissue distribution	Known splice forms	Possible function
NOX1 (MOX1, NOH1)	Xq22	Colon, smooth muscle	3	Mitogenic signaling/proton channel
NOX2 (gp91^{phox})	Xp21.1	Myeloid	1	Host defense
NOX3	6q25.1–26	Fetal kidney	1	?
NOX4 (Renox)	11	Kidney, vascular smooth muscle, endothelium	1	Oxygen sensing/signaling
NOX5	15q22	Pancreas, placenta, ovary, testis	5	?
DUOX1 (p138Tox)	15q21	Lung, thyroid, testis, prostate	2	Thyroxin synthesis/extracellular matrix
DUOX2	15q21	Colon, pancreas, testis	2	Extracellular matrix
NOXO1	?	Colon, testis	?	p47^{phox} analogue (for NOX1?)
NOXA1	?	Colon	?	p67^{phox} analogue (for NOX1?)

group with masses of about 180 kDa. The extra mass in this group is contributed by two additional domains, a central domain of two EF-hand calcium binding motifs and an N-terminal peroxidase domain [185].

All the members of the NOX family have many structural features in common with gp91^{phox}. These include six conserved transmembrane helices in the N-terminal half of the protein, the third and fifth containing conserved pairs of histidine residues 13 or 14 amino acids apart, equivalent to the axial haem ligands of gp91^{phox} (NOX2). A conserved arginine residue is present at the equivalent position of gp91^{phox} R54 thought to form a hydrogen bond with a haem propionate side-chain described above [60]. FAD- and NAD(P)H-binding motifs are also conserved (Fig. 2). The exonic structure of *NOX1* and *CYBB* (the gp91^{phox} gene) are remarkably similar, testifying to their common origin, although the intron sizes are very different, suggesting relatively ancient gene duplication [166].

In general these proteins are expressed in epithelial cells, and by analogy with gp91phox, would be expected to transport electrons across membranes. Oxygen is probably the recipient of these electrons, and it might be reduced to form superoxide or H_2O_2. Because the levels of expression of these systems are much lower than those of the NADPH oxidase in phagocytes, and because natural lesions have not been identified in these systems, gene targeting experiments may be required before their functions and properties are clearly resolved.

The transmembrane location of these molecules in epithelial linings of the gut and kidneys, where large ionic fluxes occur, suggests that they might be involved in these fluxes, as has been shown for the neutrophil system.

10.3. Specific NOX systems

10.3.1. Nox1

The best characterized of the NOXs is the O_2^--generating NOX1 (originally named *M*itogenic *OX*idase, MOX1) described by Suh et al. [287]. Their work was prompted by the fact that: (1) many tumor cells show increased production of O_2^- and other ROS; (2) cells exposed to growth factors respond by increasing ROS production, and (3) cells exposed to ROS show increased proliferation (a well studied example being vascular smooth muscle and endothelial cells). Independently, Bánfi et al. [18] cloned the same gene based on a sequence associated with H^+ channels (gp91^{phox} is thought by some groups to conduct protons). Bánfi et al. also reported the identification of two further gp91^{phox} homologs, and the Nox family has since grown to seven distinct Nox genes, several of which have multiple splice forms (Table 2).

NOX1 mRNA is expressed in colon, prostate, uterus and vascular smooth muscle, but not in leukocytes (Table 2). Lambeth's group showed that platelet-derived growth factor induced NOX1 expression in smooth muscle cells, and expression of NOX1 in normal embryo fibroblasts (NIH 3T3 cells) increased O_2^- production and cell growth. Furthermore, cells overexpressing NOX1 take on a transformed appearance, show anchorage-independent cell growth and induce aggressive tumors in athymic mice. This work showed that NOX1 might play a fundamental role in the control of cell growth. Subsequently, they have shown that NOX1 expression is a powerful trigger of angiogenesis, promoting tumor growth [10]. *NOX1* is located at chromosome Xq22 and codes for a protein of predicted length of 565 amino acids that shares 56% identity with gp91^{phox}. Recent work suggests it may be regulated by its own versions of p47^{phox} and p67^{phox}, NOXO1 and NOXA [114].

10.3.2. Other NOX family members

The second homolog described, NOX4 (originally Renox, *ren*al *ox*idase), is expressed in the renal cortex and proximal convoluted tubule epithelial cells and was suggested to be an oxygen sensor [115,279]. Expression of NOX4 in fibroblasts resulted in increased O_2^- production and a senescent phenotype (in contrast to the transformed phenotype induced by NOX1 expression). It was proposed that NOX4 regulates the expression of erythropoeitin through the generation of ROS that directly affect the activity of transcription factors. Subsequently, NOX4 has been found to be more widely expressed, and the discovery of an unrelated flavin and haem containing oxygen sensor (related to cytochrome b_5 fused to cytochrome b_5 reductase

[329]) that is ubiquitously expressed has cast doubt on the role of NOX4 as an oxygen sensor. Both NOX1 and NOX4 are expressed in the vasculature where there is an abundance of recent literature supporting a central role for ROS in both normal physiology and also pathology (reviewed in [Refs. 121,224,313,323]). NAD(P)H oxidases are major sources of O_2^- in vascular cells and myocytes. In response to growth factors and cytokines, they produce O_2^- and H_2O_2, which act as second messengers to regulate multiple signaling pathways [319]. These vascular oxidases appear to be essential to the physiology of vascular cells, regulating growth, cell migration and extracellular matrix composition. Dysfunction of ROS production has been linked to hypertension, uncontrolled growth and inflammation, and atherosclerosis. For example, excess ROS production has been demonstrated in hypertension and aging [129], while angiotensin-induced hypertrophy has been shown to be ROS-dependent [327].

A substantial literature supports a role for $p22^{phox}$ (in particular), $p47^{phox}$ and, to a lesser extent, $p67^{phox}$, in the regulation of NOX1 and NOX4 activities, but this is hard to reconcile with the data from $p22^{phox}$-, $p47^{phox}$- or $p67^{phox}$-deficient CGD patients who appear to suffer no obvious phenotypic abnormalities outside of their obvious susceptibility to infection. The latter observation indicates that $p22^{phox}$, $p47^{phox}$ and $p67^{phox}$ are not absolute requirements for NOX1 and NOX4 function and that other, as yet unidentified, isoforms of these neutrophil factors probably exist (such as NOXA1 and NOXO1, Section 4.2).

Presently, there is little information regarding the requirements of NOX3 and NOX5 for other regulatory subunits. In the case of the "long" NOX family members, DUOX1 and DUOX2, the presence of EF-hands presents the likelihood of Ca^{2+} regulation, and both biochemical data [81,101,188], disease studies [209] and thyroid expression [34,71,92] support an essential role for one or both of these proteins in thyroid hormone synthesis. Nevertheless, significant expression outside thyroid tissue suggests these proteins may perform other roles.

10.4. Functions of these proteins in other organisms

In *C. elegans*, the homologous DUOX has been shown to play an important role in cross-linking of the extracellular matrix. RNAi showed that reducing the activity of this enzyme results in abnormal development and excessive fragility of the cuticle [94]. Similarly, inhibition of DUOX activity in drosophila causes fragile wings that become torn and ragged (Lambeth, J.D., personal communication).

A related protein has recently been shown to be important for root development in plants [104]. Plants such as *Arabidopsis* contain multiple (>6) isoforms of these proteins [164,298] and although some are known to be involved in disease resistance [120] and iron uptake [246] (similar to yeast), it is highly probable some serve signaling functions [217].

Acknowledgements

We thank the Wellcome Trust and National Institutes of Health for support.

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