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Review

# NADPH oxidase of neutrophils

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## Contents



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## 1. Introduction

A number of reviews have been written on this subject and related topics in the last 10 years to which the reader will be referred in relevant sections. However, Edwards [62] provides a good overview of neutrophil physiology and Rossi [170] has provided an excellent account of the early work. The emphasis placed in this review reflects the research interests and personal bias of the authors; a paper-by-paper account of all that has been published, in any field, is likely to be dull to the point of indigestibility.

All circulating blood cells, despite their widely differing morphology, are derived from one pluripotent, haematopoietic stem cell population located in the red bone marrow of bones (vertebrae, ribs, sternum, skull and long bones) which comprise 2–3% of body weight. Erythrocytes, responsible for the transport of $O_2$ to and $CO_2$ from the tissues, are the predominant cell type, outnumbering leucocytes (white blood cells) by 1000:1. Collectively, the leucocytes are responsible for the defence of the body against infection and are subdivided into neutrophils (3700 cells/mm$^3$), lymphocytes (2500 cells/mm$^3$), monocytes (400 cells/mm$^3$), eosinophils (150 cells/mm$^3$) and basophils (30 cells/mm$^3$). The different classes of lymphocytes produce antibodies in response to an infection and serve as the immune system memory, whereas neutrophils, monocytes and eosinophils participate in the cellular immune response by engulfing, killing and digesting invading organisms. Neutrophils and monocytes are bactericidal, whereas eosinophils deal with parasites. The cytoplasm of neutrophils, eosinophils and basophils (collectively termed granulocytes) is rich in secretary granules which can be released following activation. Basophils contain histamine and serotonin which, when released, give rise to the immediate hypersensitivity reaction (allergic response). The cytoplasmic secretary granules of neutrophils and eosinophils contain proteinases, cytotoxic proteins and chelators utilised during the killing and digestion of foreign bodies. Platelets, derived from megakaryocytes, participate in blood clotting and wound healing. All blood cells have a limited lifespan, which varies from many years for lymphocyte memory cells, through 120 days for erythrocytes to a few days in the case of neutrophils, and therefore have to be continuously replaced at an appropriate rate from the pool of maturing cells within the bone marrow [4,62]. Errors in the regulation of this replacement, both natural and induced, result in a number of disorders including leukaemias and lymphoma.

Neutrophils are the initial participants in the cellular defence of the body. They contain a characteristic large, multi-lobed nucleus (thus their other name, polymorphonuclear leucocytes), a cytoplasm rich in three types of granule (azuorphilic, specific and tertiary), [11,22,139,191] but little endoplasmic reticulum, golgi apparatus and few mitochondria. Differentiation and maturation in the bone marrow takes 10 or 11 days. Once in the blood, unstimulated neutrophils are either in free circulation or marginally and reversibly attached to the endothelial cells of the blood vessel wall. Upon bacterial invasion, the neutrophils exit the bloodstream through transiently generated gaps between the endothelial cells (diapedesis) and migrate actively towards the site of infection, where they engulf (phagocytosis) and kill the bacteria. The process of phagocytosis continues until the bacterium is completely internalised, surrounded by membrane in the phagosome, into which the contents of the granules are discharged. A large burst in oxygen consumption, the respiratory burst, occurs concomitantly. The encounter is normally lethal for both bacterium and neutrophil. The resulting debris is cleared by macrophages which arrive later.

## 2. Respiratory burst

A large burst in oxygen consumption by neutrophils during phagocytosis was first observed by Baldridge and Gerard [12]. It has been shown to be insensitive to inhibitors of the respiratory chain of mitochondria (HCN, antimycin), and to be associated with an increase in metabolism through the hexose monophosphate shunt. It is now accepted that this increased oxygen consumption is due to the activity of the NADPH oxidase which catalyses the reaction:

$$NADPH + 2O_2 \rightarrow NADP^+ + 2O_2^{\cdot -} + 2H^+$$

The oxidase activity which is quiescent in resting cells may be stimulated in vitro within 15 to 60 s by a wide variety of compounds, including phorbol esters (PMA), heat-aggregated IgG (HAGG), unsaturated fatty acids and analogues of bacterial peptides (fMLP). The burst is tran-

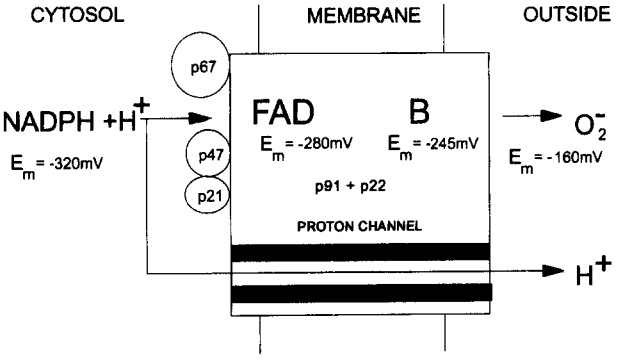


Fig. 1. Diagrammatic representation of the NADPH oxidase components and associated $H^+$-channel. The NADPH oxidase is composed of a heterodimeric, low-potential cytochrome *b* (gp91-*phox* + p22-*phox*) and cytosolic proteins which translocate to the membrane upon activation (p67-*phox*, p47-*phox* and p21$^{rac}$). The generation of superoxide involves the transfer of an electron from cytosolic NADPH, through FAD and haem, to external oxygen. This process is electrogenic and requires the efflux of $H^+$ ions through a channel to act as charge compensator. The redox potential of the cytochrome is atypically low for a *b*-type cytochrome but enables the reduction of oxygen to superoxide.

Table 1
$K_m$ and turnover numbers for the NADPH oxidase in whole and fractionated cell systems

| System | Substrate | $K_m$ ($\mu$M) | Turnover number (cyt $b$/s) | Reference |
|---|---|---|---|---|
| Neutrophils | NADPH | | 6.06 | [84] |
| Neutrophils permeabilised | NADPH | 20–30 | | [137]a |
| Cytoplasts | NADPH | | 26.67 | [84] |
| Cell-free reconstitution | NADPH | 28.4 | 65 | [99] |
| | NADH | | | |
| B-lymphocyte cell lines | NADPH | 30.5 | 13.75 | [77] |
| | NADH | 341 | 8.33 | |
| Detergent-extracted oxidase | NADPH | 45 | 18.38 | [41] |
| | NADH | 460 | n.d. | |
| Relipidated cytochrome $b$ | NADPH | 75 | 23.8 | [114] |
| | NADH | 1860 | n.d. | |

sient in nature, reaching a maximum rate at 3 min after stimulation and being undetectable above background after 30 to 60 min post stimulation [170,14]. The oxidase activity of whole cells shows a high $Q_{10}$, being virtually inactive at 25°C but reaching maximal activity at 38°C [84]. The basis of the enzyme mechanism is the utilisation of electrons from cytosolic NADPH to perform the single electron reduction of external oxygen ($O_2$) to the free radical, superoxide ($O_2^{\cdot-}$); Fig. 1. Superoxide is highly reactive and very short-lived. In solution it rapidly dismutes to $H_2O_2$, which can by a number of further reactions lead to the generation of $OH^-$, hydroxyl radical, singlet oxygen and hypochlorous acid. The $K_m$ values and turnover numbers for the oxidase in various systems with NADPH or NADH are shown in Table 1.

## 3. Chronic granulomatous disease

Chronic granulomatous disease (CGD) is an inherited condition in which the affected individuals express a increased susceptibility to bacterial and fungal infections. It occurs with an incidence of 1:250000 to 1:500000 of the European population and prior to the discovery of antibiotics lead to death following infection within the first year of life. The discovery that neutrophils from CGD patients failed to mount a respiratory burst [98,161] identified the NADPH oxidase as the genetic lesion involved in CGD and thereby significantly advanced the study of the NADPH oxidase and the diagnosis and treatment of CGD patients. Both X-linked and autosomal recessive patterns of inheritance have been demonstrated for this disease (Table 2). The use of cells from CGD patients has greatly aided the study of the different components of the NADPH oxidase. Chronic granulomatous disease and its genetics have been extensively covered in a recent reviews [44,168,203].

Table 2
Different lesions giving rise to chronic granulomatous disease

| Type CGD | Genetic lesion | % of total GCD | Gene locus | Cellular location |
|---|---|---|---|---|
| X-linked cyt $b$ −ve | gp91-phox | 60 | Xp21.1 | membrane |
| Autosomal recessive cyt $b$ −ve | p22-phox | 5 | 16q24 | membrane |
| Autosomal recessive cyt $b$ +ve | p47-phox | 30 | 7q11.23 | cytosol |
| Autosomal recessive cyt $b$ +ve | p67-phox | 5 | 1q25 | cytosol |

## 4. Assay for NADPH oxidase

There are a number of assays for the detection of superoxide generation and hence the activity of the NADPH oxidase. In all cases it is important that the progress of $O_2^{\cdot-}$ generation be followed continuously and that with whole cells and cytoplasts the temperature be maintained at 38°C. The following methods are in current use:

(a) The superoxide dismutase (SOD)-inhibitable reduction of cytochrome $c$, in which the unpaired electron of $O_2^{\cdot-}$ reduces cytochrome $c$ and causes an increase in absorbance at 550 nm. This may be done either by conventional spectrophotometry or using a micro plate reader.

(b) Chemiluminescence arising due to the interaction of $O_2^{\cdot-}$ or a dismutation product, with luminol or lucigenin which results in the emission of light, using a luminometer. This assay system has also been adapted for use on a microplate reader.

(c) Nitroblue tetrazolium reduction (NBT) is usually performed on a few cells on a microscope slide and counterstained with eosin. The basis of the assay is that $O_2^{\cdot-}$ or, more likely a product, donates electrons to the water-soluble NBT, resulting in the formation of the water-insoluble, coloured formazan. The assay is the test used in the identification of patients and carriers of CGD.

(d) Oxygen consumption detected using an oxygen electrode shows a dramatic increase following stimulation of the oxidase. Care has to be exercised to ensure that $O_2$ consumption by other means, e.g., mitochondria, is excluded by using suitable inhibitors.

(e) Changes in fluorescent properties of a number of dyes have been exploited for the detection of the activity of the oxidase, e.g., scopieotin, dihydrorhodamine 123. However, these are assays for $H_2O_2$ production and therefore are indirect assays and not absolutely specific for $O_2^{\cdot-}$.

(f) Species-specific spin trap probes, whose presence is detected by the use of EPR.

In all cases it is necessary to initiate the activity of the oxidase by the addition of an appropriate concentration of

a stimulus and it is prudent to ensure the specificity of the activity by use of an inhibitor. The method of choice is certainly the reduction of cytochrome *c*, as this is least likely to result in artifacts.

## 5. Cell types producing superoxide

The activity of the oxidase can be measured, by one of the above assays, in neutrophils, eosinophils, macrophages either isolated from whole blood or recovered from the peritoneal cavity following the elicitation of an immune response (peritoneal cells), in neutrophils isolated from the inflamed joints of a rheumatoid patients (synovial fluid neutrophils) and in macrophages recovered by lavage of the lungs (alveolar macrophages). With these cells superoxide generation can be elicited in isolated whole cells, enucleated cells (cytoplasts), permeabilised cells, in a cell-free reconstitution system and a modified reconstitution system using a combination of purified and recombinant protein components. Neutrophils are normally isolated from whole blood or the buffy coat fraction (concentrated white blood cells). They are readily isolated in high numbers ($10^9$ per pint blood) by means of centrifugation through a selective density gradient.

Cytoplasts are enucleated, agranular, right-side-out derivatives of cells which, despite lacking the internal organelles of their parent cell retain their important functional characteristics. Cytoplasts can be generated from neutrophils in large numbers on a discontinuous step gradient of Ficoll. They express a stimulatable NADPH oxidase activity and retain an ability to undergo chemotaxis and phagocytosis [169]. As they are single-compartment cells, they have been exploited in studies in which the unequivocal cellular location of the dyes was important [82] and in studies dissecting the relative contribution of superoxide and the granular contents to the process of killing [148].

The technique of electroporation [71,72,112] and a number of bacterial toxins (streptolysin O, $\alpha$-toxin) [13,100] have been utilised to permeabilise cells, facilitating access to the interior. However, none of the permeabilisation techniques is 100% efficient (some whole cells always remain) and the holes also permit the leakage of cytosolic components and cofactors out of the cell. In fact, recent studies have made use of this loss of cytosol to explore the requirements for the maintenance of the oxidase activity [32].

The use of the cell-free reconstitution system was first reported in 1984 [23,91] and involves the recombination of the membrane and cytosolic fractions isolated from unstimulated cells following disruption by sonication [158]. The elicitation of oxidase activity requires the presence of GTP-$\gamma$-*S* [66,123,155], $Mg^{2+}$ and is activated by the addition of an unsaturated fatty acid (arachidonate) or SDS [24,45,123,186]. The membrane and cytosolic fractions from different sources can be recombined in a heterologous cell-free system [35]. A further refinement of the cell-free reconstitution system has been the development of the use of purified or recombinant, relipidated membrane components of the oxidase combined with recombinant cytosolic proteins to demonstrate the absolute requirement or enhancement of oxidase activity achieved by the inclusion of a known protein [1,2,135,172,173]. The broken cell assay systems are relatively unstable at 37°C and are therefore normally monitored at 25°C.

## 6. Cells used in the study of the oxidase by tissue culture

Of blood cells which express the oxidase only monocytes can be maintained in culture for some time (7–14 days). They grow in size but are incapable of cell division. Other blood cells survive only a matter of few hours in vitro and must be used the same day. However, there are a small number of immortal cell lines which either constitutively express the oxidase or in which the expression can be induced. Some of these are:

(a) Isolated B lymphocytes immortalised following transformation with Epstein-Barr virus (EBV-transformed lymphocytes) can be maintained as a suspension in culture and express the components of the oxidase constitutively [208]. Low levels of oxidase activity can be triggered by a number of compounds, including those that cross linking Fc receptors, interleukin 1$\alpha$, phorbol esters [77,127]. The lag time following addition of the stimulus is longer than that observed for the neutrophil oxidase and superoxide generation is sustained for longer (2 h).

(b) A human myeloid cell line, HL60, isolated in 1977 from a patient with acute promyelocytic leukaemia [36]. The cells are characterised as promyelocyte, grow continuously in suspension and can be induced to differentiate into neutrophil by addition of dimethylsulfoxide (DMSO) or dimethylformamide (DMF) or into macrophages by 1,25-dihydroxyvitamin $D_3$, PMA or sodium butyrate [21,37,38,81]. The process of differentiation into neutrophils requires 5 to 7 days and is associated with an increase in the expression of the components of the oxidase correlated with an increase in the activatable superoxide generation [121,130,143,167]. Unfortunately, the differentiated cells are no longer immortal.

(c) PLB-985 are a diploid human myelomonoblastic cell line derived from patient with relapsed myeloid leukaemia [205,216]. This cell line has very similar properties to those described for HL60 cells.

(d) U937 are exclusively a monocyte cell line in which the expression of the oxidase components and superoxide generation can be induced by PMA, all-*trans*-retinoic acid, vitamin $D_3$, interferon-$\gamma$ together with tumour necrosis factor-$\alpha$ [81,109,195].

(e) THP-1 are also a monocyte cell line derived from young patient with acute monocytic leukaemia which can

be induced to express the oxidase [204]. They have been utilised to prepare cDNA libraries.

## 7. Components of the oxidase

The identification of the components of the oxidase was facilitated by the availability of cells from CGD patients and the availability of complementation assays (cell fusion [76] and cell-free reconstitution system). It is now widely accepted that the oxidase consists of a membrane-spanning, heterodimeric cytochrome *b* consisting of a large β-subunit (gp91-*phox*) and a smaller α-subunit (p22-*phox*) associated with two proteins located in the cytosolic fraction of unstimulated cells (p47-*phox* and p67-*phox*) (Fig. 1). The cDNA encoding these proteins have been cloned and sequenced and the chromosomal location has been identified [52,53,80,119,126,154,176,198,199,209,215]. The corresponding proteins have been purified ; cytochrome *b* [180], p67-*phox* [158]a,[195]a and p47-*phox* [158]b,[195]b.

### 7.1. Cytochrome b

The absence of a characteristic *b*-type cytochrome difference spectrum (reduced − oxidised) from neutrophils isolated from a number of CGD patients suggested a role for the *b*-type cytochrome in the NADPH oxidase [179,180]. The differentiation of HL60 cells into neutrophil-like cells is associated with a dramatic increase in their ability to generate superoxide in response to stimuli and this is exactly paralleled by an increase in cytochrome *b* [121,167].

The redox potential is atypically low (−245 mV) for a *b* cytochrome, but this enables the reduction of oxygen to superoxide [40,214]. In a detergent-extracted active oxidase complex, under anaerobic conditions, in the presence of NADPH the cytochrome is only some 10% reduced [138]. The rate of electron flow through the *b* cytochrome has been demonstrated to be matched by the rate of generation of superoxide [41]. It is thought that the low-level steady-state reduction of the *b* under anaerobic conditions indicates a requirement for oxygen binding before functional reduction can occur [41].

In the neutrophil, the cytochrome *b* is localised in the plasma membrane and in the membrane of the specific granules [181] in a ratio of 30:70%. Some authors have found even higher amounts in the specific granules [20]. There is evidence that some of the cytochrome in the specific granules translocates to the plasma membrane or membrane of the developing phagolysosome following stimulation [34,182]. This suggests that the granules act as a reservoir of cytochrome to be called upon when required, although the extent to which this occurs may be small. This hypothesis is supported by the fact that the specific granule membrane cytochrome *b* has been demonstrated to be functionally competent when combined with the cytosolic fraction [29]. Whether the specific granular cytochrome pool becomes activated following stimulation but before incorporation into the plasma membrane is not known at present, but seems unlikely.

The X-linked gene responsible for CGD was cloned by reverse genetics from a differentiated HL60 cDNA library [176]. The predicted product from the cloned gene was a polypeptide of 468 amino acids. The lack of sequence similarities with other cytochromes cDNAs and the ab-

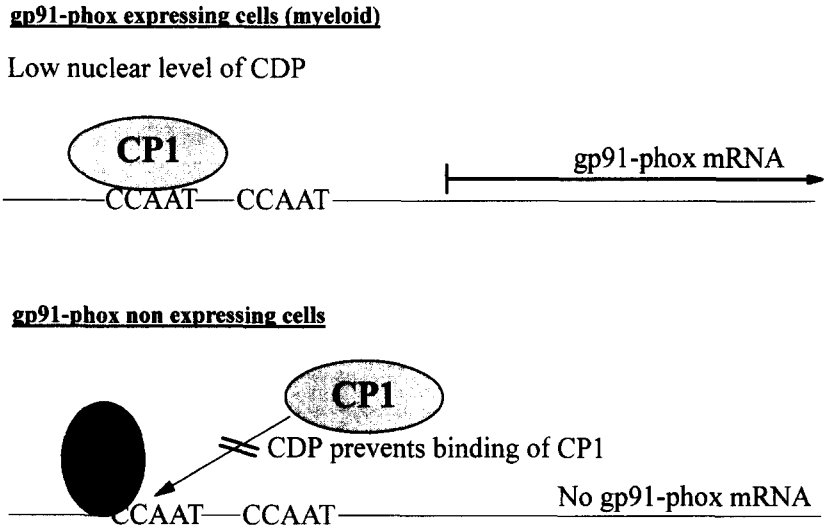


Fig. 2. Proposed mechanism for the restriction of gp91-*phox* expression to myeloid cells. The 1.5 kb upstream region of gp91-*phox* promoter contains a duplicated CCAAT box. In myeloid cells the expression of gp91-*phox* is facilitated/promoted by the binding of the DNA-binding protein, CP1, to the distal CCAAT box. In non-myeloid cells the expression of gp91-*phox* is suppressed by the interaction of CDP (CCAAT displacement protein) with the promoter, excluding the binding of CP1. Nuclear extracts of myeloid cells have much reduced levels of CDP compared to those from non-myeloid cells. This 1.5 kb region contains sufficient information to direct the expression of reporter genes to only cells of myeloid lineage.

sence of an obvious haem binding site was not consistent with a cytochrome. Independently of this, the cytochrome was purified from human neutrophils and shown to be a heterodimer of $\alpha$-subunit (22 kDa) and a $\beta$-subunit (broad band 70–110 kDa indicative of glycoprotein) [153,198]. The N-linked glycosylation of the $\beta$-subunit is reported to be removed by endoglycosidase F treatment, leaving a protein which runs with an apparent molecular mass of 55 kDa on SDS-PAGE [80]. The extent of the reported glycosylation appears to vary between individuals [110] and between species from high in human neutrophils to very low in guinea pig macrophages [157]. The N-terminal amino acid sequence of the $\beta$-subunit [198] corresponded to the sequence of the CGD gene as elucidated by Orkin and co-workers [176]. It was established that the in vivo $\beta$-subunit N terminus is at nucleotide 16 of the cDNA sequence, some 306 nucleotides upstream of that originally suggested. Independently, the predicted protein sequence of the X-CGD gene was used to raise anti-peptide antibodies. Western blots identified a 90 kDa protein corresponding to the X-CGD gene [52]. The corrected 5′ untranslated sequence [189] suggests that the protein is not synthesised with a leader sequence, predicting a cytosolic N-terminus. The initiating Met is probably removed following translation to give the native protein. The hydropathy plot of the full-length $\beta$-subunit suggests that it may cross the membrane 4 or 5 times and has a large hydrophobic C terminal domain. However, the precise three-dimensional structure of the protein is at present unknown.

The $\beta$-subunit, gp91-*phox* appears to be exclusively expressed in cells of the myeloid line, as no clear demonstration of gp91-*phox* (mRNA or protein) in other cells has been reported [154,189], in contrast to p22-*phox*, p47-*phox* and p67-*phox* [103]. The regulation of its restricted expression has been shown to be due in part to regions in the 1.5 kDa 5′-untranslated region of gp91-*phox* gene [189]. The region contains a duplicated CCAAT box between nucleotides $-106$ and $-124$ and a TATA box at nucleotide $-30$ (Fig. 2). Deletions made in this region revealed an interaction between the DNA binding protein, CPI, and the distal CCAAT box in cells expressing gp91-*phox*. High levels of a second DNA-binding protein, CCAAT displacement protein (CDP) are found in the nuclear extracts of cells not expressing gp91-*phox*. In the presence of CDP expression of gp91-*phox* is repressed, since the binding of CDP and CP1 is mutually exclusive. The absence of CDP:DNA binding, usually through low levels of CDP expression, is a prerequisite for gp91-*phox* expression. Transgenic mice given the construct containing the 1.5 kb–2.6 kb upstream region of gp91–*phox* attached to one of two reporter genes, human growth hormone or SV40 early region showed expression restricted to 10% of the monocyte. Constructs with deletions in the CCAAT region gave greater levels of gene expression in the transgenic mice [190]. It can therefore be concluded that 1.5 kb upstream region of the gp91-*phox* is sufficient to target its expression to only myeloid cells and that repression of expression involves interaction between the DNA binding protein CDP and the CCAAT box region. As expression of the reporter genes could not be detected in the granulocytes and expression was only found in 10% of the monocyte population it can be concluded that this 1.5 kb upstream region does not contain all that is required to switch on expression.

The $\alpha$-subunit of the cytochrome, p22-*phox*, was cloned and sequenced by Parkos et al. [154] and has a predicted molecular mass of 22 kDa. Comparison of the cDNA and protein sequence demonstrated that the protein is not synthesised with a leader sequence. The hydropathy plot suggests that the protein may cross the membrane twice. That the protein protrudes on both faces of the membrane is suggested by the binding of a number of antibodies directed against defined sequences within the protein [102]. Although the mRNA for the $\alpha$-subunit has been reported to be present in uninduced and DMF induced HL60 cells, EBV-transformed B lymphocytes, HeLa cells, HepG2 (hepatic), K562 (erythroleukaemic) and human endothelial cells, the stable expression of the protein was detected only in DMF-induced HL60 cells and therefore appears to be restricted to cells expressing mRNA for the $\beta$-subunit, i.e., myeloid cells [154]. CGD patients with a genetic defect in p22-*phox* gene are rare and show an autosomal recessive pattern of inheritance (Table 2) [168]. It has been reported that CGD patients with a defect in either cytochrome subunit gene lack the stable expression of both subunits [180,210]. This has been taken to suggest that stable expression, i.e., synthesis and insertion into the membrane, requires the regulated expression of both. However, the level of expression of the mRNA was not reported so it is not possible to decide whether the lack of expression of the unaffected protein is regulated by the affected protein at the level of transcription or translation or whether the expressed protein just turned over more rapidly. In contrast, stable expression of gp91-*phox* occurs in HL60 cells lacking p22-*phox* expression in the presence of a haem synthesis inhibitor and in Chinese hamster ovary cells transfected with a plasmid containing the cDNA for gp91-*phox*. No expression of p22-*phox*, p47-*phox* or p67-*phox* was detected [83]. In EBV-transformed B lymphocyte cell lines established from CGD patients with a genetic lesion in p22-*phox* gene, the expression of gp91-*phox*, although lower, is not absent [83,128,159]. That this might be the case in vivo is suggested by the presence of gp91-*phox* intermediates in CGD patients lacking expression of p22-*phox* [128,159]. Therefore, the expression gp91-*phox* does not require that of p22-*phox*.

### *7.1.1. Haem moiety binding site*

Low-temperature (4.2 K) EPR spectra of the partially purified pig cytochrome showed peaks at $g = 2.85$, 2.21 and 1.67 indicative of a haem in low-spin configuration, with bis(imidazole) co-ordination [101,206]. However, the

determination of which subunit carries the haem moiety presents a problem, since the sequence of neither subunit of the cytochrome contains regions of homology with known cytochromes and other haem-binding proteins. The sequence of the $\alpha$-subunit contains only one histidine; therefore, a single p22-*phox* subunit cannot contain the haem binding site.

In an attempt to determine the haem-binding subunit, Nugent et al. [144] used ionisation inactivation to determine the size by following the loss of the characteristic cytochrome difference spectrum. It was found that the target corresponded to the destruction of a protein of 21 kDa, possibly p22-*phox*. In agreement with this observation, HL60 cells differentiated in the presence of the haem synthesis inhibitor succinylacetone fail to generate superoxide in response to a stimulus and do not show a typical cytochrome spectrum. Although these cells express gp91-*phox*, they have no detectable stable expression of p22-*phox* reacting with polyclonal antibodies [83]. Therefore, the translation or stable expression of the p22-*phox*, but not gp91-*phox*, requires the presence of haem, suggesting that this subunit contains the haem binding site. If p22-*phox* does contain the haem pocket, then the haem moiety must be held between either a dimer of $\alpha$-subunits or between a dimer composed of $\alpha$- and $\beta$-subunit or even in some novel fashion. The number of haem moieties per oxidase complex is uncertain. Quinn et al. [162] report that the oxidase is a multi-haem complex.

### 7.2. Cytosolic factors

Prior to the development of the cell-free assay system, complementation assays with poly(ethylene glycol)-induced fusion of monocytes from different CGD patients demonstrated that there were at least three different genetic defects that could give rise to CGD [76]. Therefore, the genetic lesions giving rise to CGD were not restricted to the cytochrome in the membrane. Segal et al. [178] identified a 47 kDa protein which was not phosphorylated upon activation of neutrophils from autosomal recessive CGD patients. The activity of the cell free reconstitution system was known to be enhanced 3-fold by the inclusion of GTP or GTP-$\gamma$-*S* [66,123,152,186], which indicated the involvement of a GTP-binding protein in the oxidase. In an attempt to purify the oxidase-associated GTP-binding protein(s), Clark and co-workers [210] fractionated cytosol by passing it through a GTP-column and eluting the bound protein with GTP. Polyclonal antibodies raised to the eluant of a normal donor were used to detect the absence of expression of proteins of apparent molecular mass of either 47 kDa or 67 kDa in the neutrophil cytosols of cytochrome-*b*-positive CGD patients [145,210]. Genetic defects in p47-*phox* and p67-*phox* are reported in 30% and 5%, respectively, of all CGD patients.

The amino acid sequence of neither p47-*phox* nor p67-*phox* contains regions of homology to known flavin- or NADPH-binding sites and no detectable FAD has been reported to be associated with either isolated protein [27]. It is therefore probable that neither of these cytosolic proteins acts as the NADPH-oxidase-associated flavoprotein, nor do they contain the NADPH binding site. Both do, however, contain two regions of homology with the src tyrosine kinase SH3 domains. The possible roles for the SH3 domains in the oxidase will be discussed in greater detail below.

It has been widely reported that there is a time-dependent and ongoing increase in the amount of both p47-*phox* and p67-*phox* in the membrane fraction of the cell following activation of the oxidase, which has been ascribed to a translocation of both proteins from the cytosol to the membrane [30,31]. However, at best the proportion of either recovered in the membrane fraction is 2% of the total immunodetectable protein, even 10 min after stimulation and long after the oxidase has achieved maximal activity (around 1 min or less) [3]. This raises the question as to why there is an excess of cellular p47-*phox* and p67-*phox* protein. Dusi et al. [60] have shown that with PMA-, fMLP- or Con-A-stimulated human neutrophils p47-*phox* and p67-*phox* are continuously recruited over the period of study (7 min), whereas the rate of $O_2$ consumption had reached a constant rate within 1 min. It is possible that a given active oxidase complex is capable of superoxide production for a limited period of time and that the continuous generation of superoxide requires continuous recruitment of cytosolic factors which represent the expendable components of the complex.

The translocation of both p47-*phox* and p67-*phox* following activation of the oxidase does not occur in cells which lack expression of the cytochrome *b* [95]. This can be interpreted as either that the signal necessary for or that triggers translocation is not present if a functional oxidase cannot be formed, or, much more likely, that translocation to the membrane occurs but as the membrane lacks the attachment site for the cytosolic factor(s) they do not become stably associated and fall back into the cytosolic fraction. It is possible to speculate that the attachment site is on either or both of the subunits of cytochrome *b* (see below). The translocation of p67-*phox* does not occur in cells from CGD patients lacking p47-*phox*. However, the translocation of p47-*phox* is unaffected by the absence of p67-*phox* [61,94,149]. It seems that the translocation of the two cytosolic factors is a co-ordinated event, either because the two factors translocate together as a complex or that the attachment of p47-*phox* to the membrane forms the binding site for p67-*phox*. That the second of these possibilities is probably correct is supported by the recent observations of Abo et al. [3], in which the translocation of both p47-*phox* and rac2a occurred at substimulatable concentrations of fMLP or PMA and that the activity of the oxidase correlated with the subsequent translocation of p67-*phox*. Therefore, the translocation of p47-*phox* precedes and is necessary for the translocation of p67-*phox*.

The stoichiometry of the concentration of the components of the active oxidase is unknown.

However, relipidated purified cytochrome alone is capable of generating superoxide [114,115], which calls into question the function of the cytosolic factors. Is it possible that in vivo the cytosolic factors are required to hold the conformation of the cytochrome in a form corresponding to that of the relipidated cytochrome? At this time the function(s) of both cytosolic factors has not been clearly identified.

The second largest group of CGD patients is that which arises as a consequence of a genetic lesion in p47-*phox*. Casimir et al. [26] have identified a hot spot for mutations located at the first intron exon junction which contains a GCGC repeat. A mismatch results in a deletion of one of the GC pairs and shifts into frame a stop codon which terminates translation prematurely. This deletion mutation was reported to be found in three unrelated CGD patients lacking the expression of the p47-*phox* protein.

### *7.3. Low-molecular-weight GTP-binding protein*

A wide range of ras monomeric, GTP-binding proteins have an important role in cellular functions, including protein trafficking through the cell and signal transduction pathways involving receptor tyrosine kinase. Like heteromeric GTP-binding proteins, they are active in the GTP-bound form. The members of this family and their associated GTPase-stimulating proteins (GSP) have been reviewed recently by Hall [75], Goud and McCaffrey [70], Pfeffer [156], Rothman and Orci and [171] Bokoch and Der [16] and their role in the oxidase by Bokoch [17,18]; therefore, we will give only a brief account here.

These proteins are monomeric, have an apparent molecular mass of 21 kDa, and are members of a family that exhibits a high degree of sequence homology, the major differences being in the C-terminal ends of the proteins. Members of both the ras and rac subfamilies undergo a post-translational modification consisting of prenylation of the cysteine located four amino acids from C-terminus followed by removal of the three C-terminal residues. The unaltered proteins are found predominantly in the cytosol of unstimulated cells, associated by their prenyl tail with GDI, an inhibitory protein of the GDP/GTP exchange.

An involvement with the NADPH oxidase for this class of proteins was first suggested by the co-purification of cytochrome *b* and a 21 kDa protein, immunologically identified as rap1a [163]. Later and independently, a cytosolic factor ($\sigma$) in addition to p47-*phox* and p67-*phox* was shown to be required to obtain maximum superoxide generation in a cell-free reconstitution system [23,24]. $\sigma$-Factor was subsequently shown to consist of $p21^{rac1}$ and its associated GDP-dissociation inhibitor *rho*GDI [1]. The purified rac1 protein, added in the GTP form, increased the activity of a purified cytochrome with recombinant p47-*phox* and p67-*phox* by up to 3-fold. This degree of activation is in remarkable agreement with that reported for the addition of GTP-$\gamma$-*S* alone to the cell-free reconstitution system [66]. This protein was later identified as rac2A, which is expressed in cells of myeloid origin whereas rac1a is more widely expressed.

From the work of Abo et al. [3] it appears that, in the resting unstimulated neutrophil, rac2a is complexed with its GDP/GTP exchange protein (*rho*GDI), in the GDP-bound form, located in the cytosol. Following activation of the oxidase, rac2a is found dissociated from its GDI, probably in the GTP-bound form, and undergoes a translocation to the membrane and specific granules [3,131,164]. As with the other cytosolic factors discussed above, less than 1% of total cellular rac2a was relocated [3]. How this is triggered is not known and the function of rac2a in the activation and activity of the oxidase is uncertain. It has been suggested that he dissociation of rac2a from its GDI is triggered by phosphorylation or by the addition of arachidonate [1,183]. Compactin is a potent inhibitor of 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA), which is on the pathway for the biosynthesis of the substrates for prenylation. HL60 cells differentiated in the presence of DMSO and compactin lack the ability to generate superoxide and the cytosol from such cells is incapable of functioning in a cell-free reconstitution system. The further addition of mevalonic acid in the culture medium restored the ability to generate superoxide [19]. These experiments provide strong evidence that a prenylated rac protein is required in order to achieve maximal rates of superoxide generation.

### *7.4. p40-phox*

Segal and colleagues have recently described an additional protein which 'enhances' the activity of the purified recombinant cell-free system above that already described [213]. The protein bears a high degree of homology to p47-*phox* and also has two SH3 domains. It is difficult at present to judge the extent of involvement of this protein in the oxidase and to assign a function to it. However, the protein was identified immunologically and reported to translocate to the membrane upon activation of the oxidase in whole cells and in cell-free reconstitution system. The nature of the involvement of this protein in the oxidase will be resolved with time. At present, no CGD patients with a genetic defect in this protein have been reported.

### *7.5. Flavoprotein*

The involvement of a flavin moiety in the active complex has long been recognised. The addition of FAD but not FMN was reported to enhance the activity of the detergent-extracted oxidase [5], whereas inactive analogues of FAD were found to be mild inhibitors of oxidase activity in these preparations [151,152]. Some authors have shown that FAD is required in the cell-free reconstitution

system. An EPR signal corresponding to a semi-flavin radical has been observed in an active oxidase [104].

The compound diphenylene iodonium (DPI) is an inhibitor of complex I of mitochondria showing time-dependent inhibition with a $K_i$ of 100 $\mu$M [164]a. This compound was subsequently shown to be a more potent inhibitor of the detergent-extracted active NADPH oxidase preparation from pig neutrophils ($K_i$ 10 $\mu$M) and showed a similar time-dependent inhibition [42]. DPI was subsequently shown to inhibit the NADPH oxidase of rat peritoneal macrophages [78], human neutrophils and cytoplasts (enucleated cells) [82], EBV-transformed B lymphocytes [77,127], differentiated HL60 cells [83] and a cell-free reconstitution system derived from human neutrophils [58,99]. A number of analogues which were reported inhibitors of mitochondria complex I were also shown to inhibit NADPH oxidase, though with a higher $K_i$ value than DPI [43].

As described above, the steady-state difference spectrum of an active detergent-extracted oxidase preparation, recorded in the presence of NADPH, shows only a 10% reduction of cytochrome *b* and a flavin trough corresponding to flavin reduction [41]. These were both absent from spectra which were recorded in the presence of NADPH and DPI (10 $\mu$M). This was interpreted as an inability of the electrons to enter the oxidase complex and therefore that DPI must act at or before the flavoprotein. It was assumed that, since DPI was capable of inhibiting the oxidase in the already activated detergent-extracted preparation, it must act directly upon the oxidase complex itself rather than a component of the signal transduction pathway for the activation of the oxidase. DPI was less effective an inhibitor in the presence of excess (2 mM) NADPH, suggesting that there was competition for binding between DPI and NADPH. Therefore, DPI was assumed to inhibit the NADPH oxidase at the level of or before the flavoprotein component [42].

In an attempt to identify the NADPH-oxidase-associated flavoprotein, Cross and Jones [42] incubated $^{125}$I-DPI with pig neutrophil extracts. After SDS-PAGE, a number of bands showed labelling, but attention was focused on a protein of apparent molecular mass 45 kDa [42]. A band of similar molecular mass was subsequently demonstrated in rat peritoneal macrophages [78], fibroblasts [134], erythropoietin-producing hepatoma cells (Hep G2) [69] and human neutrophils. As a consequence of the site of action of DPI and its labelling pattern, the 45 kDa band was assumed to be the flavoprotein of the NADPH oxidase. However, these experiments were conducted at a time when it was not known that DPI must first be converted to a radical before it can inhibit the oxidase (see below) [146,147,200].

The inhibition of both the mitochondrial complex I and NADPH oxidase by DPI is time-dependent at all concentrations tested [164]a. It has been demonstrated that DPI, pre-incubated with cells for even long periods of time (1 h), failed to inhibit the initial burst of oxidase activity. Further, the respiratory burst of cells pre-incubated and removed from the DPI solution prior to stimulation was unaffected [79]. These observations may be taken to suggest that a change in conformation of the oxidase is a pre-requisite for inhibition or that the inhibitory compound is not DPI per se but that it requires chemical modification. That the latter is the case is supported by a number of observations [58,82,146,147,200]. It appears that DPI must first be converted to a radical (by the turnover of the oxidase, exposure to reducing agents) before reaction can occur. Reaction may be with a number of targets varying from FAD, haem or proteins.

Vignais and colleagues have also investigated the interaction of $^{125}$I-labelled DPI and iodonium biphenyl with proteins of bovine neutrophils and cell-free reconstitution system. They reported a modification in the cytochrome *b* spectrum in active oxidase treated with iodonium biphenyl or DPI [57,58]. $^{125}$I-DPI labelled a large number of proteins on SDS-PAGE in resting cells, but there was a marked increase in labelling of a 22 kDa band which was associated with activity of the oxidase [58]. As suggested by its apparent molecular weight and the correlated modification of the cytochrome *b* spectrum the protein was identified as the $\alpha$-subunit of the cytochrome *b* (p22-*phox*).

The DPI-dependent inhibition of FAD reduction by NADPH observed by Cross and Jones [42] is not inconsistent with the site of action of DPI postulated by Vignais and co-workers, since interaction of cytochrome *b* with oxygen is a necessary pre-requisite for the reduction of FAD by NADPH [41].

Although a specific site of action for DPI in complex 1 has been reported, [164]a, the DPI radical is highly reactive. This makes the determination of its site of action within the oxidase extremely difficult and uncertain. There is no doubt that DPI is an extremely effective and very useful inhibitor of superoxide generation, but whether this is due to modification of a specific target or whether its effects are merely an accumulation of modifications, the sum of which renders the enzyme non-functional, is not known. The non-specific labelling of proteins by $^{125}$I-labelled DPI is due presumably to the generation of radicals by endogenous reductants; see [57,58].

A number of enzyme systems having a cytochrome and flavin (FAD) have been shown to be inhibited by DPI, e.g., cytochrome *P*-450, endothelial and macrophage nitric oxide synthase, in addition to mitochondrial NADH dehydrogenase. Therefore, DPI is a good inhibitor of FAD cytochrome enzyme systems which permit the generation of DPI radical [147].

### *7.5.1. Cytochrome b as the flavoprotein*

Within the amino acid sequence of gp91-*phox* Segal et al. [184] and Rotrosen et al. [173] identified regions of homology to the NADPH and FAD binding sites of a number of other enzymes, the highest degree of homology

being with ferredoxin-$NADP^+$ reductase. In support of the hypothesis that the β-subunit of the cytochrome is also the flavoprotein (flavocytochrome), Segal et al. [184] reported a correlation between the levels of haem and the flavin in preparations containing widely different amounts of gp-91-*phox*; from inactive and active neutrophils, active neutrophils; from an X-linked CGD patient; an autosomal recessive patient; uninduced HL60 cells, which was taken to suggest a coexpression of their binding proteins. Unfortunately the authors did not study induced HL60 cells, which show increased expression of the components of the oxidase and so should show a parallel increase in haem and flavin moieties. Their purified cytochrome had no flavin associated with it but could be reflavinated to 20% of that of membranes, though the extent of reflavination and the ability to generate superoxide was not investigated. The photolabile NADPH analogue 2-azido-NADP labelled a broad band in the region of the β-subunit on an SDS-PAGE, only when the oxidase was turning over. They concluded that the cytochrome was a flavocytochrome and that the β-subunit contains both the FAD and NADPH binding sites.

Photolabelling of neutrophil membranes with [$^3$H] arylazido NADPH [56]a or deflavinated membranes with [$^3$H]arylazido FAD [56]b have provided convincing evidence that the oxidase is an NADPH-dependent flavocytochrome *b*. The predominantly photolabelled species was a 90–100 kDa protein which was established unambiguously to be the β-subunit (gp91-*phox*) by specific immunodetection and deglycosylation.

In further support of the hypothesis, attention was drawn to a CGD patient who showed an X-linked pattern of inheritance and whose neutrophils failed to generate superoxide in response to a variety of stimuli but had a normal level of cytochrome *b* [173,184]. Orkin and colleagues [54] had demonstrated that the patient had a single-point mutation proline 414 → histidine in the sequence GAGIGYT*P*-FAS, but could not explain why this rendered the protein non-functional, except that it may result in an altered protein conformation. However, the identification of this region of the protein as the possible NADPH-binding site may be a more probable explanation for the patient's clinical symptoms.

If the cytochrome is a flavocytochrome, as is widely accepted, this would explain the lack of a class of CGD patients lacking the flavoprotein only. It also poses the question: if the cytochrome contains the flavin, NADPH and haem binding sites what is the role and function(s) of the cytosolic factors? The flavocytochrome contains all that is necessary for the transfer of electrons from NADPH to oxygen; therefore can it generate superoxide in the absence of the cytosolic factors? It has recently been reported that the purified cytochrome from guinea-pig macrophages, relipidated with octyl glucoside, was capable of generating superoxide at a rate of 23.8 $s^{-1}$, following the activation by LDS and in the presence of NADPH and FAD [114,115]. Resting cells do not generate superoxide, despite the fact that the substrate, electron acceptor and the prosthetic groups are together in the membrane. Therefore, in vivo the cytosolic factors may be necessary for altering the conformation of one or both the subunits of the cytochrome upon activation.

### 7.6. $H^+$ channel

The activation of the NADPH oxidase in human neutrophils and macrophages is associated with a rapid depolarisation of the membrane potential from −60 mV to around −25 to −20 mV. The depolarisation occurs with a wide range of activators of the oxidase, is complete within 1 min following the addition of the stimulus and is stable for periods of more than 10 min. Some authors have also observed a small initial hyperpolarisation (5 mV) prior to the subsequent depolarisation. Initially it was proposed that the change in membrane potential was an effect of the signal transduction pathway leading to the activation of the oxidase [113,136,196,197], possibly arising as a consequence of a triggered influx of $Ca^{2+}$ ions [211]. However, the failure to observe the depolarisation in stimulated neutrophils from various CGD patients [118,187,188] is not consistent with this hypothesis, since the lesion lies in one of the components of the oxidase complex and not in the signal transduction pathway.

Cytoplasts are right-side-out, single-compartment enucleated neutrophils which retain the functions of the parent cell, including a fully active PMA- and fMLP-activatable NADPH oxidase [82,164]. They retain a resting membrane potential at −60 mV and show a rapid depolarisation of greater than 40 mV, upon activation of superoxide generation. The extent of the depolarisation following the addition of PMA was shown to be considerably reduced in the presence of DPI, an inhibitor of the oxidase. The addition of DPI, following the PMA, resulted in a partial repolarisation of the membrane potential [82,84]. These observations strongly suggested that the activity of the oxidase contributed directly to the changes in the membrane potential.

To generate superoxide, the oxidase passes an electron to external oxygen using internal substrate (glucose) through NADP-linked dehydrogenase as the donor, i.e., it transfers an electron across the membrane. NADPH is a hydride ion donor, but cytochrome *b* is an electron acceptor as is dioxygen. What happens to the $H^+$ ion? If it is transferred to the external environment, the process is electroneutral. If, however, it is retained on the inside, then the process shows a net transfer of a negative charge outwards, consistent with depolarisation. It was therefore proposed that the depolarisation of the membrane potential was due to the fact that the transfer of an electron from internal NADPH to external oxygen was not synchronised with the transfer of a compensating charge, either negative inward or positive outward. Therefore, the generation of superoxide by the NADPH oxidase is an electrogenic

process [82]. It follows from this hypothesis that if a neutrophil, macrophage etc. is incapable of generating superoxide in response to a stimulus, it is incapable of exhibiting a membrane potential depolarisation and this therefore provides an explanation for the reported failure to observe a depolarisation of membrane potential in neutrophils from CGD patients. As the depolarisation of the membrane potential reaches a steady state, not only must the movement of a compensating charge occur, but eventually, about 1 min after stimulation, it must exactly balance that of the transfer of electrons.

Thomas and Meech [201] have presented evidence for the existence of an $H^+$ channel in snail neurones. This channel was blocked by addition of $Zn^{2+}$ or $Cd^{2+}$. In the presence of $Zn^{2+}$ or $Cd^{2+}$ the PMA-stimulated membrane potential depolarisation was accelerated and greatly extended ($-60$ mV to $-10$ mV), suggesting that these metal ions delayed the achievement of the balance between the flux of the electrons and the proposed charge compensator and suggesting the involvement of an $H^+$ channel. That an efflux of $H^+$ might act as the charge compensating ions was further suggested by the observation that the extent of the PMA-triggered membrane potential depolarisation could be dramatically altered by the extent of transmembrane pH gradient. The depolarisation was smaller if the cytoplasts were stimulated in an alkaline medium and greater if stimulated in an acid solution. The larger the driving force for the efflux of $H^+$ ions, the smaller the extent of the membrane potential depolarisation and similarly in the presence of an inward pH gradient the potential depolarisation was greatly enhanced [82]. These observations all suggested that $H^+$ were directly involved in the charge compensation for the electrogenic generation of superoxide, i.e., an efflux of $H^+$ through a channel compensates for the activity of the oxidase (Fig. 1). The proposed presence of an $H^+$ channel facilitates the maintenance of superoxide by compensating for the efflux of electrons while also preventing a dramatic fall in internal $pH_i$. However, initially the two transmembrane fluxes are asynchronous, one lagging behind that of the other.

The internal pH ($pH_i$) of neutrophils and a number of other cells has been investigated by preloading the pH-sensitive fluorescent dye, BCECF (2′,7′-bis(2-carboxyethyl)-5-(and -6-)carboxyfluorescein) into the cytosol by means of its lipid-soluble acetoxyester. Cleavage by cellular esterases exposes four negative charges which effectively traps the dye in the cell [166]. The response of the dye can be calibrated by alterations in the external pH ($pH_o$) of the cells in the presence of the electroneutral $H^+$:$K^+$ exchanger, nigericin [202]. This strategy has been exploited by a number of workers to monitor changes in $pH_i$, of many cells including neutrophils [73,74,117,212] and cytoplasts [85]. Neutrophils maintain a resting $pH_i$ of 7.3 (approx. 0.1 pH units below that of $pH_o$) [73,74,85]. However, stimulation of the oxidase is associated with a small fall in $pH_i$ (0.1 pH unit in 1 min) followed by a gradual alkalinisation to 0.2 pH units above that of the resting cell, over the following 5 min [73,85]. As the generation of superoxide utilises NADPH, the stimulation of the oxidase is associated with an increase in pentose phosphate pathway activity and hence an increased release of $CO_2$. Amiloride, and a number of derivatives, are inhibitors of $Na^+$ ion fluxes through channels or cotransporters including the $Na^+$:$H^+$ exchanger. The extent of the acidosis following the triggering of the oxidase was slightly increased in the presence of amiloride, or one of it derivatives, and no alkalinisation was observed over 5 min, suggesting that the $Na^+$:$H^+$ exchanger contributes to the recovery from the acid load imposed by the generation of superoxide [74,85].

It follows from the above hypothesis of an electrogenic oxidase and an associated $H^+$ channel that in the presence of inhibitors of the channel or if an alternative ion is permitted to act as the charge compensator for the transport of electrons, then the fall in internal pH following the activation of the oxidase should be greatly enhanced. The stimulation of superoxide generation in cytoplasts was shown to result in a fall in $pH_i$ and this occurred more rapidly and to a greater extent in the presence of $Zn^{2+}$ or $Cd^{2+}$ ions (0.25 pH units), in the presence of $K^+$ ionophore valinomycin (0.3 pH unit) or in the presence of both valinomycin and $Zn^{2+}$ or $Cd^{2+}$ ions (0.5 pH unit) [85]. These observations parallel the changes observed in the membrane potential and strongly suggest that the efflux of $H^+$ ions occurs through a channel which acts as a charge compensator for an electrogenic oxidase. The changes in $pH_o$ were also consistent with the existence of an $H^+$ channel, blocked by $Zn^{2+}$ or $Cd^{2+}$ [85].

The activity of the oxidase was subsequently shown to be tightly coupled to the efflux of $H^+$ ions through the channel. The inhibition of the channel results in inhibition of the superoxide generation. That this was not due to the effect of lowered $pH_i$ was demonstrated by the restoration of superoxide generation following the provision of an alternative charge compensating ion [86]. Therefore, the generation of superoxide by the oxidase is dependent upon the movement of a compensating ion, normally $H^+$, through a channel. However, there is not an absolute requirement that the compensating ion be $H^+$; $K^+$ and valinomycin acted as the charge compensator for the oxidase until other factors become limiting. Therefore, the mechanism for the transfer of an electron through the oxidase does not require the efflux of the $H^+$, nor does the $H^+$ channel depend on oxidase activity.

Resting human neutrophils maintain a $pH_i$ within 0.1 pH unit of $pH_o$, despite having a large membrane potential (negative inside) which would favour the influx of $H^+$ ions, presumably because $H^+$ ions are pumped out by the $Na^+$:$H^+$ exchanger and the membrane is relatively impermeable to $H^+$; the channel only opens when activated either by such agents as PMA or more directly by arachidonate (see below). As described above, the activation of

the oxidase is associated with a rapid depolarisation of the membrane potential and an initial fall in $pH_i$, which provide a driving force (protonmotive force, $\Delta p$) for $H^+$ efflux.

The activity of the channel can be monitored in the absence of the oxidase activity, thereby easing the study of its activation and regulation. For example, exposing cells suspended in a high $Na^+$ medium to an inward pH gradient by rapidly dropping the $pH_o$ ($pH_o < pH_i$) and/or generating a strongly negative membrane potential by the addition of valinomycin results in a large driving force for the influx of $H^+$ ions. Alternatively, in a medium high in $K^+$ ions and in the presence of valinomycin, conditions under which the membrane potential approaches zero, the imposition of an outward pH gradient ($pH_o > pH_i$) by the addition of Tris base provides a driving force for the efflux of $H^+$ ions. The possibility of either the oxidase or the $Na^+$:$H^+$ exchanger contributing to the pH changes can be prevented by the addition of excess DPI and amiloride [87]. By monitoring the channel function in the absence of the activity of the oxidase, it was demonstrated that the channel was not opened by depolarisation of the membrane potential, the imposition of a large pH gradient, in either direction, or by the imposition of combination of the two, $\Delta p$. However, in all cases the appropriate change in $pH_i$ resulting from the predicted flux of $H^+$ ions was observed following the addition of arachidonate [87]. As arachidonate failed to act as an $H^+$ carrier (protonophore, uncoupler) in liposomes [87] it was postulated that arachidonate activates the $H^+$ channel as with the oxidase itself. Arachidonate may open the channel by a direct action upon the channel protein(s) or by indirect means, e.g., increasing the fluidity of the membrane, allowing protein movement or conformation change. In the presence of arachidonate, the direction and extent of $pH_i$ changes observed is that predicted by the protonmotive force and the channel can conduct $H^+$ ions in either direction.

Kapus et al. [106] have reported the measurement of a $Zn^{2+}$ or $Cd^{2+}$ inhibitable $H^+$ channel in $NH_4^+$ pulsed, acid-loaded pig neutrophils. They report that the efflux of $H^+$ ions is 2.5-fold enhanced in the presence of PMA and suggest that this results from the PMA directed opening of the channel [106]. There is no evidence to suggest that PMA acts directly upon the channel but probably acts through its activation of protein kinase C.

In recent years there have been a number of electrophysiological studies of $H^+$ conductances in rat epithelial cells [47], differentiated HL60 [50], mouse macrophages [107] and human neutrophils [48] which have all identified the presence of a current ascribed to $H^+$. The outward conductance was observed at holding potentials of +60 mV or higher, with an imposed pH gradient of 1 to 1.5 pH units. At a given potential the magnitude of current observed correlated with the size of the transmembrane pH gradient. Irrespective of the pH gradient, no current was reported at holding potentials at zero or negative potentials [48,50,107,108]. It has been concluded on this evidence that the $H^+$ conductance is voltage-gated. The $H^+$ conductance was partially blocked by 2 mM to 100 $\mu$M $Cd^{2+}$ or $Zn^{2+}$ in a reversible manner [48,50]. The current was restored at higher holding potentials; that is, the presence of either $Cd^{2+}$ or $Zn^{2+}$ the current voltage (IV) curve shifts to the right [48]. Unusually for a channel, there was 1–2 s delay in opening following the imposition of the step to positive holding potentials and, also unlike other cation channels, showed no inactivation characteristics, i.e., prolonged exposure to high holding potentials does not cause closure of the channel [48,50,107]. The recorded current was always reported to arise from an efflux of $H^+$ ions. However, the failure to record current from an influx arises probably from technical difficulties rather than an absence of this flux. The current observed was enhanced at least 3-fold in the presence of 50 $\mu$M or 10 $\mu$M arachidonate, which shifted the current–voltage curve to the left [48,108]. That is, the opening of the channel was greater in the presence of arachidonate, which allows opening at lower potentials than in its absence. This electrophysiological data suggest a rate of flux of $10^4$ $H^+$/s, which is faster than a carrier but slower than normally expected for a channel see review [49].

From the above description, it will be apparent that there are strong similarities between the putative voltage-gated $H^+$ channel described in the electrophysiological studies and the arachidonate-activatable $H^+$ channel described in $pH_i$ studies of populations of human neutrophils. Both channels are inhibited by the presence of $Zn^{2+}$ or $Cd^{2+}$, they are activated or enhanced by the addition of arachidonate and are present in phagocytes and cells expressing the NADPH oxidase. The delay in opening of the $H^+$ channel apparent in electrophysiological measurements may be the reason why the membrane is depolarised on initiating superoxide generation, since the outward conduction of electrons would precede $H^+$ transport. The membrane potential depolarisation was originally ascribed to the opening and or initiation of $H^+$ efflux occurring after the initiation of superoxide generation. However, it is equally true to say that if the channel is opened at exactly the same time as or before superoxide is generated, then an influx of $H^+$ ions would occur, driven by the negative membrane potential, which it turn would give rise to depolarisation. This aspect is currently under investigation.

One marked difference between the results obtained by the two approaches is that the electrophysiological studies indicate a voltage-gated channel [48,50,107], whereas in the $pH_i$ studies with BCECF voltage depolarisation failed to open the channel; this opening occurred only in the presence of arachidonate [87]. In the electrophysiological studies, no current was observed at holding potentials of 0 mV or lower, whereas in the $pH_i$ studies arachidonate-dependent $H^+$ flux occurred at or near zero membrane potential (high $K^+$ medium together with valinomycin) the

direction depending only on $\Delta$pH. There are problems which relate to the methodology of patch clamping. The first of these is temperature. Patch clamping experiments are carried out routinely at room temperature. Both the activation (by PMA, for example) and the activity of the NADPH oxidase of neutrophils have a high $Q_{10}$. The BCECF experiments were carried out at 37°C. Second, in patch clamping in the cell-attached mode the cell cytosol is either replaced by or precipitated by the contents of the electrode. What effects this may have on channel activity is impossible to predict. The cytosol in the BCECF experiments was in the native state. The overall similarity in results obtained ($Cd^{2+}$, $Zn^{2+}$ inhibition, arachidonate elucidation) make it reasonable to assume that they are reflections of the same channel activity and the differences arise from artifacts.

Although the possibility that a rapid and transient depolarisation to severely positive potential does occur, it has not been observed by the use of various dyes. It seems unlikely that the positive potentials required in the electrophysiological studies are obtained in vivo.

*7.6.1. Identification of the protein(s) which form and regulate the $H^+$ channel*

The $H^+$ channel activity is vital for the activity of the oxidase. Full understanding of the oxidase therefore requires the identification of the protein(s) that function as and/or regulate the opening and functioning of the channel. By definition it must be a transmembrane protein present in the plasma membrane of cells with oxidase activity, but not necessarily only those cells. The fact that both the oxidase and the channel are activated by arachidonate could be taken to suggest that the channel is part of the oxidase complex. However, this does not necessary follow, as the non-synchronisation of initiation of the two processes maybe taken to suggest that their activation is not a single event.

Patch-clamping studies have identified an $H^+$ channel in differentiated HL60 cells [50]. In a later paper it was reported that undifferentiated HL60 cells did not have this activity [160]. The channel activity has also been shown to increase upon DMSO and DMF induced differentiation of HL60 cells to neutrophil-like cells as monitored by $\Delta$pH changes and this paralleled the ability to generate superoxide [83]. However, it should be noted that an increase in expression of a large number of proteins occurs.

Grinstein and colleagues have monitored the $pH_i$ changes of neutrophils isolated from CGD patients lacking the expression of various oxidase components. They deduced that the channel is absent in the cells of patients lacking the expression of gp91-*phox*, or p22-*phox*, or p47-*phox* and was 50% of normal in a carrier of X-linked CGD [140]. It follows from their data that the activity of the channel is correlated with the activity of the oxidase. However, the authors failed to distinguish between the ability to generate superoxide and $H^+$ channel activity. According to their criteria, any defect in the superoxide-generating system would be seen as a defect in channel activity. These authors subsequently completely eliminated the possible involvement of gp91-*phox* in the channel on the basis that neutrophils from a special X-linked CGD patient with a 10% expression of gp91-*phox* showed 70% of normal channel activity [141]. As the activity of the channel did not correlate with the level of gp91-*phox* expression, they concluded that it was not involved. However, Orkin and colleagues have demonstrated that expression of gp91-*phox* at 10% of normal was sufficient to achieve normal levels of superoxide generation when using gene therapy to restore oxidase activity in PLB-985 cells with a generated X-linked CGD phenotype [216]. By inference it does not follow that the alterations in the level of protein expression will be absolutely matched by alterations in either or both oxidase and channel activity. Consequently, it would be highly unsafe to conclude that gp91-*phox* was not involved with the channel.

Kaldi et al. [105] utilised a functional assay to explore the possible expression of the $H^+$ channel in pig lymphocytes. They identified a PMA- and arachidonate-activatable $H^+$ channel activity in cells despite reporting that the cells do not generate superoxide. They concluded that the channel was not an integral part of the oxidase, i.e., not one of the protein components [105]. However, Maly et al. [129] detected superoxide generation and the presence of cytochrome *b* in tonsillar lymphocytes and B cell lines. Therefore, as Kaldi et al. [105] did not monitor the expression of gp91-*phox*, they cannot eliminate its involvement.

Fully functional arachidonate activated $H^+$ channel was demonstrated in EBV-transformed B lymphocyte cell lines derived from normal and CGD patients lacking the expression of p47-*phox* and p67-*phox* but was absent in those cells lacking gp91-*phox* [83]. Therefore, in our hands, neither cytosolic factor regulates the activity of the channel, but we cannot rule out the involvement of other, as yet unidentified, protein(s) in the regulation of the channel [83]. The inhibition by succinyl acetone of haem synthesis during the DMSO-induced differentiation of HL60 cells results in the absence of a stable p22-*phox* expression, while gp91-*phox* is unaffected. Since these cells exhibit a fully functional $H^+$ channel and the EBV lymphocytes lacking gp91-*phox* do not, it can be postulated that it is the absence of gp91-*phox* in the X-linked CGD cells which gives rise to the failure of channel activity. Chinese hamster ovary cells (CHO) transfected with gp91-*phox* cDNA show the expression of an arachidonate-activated $H^+$ channel which correlates with the expression of gp91-*phox*. This channel is absent from control cells. It was shown immunologically that p22-*phox*, p47-*phox* and p67-*phox* were absent from both control and induced cells. These experiments provide clear evidence that gp91-*phox* is the only component of the oxidase involved in the functional channel. At present it cannot be excluded that the channel

is an unidentified protein expression of which is tightly coupled to and regulated by the expression of gp91-*phox*, even in Chinese hamster ovary cells [83].

Grinstein and Curnutte have recently suggested that the assembly of a fully functional oxidase is a prerequisite for channel activity [141]. This is not consistent with the results with Chinese hamster ovary cells reported above or with their own demonstration of a fully functional channel in cells from a CGD patient lacking expression of p67-*phox*. The statement by Grinstein and co-workers that a CGD patient lacking gp91-*phox* shows channel activity is contradicted by their data (Fig. 1 of [142]).

## 8. Role of arachidonate in the activation of the NADPH oxidase

The attainment of maximal rates of superoxide generation by the NADPH oxidase takes less than 1 min with PMA and some 1–2 min with fMLP. The signal transduction pathway leading to the activation of the oxidase is considered to consist of a membrane-bound receptor, a heteromeric GTP binding protein, a phosphoinositol specific phospholipase C and a protein kinase C; see reviews [137,185]. It is commonly stated that phosphorylation of component(s) of the oxidase per se by protein kinase C is required for activation of the oxidase [6,25,68,92,95].

The addition of PMA or fMLP leads to the activation of the oxidase and to the phosphorylation of a wide range of proteins [96] In search of the specific target for phosphorylation, Heyworth and Segal [92,178] identified a 47 kDa phosphoprotein which was not phosphorylated in autosomal CGD patients, but since, as was subsequently shown, the protein is absent from these patients, that is not surprising. In normal neutrophils the protein was shown to translocate to the membrane following activation [93]. The ability of staurosporine (an inhibitor of protein kinases) to inhibit the PMA activation of superoxide generation and the phosphorylation of p47-phox supported the involvement of protein kinase C directed phosphorylation in the activation of the oxidase [96]. It was therefore concluded that phosphorylation was the immediate activator of the oxidase. Dusi and Rossi [61] have shown a staurosporine-sensitive phosphorylation of p67-phox with similar conclusions.

The amino acid sequence of p47-*phox* contains a number of potential protein kinase C phosphorylation sites. Rotrosen and Leto [175] have isolated eight different phosphorylated forms of p47-*phox* on two-dimensional electrophoresis gels, based on variation in the isoelectric point. The shift in isoelectric point is considered to be due to the stepwise phosphorylation of the protein, corresponding to eight different phosphorylation events. The addition of the 3rd or 4th phosphate group was demonstrated to be associated with the shift of the protein from the cytosolic to the membrane fraction of the cell and that the addition of the 6th, 7th or 8th phosphate group resulted in a return of the protein to the cytosolic fraction.

The PMA activation of the oxidase has a lag of 20 s and reaches a maximal rate of superoxide generation at 1 min after stimulation at 37°C. However, the phosphorylation of p47-*phox*, and other proteins, is barely detectable at 20 s and shows an accumulation over the following 10 min, when the activity of the oxidase is in decline. The stark differences in the time-courses for superoxide generation and that of p47-*phox* phosphorylation make very unlikely the correlation between the two processes claimed in Refs. [6,96,97].

If the phosphorylation of p47-*phox* or any other process is the direct activator of the oxidase, it "most probably" occurs under all conditions and circumstances in which the generation of superoxide is triggered and occurring. Badwey et al. [7,8] have shown that the retinal and 1,2-dioctanoyl-*sn*-glycerol stimulation of superoxide generation by human neutrophils was not associated with the phosphorylation of p47-*phox*. The activation of the oxidase by arachidonate is completely insensitive to staurosporine and under these circumstances there is no increase in the incorporation of $^{32}P$ into p47-*phox* or any other protein (with or without staurosporine) [89]. Under the same conditions, PMA stimulation was associated with an increase in phosphorylation of a wide range of proteins, including p47-*phox*. The failure to observe arachidonate-activated phosphorylation with and without staurosporine suggests that arachidonate and not phosphorylation is the immediate activator of the oxidase [89] and would appear to eliminate the possibility that the arachidonate activation is via PKC as has been suggested by McPhail et al. [133]. This conclusion is strongly supported by the findings of Dusi et al. [60]. They showed that translocation of p47- and p67-phox and superoxide generation were unaffected by staurosporine in $Ca^{2+}$-depleted, fMLP and Con A activated neutrophils, whereas phosphorylation was very largely inhibited.

The addition of purified protein kinase and ATP to a cell-free reconstitution system failed to activate superoxide generation [39] arguing against a kinase-directed phosphorylation of an oxidase component as the immediate activator of superoxide generation. However, arachidonate and other unsaturated fatty acids will activate the oxidase in whole cells [9,10], cytoplasts [89], the cell-free reconstitution system [23,186] and in a system consisting of purified cytochrome *b* and recombinant cytosolic factors [1]. It has been suggested that the high levels of arachidonate required in cell-free reconstitution systems (150 $\mu$M) compared with the whole cells (8 $\mu$M) is not consistent with arachidonate's role as a physiological activator. However, it has been shown recently that arachidonate performs two functions in the cell-free reconstitution system; first as a detergent to break up the membrane vesicles, allowing access to both faces of the membrane, and the second as an activator of the oxidase. When the first function is per-

formed by a mild detergent, low concentrations of arachidonate (10–15 $\mu$M) are required and are absolutely necessary for the activation of superoxide generation [99].

Phospholipase $A_2$ ($PLA_2$) is the enzyme which cleaves the fatty acid from carbon 2 of the glycerol backbone of the phospholipid generating a free fatty acid. The fatty acids on carbon 2 are normally unsaturated and predominantly arachidonate in both phosphatidylinositol and phosphatidylcholine. A number of different $PLA_2$ enzymes have been isolated (reviewed by [207]). The porcine pancreatic 14 kDa $PLA_2$ has been widely studied and its properties have been invoked in models despite its obvious lack of involvement in stimulation-associated membrane lipid metabolism. A number of workers have recently isolated a cytosolic 85 kDa $PLA_2$ [31,51,116] which, because it is phosphorylated in association with activation in a number of different cells [28,56,124,125], is a better candidate for involvement in the stimulated release of unsaturated fatty acids in neutrophils. The role of and mechanisms triggering the activation of this $PLA_2$ in neutrophils is at present unknown, but could well be phosphorylation at the hands of an activator-stimulated protein kinase.

The stimulation of the oxidase by a wide range of compounds is associated with an increase in the level of free arachidonate and other unsaturated fatty acids and in the subsequent production of leukotrienes and prostaglandins. Release is generally monitored as radioactive counts released into the external aqueous environment of $^3$H-arachidonate loaded cells. If the bulk of the liberated fatty acid is in the hydrophobic environment of the membrane it will not be recorded by this technique. This probably accounts for the small percentage total counts recovered by workers utilising this assay and hence the low assumed concentrations of free fatty acid.

The PMA stimulation of superoxide generation was inhibited by a range of compounds thought to be inhibitors of porcine 14 kDa $PLA_2$. The inhibitors were equally effective whether added before or after PMA, but if added after PMA the activity was not immediately terminated but decreased in a time-dependent manner. In all cases, superoxide generation was restored following the addition of arachidonate or SDS [90]. The mechanism of action of these inhibitors is not fully understood. They were designed around the active site of the X-ray crystallographic structure of pig pancreatic $PLA_2$ and they are effective at inhibiting the generation of free fatty acid in an in vitro assays. The common features of these inhibitors are a long hydrophobic saturated tail with a positively charged headgroup. Stearylamine, which is very similar to these compounds, inhibits the PMA activation of superoxide generation in a manner identical to the $PLA_2$ inhibitors. In the presence of stearylamine or a putative $PLA_2$ inhibitor, the generation of superoxide gradually recovers, in a time-dependent manner, suggesting the continuous generation of an activator which eventually overcomes the inhibition

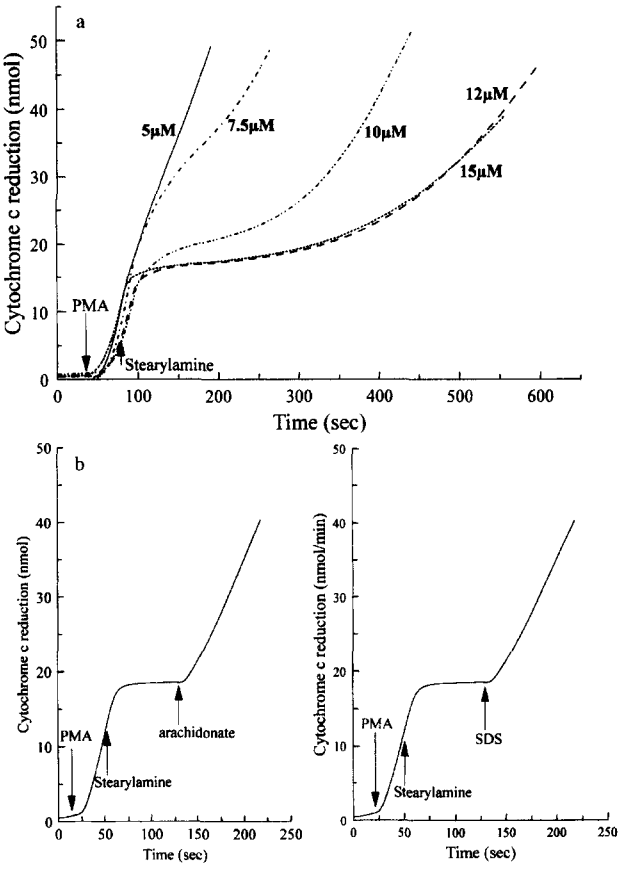


Fig. 3. Stearylamine revisable inhibition of superoxide generation by cytoplasts. The generation of superoxide by PMA-stimulated cytoplasts, monitored as the reduction of superoxide, was inhibited in a concentration dependent manner by stearylamine. However, at all concentrations tested restoration of superoxide generation occurred some minutes following the addition of stearylamine, no further additions. The higher the concentration of stearylamine the longer the time before the restoration of superoxide was observed (a). The addition of either arachidonate or SDS to cytoplasts following inhibition by stearylamine immediately restores their ability to generate superoxide (b).

(Fig. 3) (Henderson and Chappell, unpublished data). We postulate that stearylamine and the putative $PLA_2$ inhibitors are capable of binding free arachidonate, making it unavailable to the oxidase. In support of this suggestion, it has been shown by us that arachidonate and stearylamine form an extremely tight water-insoluble (in the $\mu$M range) complex, which is extractable into hexane.

Analogues of arachidonate have been described which are inhibitors of the cytosolic 85 kDa $PLA_2$. They are product analogues which competitively inhibit the enzyme by binding to the substrate binding site [193]. They are very slow-acting and in our hands fail to significantly inhibit superoxide generation. The nature of the $PLA_2$ which is involved in the activation of the neutrophil oxidase is at present unknown.

Cockcroft has proposed a role for a G protein in the activation of $PLA_2$, independent of that required to activate PLC, but both receptor-linked [33]. The theory is based on a differential effect of pertussis toxin on the

fMLP-stimulated PLC and $PLA_2$ activities. However, it is difficult to see how PMA and other stimuli acting downstream of a receptor could stimulate $PLA_2$ and hence the liberation of arachidonate. The evidence for a role for PKC in the activation of the oxidase is compelling. It is possible, as stated previously, that the target is the activation of the 85 kDa $PLA_2$.

Arachidonate and other unsaturated fatty acids are the only compounds which activate the oxidase in all assay systems, i.e., whole cells, cell-free system and in the purified recombinant protein assays; they are the only compounds which are unaffected by and can overcome the action of inhibitors of the signal transduction pathway such as staurosporine. Arachidonate stimulation of the oxidase is not associated with and does not require phosphorylation. This strongly suggests that arachidonate is the immediate activator of the oxidase. Whether it acts through direct action upon one or more of the components of the oxidase or through changes in membrane fluidity surrounding the cytochrome is at present unknown. However, arachidonate is a good candidate for a signal molecule, as it is readily liberated, has a number of pathways for its removal and is very unstable.

An analysis of the fatty acids, unsaturated and their ester, amide and saturated derivatives demonstrated that the amide and ester forms of the unsaturated fatty acids were incapable of stimulating superoxide generation in a cell-free system despite the fact that their free fatty acid forms were fully active [123,186]. This suggests that activation requires an unsaturated fatty acid with a free $COO^-$ group. The free $COO^-$ group may facilitate insertion into the membrane or it may be a requirement for the interaction with the protein components. As detergents alone are incapable of stimulating the oxidase [186], it is very unlikely that the activation by arachidonate can be exclusively ascribed to detergent properties and it is more likely that it is a direct effect upon oxidase component(s).

## 9. Assembly of the active oxidase

Received opinion is that superoxide generation requires the assembly of the oxidase complex by translocation of the cytosolic factors, p47-*phox* and p67-*phox*, to the membrane cytochrome *b* in the developing phagosome. The GTP binding protein also translocates to the membrane upon activation.

The absence of translocation in cells from X-linked CGD patients and the translocation of p47-*phox* in the absence of p67-*phox*, but not vice versa, suggests an interaction between the cytochrome *b* and p47-*phox* in the active oxidase [94,95,149]. Rotrosen et al. [174] have reported the identification of a region in the C-terminal 14 amino acids of gp91-*phox* which is required for the assembly of the active oxidase. Peptides containing the sequence RGVHFIF (amino acid 559–565 gp91-*phox*) inhibited the activity of the oxidase in a human neutrophil cell-free reconstitution system when the peptide was present before the addition of cytosolic proteins and arachidonate but not when added after assembly [111]. The inclusion of the peptide also appeared to prevent the phosphorylation of p47-*phox* in a cell-free phosphorylation system [174]. Therefore, it was proposed that the C terminal of gp91-*phox* is the binding site for p47-*phox* in the active oxidase complex. The region of p47-*phox* responsible for this purported binding was not identified. However, we have failed to reproduce the result with this peptide in a cell-free system. Solutions of the peptide were highly acidic, possibly due to the presence of trifluoracetic acid (TFA), the eluant and counterion in the automatic synthesiser. Inhibition similar to that reported by Rotrosen et al. [174] was achieved only if the pH of the peptide solutions remained acidic. This strongly suggests to us that the inhibition of the oxidase by this peptide arises from the acidity of the solution rather than the specific amino acid sequence of the peptide it contains. Neutralised peptide at high concentration was without effect. A solution of TFA alone was capable of inhibiting superoxide generation by a cell-free system ([88] and personal observation). Roos [168] has also noted that inhibition by peptides was due to the acidity of the solution and not the amino acid sequence of the peptide. Therefore, the direct interaction between p47-*phox* and gp91-*phox* is unproven.

A wide range of proteins has now been identified which contain regions of homology with domains in the src kinase, i.e., SH2 and SH3 proteins. Some proteins contain either SH2, e.g., phosphoinositol specific phospholipase C [192], non-receptor tyrosine kinases or SH3 domains, e.g., GAP and p47-*phox*, p67-*phox* and possibly p40-*phox* [59,119,126,209,213], while adapter proteins, e.g., Grb2, *drk* (*Drosophila* eye), contain both SH2 and SH3 domains [150,177]. SH2 domains have been shown to bind phosphotyrosine-containing peptides [15] following the activation of receptor and associated tyrosine kinases, leading to the activation of ras [63,67,122,177]. A proline-rich consensus sequence (XPXXPPP$\varphi$XP) has been identified as the SH3 domain binding site in a number of proteins and the overall three-dimensional structure of the src SH3 domain with and without the peptide bound has been determined [165]. The cytosolic factors p47-*phox*, p67-*phox* and the recently reported p40-*phox* each contain two predicted SH3 domains. The C terminal of the amino acid sequence of p22-*phox* has a region of high homology to the proline-rich sequence which may be an SH3 binding site. Similar sequences are contained within p47-*phox* and possibly p67-*phox* but not gp91-*phox* [132]. In other systems, the SH2 and SH3 domains have been proposed to be involved in protein interactions following activation. Therefore, it could be suggested that the SH3 domains may bind the subunits of the oxidase complex and therefore are involved in and direct the assembly of the active oxidase.

### 9.1. Possible role of SH3 domains in subunit interactions

Leto and co-workers [46] have investigated the role of the SH3 domains in the functioning of p67-*phox*, both in a cell-free reconstitution system and in an EBV-transformed cell line established from a CGD patient lacking p67-*phox* expression. Mutant protein in which either of first SH3 domain or the second or both were deleted were constructed. Also proteins containing only the N-terminal 246 and the C terminal 296–457 amino acids were prepared. In the cell-free system superoxide generation in the presence of a mutant protein was more than 50% that of normal, even with the protein lacking both SH3 domains. However, in the whole cells none of the mutant proteins was capable of supporting superoxide generation at a level greater than 10% of control. In both assay systems the deletion of the second, C terminal SH3 domain as opposed to the first, resulted in greater degree of inhibition, but whether this is due to a failure to bind because of the absence of an SH3 domain or because of a change in conformation is not clear. The difference between the results obtained from the two systems is striking and requires an explanation. The cell-free system lacks some of the components and certainly the organisation of the cytoskeleton and other features. It has been suggested that the cytosolic factors are associated with the cytoskeleton and that the SH3 domains are involved in the binding to proteins of the cytoskeleton [119]. Therefore, it could be suggested that the role of the p67-*phox* SH3 domains is in its directed translocation to the plasma membrane via the cytoskeleton rather than in the interaction between subunits.

A number of workers have utilised isolated SH3 domains, expressed as glutathione-*S*-transferase fusion proteins, immobilised on glutathione Sepharose, to investigate their ability to bind other proteins and the various proline rich regions. Finan et al. [64] demonstrated that the p67-*phox* C terminal SH3 domain bound to p47-*phox* when exposed to the detergent extract of differentiated HL60 cells. The peptide KPQPAVPPRPSADL of the p47-*phox* C terminal proline-rich region was reported to inhibit completely this interaction at 750 $\mu$M ($K_i$ 100 $\mu$M). The underlined sequence is minimum core required for inhibition. Similar interactions between glutathione-*S*-transferase fusion protein containing both p47-*phox* SH3 domains and the C terminus of p22-*phox* and p47-*phox* itself (residues 70–83 and 358–390) were reported by Leto et al. [120]. The fusion proteins containing the SH3 domains individually bound both p47-*phox* and p22-*phox*, but less avidly. The interaction with C terminus p22-*phox* was inhibited by the proline-rich peptide p22-*phox* (residues 149–162) and not observed with the p22-*phox* mutant $Pro^{156} \rightarrow Gln$ [55] located in the proline-rich region. In an erythroleukaemic cell line, K562, cotransfected with p22-*phox* and p47-*phox* a similar absence of translocation, following PMA stimulation, was observed with this p22-*phox* amino acid substitution mutant. Interestingly, normal PMA-induced translocation of p47-*phox* occurred in the presence of p22-*phox* but absence of gp91-*phox*, strongly indicating that gp91-*phox* is not essential for p47-*phox* binding to the membrane. In agreement with the above, Sumimoto et al. [194] also demonstrated an interaction between the isolated C-terminal SH3 domain p67-*phox* and C-terminal proline-rich region (residues 358–390) of p47-*phox*. Therefore, SH3 domain–proline-rich region interactions have been described as occurring between p47-*phox*:p22-*phox*; p47-*phox*:p47-*phox*; p47-*phox*:p67-*phox*. Since superoxide generation requires activation, these data do not necessarily demonstrate interactions in the assembled, active oxidase but more likely describe the state in the unstimulated cell, in a possible cytosolic complex. Of these, the p47-*phox*:p22-*phox* interaction is probably most relevant in PMA-activated cells.

The src kinase C terminal proline-rich sequence is reported to be bound to its own SH3 domain in the inactive kinase. Activation is thought to break the interaction, thus allowing binding to other proteins and potential substrates. It is therefore possible that proline-rich sequences of the p47-*phox* could bind its SH3 domains in the unstimulated cell. Thus, it could be suggested that activation would involve an alteration in p47-*phox* conformation, exposing its SH3 domains and thereby promoting its interaction with the C-terminal proline-rich region of p22-*phox*. That stimulation of the oxidase may indeed induce conformation change of this nature is suggested by the selective binding of a monoclonal antibody to the p47-*phox* SH3 domain. Although the monoclonal antibody recognised glutathione-*S*-transferase fused to SH3-SH3 p47-*phox* domains (residues 154–285) in the presence or absence of arachidonate [194], its ability to bind p47-*phox* was totally dependent upon the presence of 50 $\mu$M arachidonate or 100 $\mu$M SDS. The glutathione-*S*-transferase–SH3 fusion protein was shown to bind to p47-*phox* (residues 286–390) C-terminal, proline-rich region. This suggests that in the unstimulated native p47-*phox* the SH3 domains are inaccessible to the monoclonal antibody due to intramolecular interaction(s). In agreement with this, Leto et al. [120], Sumimoto et al. [194] demonstrate an interaction between the glutathione-*S*-transferase–SH3 p47-*phox* and the GST-C terminal p22-*phox* fusion protein (residues 145–170) which was not observed with the $Pro^{156} \rightarrow Gln$ mutant. The SH3 binding site was narrowed down to a 10-amino-acid core, PPSNPPPRPP. Binding of p47-*phox* to p67-*phox* was also described to be arachidonate- or SDS-dependent.

Fuchs et al. [65] have recently described interactions between p40-phox and both p47-phox and p67-phox. The SH3 domain of p40-phox (base 565 to the stop codon) was shown to interact with p47-phox and C-terminus p40-phox with p67-phox.

We can therefore conclude that there is some evidence that the SH3 domain–proline-rich regions probably play a

role in the regulation of the oxidase component interactions, both in the inactive and stimulated cells, and that arachidonate plays an important role.

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