Mononuclear Phagocytes

Characteristics, Physiology and Function

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Ralph van Furth

Department of Infectious Diseases
University Hospital
Leiden, The Netherlands

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Characterization of phagocyte NADPH oxidase

R. Rossi, P. Bellavite, M.C. Serra, and E. Papini

Introduction

During phagocytosis and when stimulated by suitable membrane-perturbing agents, granulocytes, monocytes and macrophages undergo a dramatic increase in oxygen consumption (the so called respiratory burst) with concomitant production of large amounts of superoxide anion (O_2^-), of hydrogen peroxide (H_2O_2) and of other oxidizing radicals. It is widely accepted that biological significance of the respiratory burst is that of providing a battery of reactive compounds that phagocytes can use to kill virus, bacteria and other cells (1–5).

It is now generally agreed (6–11) that the enzyme (or enzyme system) responsible for the respiratory burst is a NADPH oxidase, as was first described by us about twenty years ago (12, 13). The enzyme catalyzes the one-electron reduction of oxygen to superoxide utilizing the reducing equivalents provided by NADPH. Since two electrons are released by each NADPH oxidized, the reaction stoichiometry is the following (14, 15): $NADPH + 2O_2 \rightarrow NADPH^+ + H^+ + 2O_2^-$

The present communication deals with the properties and the nature of NADPH oxidase as can be inferred from recent studies carried out in our and other laboratories utilizing subcellular fractions and partially purified enzyme.

Properties of NADPH oxidase

The main properties of NADPH oxidase can be summarized as follows: 1) NADPH oxidase is completely inactive in resting cells and becomes active following suitable perturbations of plasma membrane such as the exposure of phagocytes to opsonized particles, to inflammatory mediators C5a and C567, to fatty acids, to phorbol esters, to formylated chemotactic peptides, to lectines, etc. The activated enzyme can be measured in subcellular fractions (membranes) and it has also been obtained in solubilized form (8, 15, 16); 2) NADPH oxidase is present in all phagocytes and it is localized, at least in its active form, on the plasma membrane (17, 18); 3) It is insensitive to KCN, NaN_3, antimycin A, rotenone, myxothiazol (9, 15); 4) It is inhibited by parachloromercuribenzoate, Cibacron blue, batophenanthroline sulfonate, FAD analogues and it is inactivated by many detergents and high ionic strenghts (8, 15, 19, 20); 5) The Km for NADPH is 0.02–0.05 mM, for NADH is 0.5–1.0 mM, for O_2 is about 0.03 mM (9, 11, 21, 22). In macrophages the Km for NADPH can vary according to the different immunological types of these cells (23).

Nature of the oxidase: an electron-transport chain?

The present status of the knowledge on the nature oxidase seems to indicate that the oxidation of NADPH with production of O_2^- is catalyzed by a system involving various components, so forming an electron transport chain located on the plasma membrane. The candidate components are a flavoprotein, a cytochrome b, quinones and possible other cofactors. A schematic representation on this

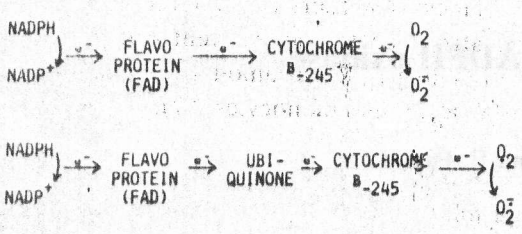

Fig. 1. Proposed models of NADPH oxidase.

system, as it has been proposed (8, 24–28), is shown in Figure 1.

However, true evidence on the precise nature of the enzyme and on the participation of single components is lacking, because till now any attempt to purify the active system has been unsuccessful. In the following sections detailed and critical analyses of the evidences concerning the involvement of the various candidates in such an electron transport chain are presented.

Flavoprotein

The oxidase has thought to be a flavoprotein since 1964 (29) but this early indication was referred to a cytoplasmic NADH oxidase, whereas the O_2^- forming enzyme has proven to be a membrane bound NADPH oxidase. The flavoprotein (FAD) nature of the oxidase is widely accepted on the basis of the considerations and of the findings reported in Table I.

In our opinion, however, the requirement of FAD for the oxidase activity is not truly demonstrated. In fact, with regard the data reported in Table I, it could be observed that theoretically, FAD is not the only way to link two electron donors with one electron acceptor; alternative models involving metal ions could be advanced (35). It cannot be excluded that the higher activity of the Triton X-100 solubilized oxidase is an artifact. It is known that detergents can alterate the mid point potential of cytochrome b (36) and this could induce a derangement in the electron flux from NADPH to O_2 so that another oxide-reducing component could become necessarry. The inhibition of O_2^- formation by FAD analogues could be due to interference with the NADPH-binding site and not to the substitution of native FAD. This possibility would be in agreement with the fact that Cibacron Blue, which has many similarities with the FAD molecule and which binds to nucleotide-requiring enzymes, inhibits O_2^- formation by NADPH oxidase (37). Direct DCIP reduction by NADPH oxidase has not been confirmed in our (see below) and in other (20) laboratories. It is likely that the reduction shown by others (32, 33) is due to O_2^-.

The absence or the decrease of FAD in CGD neutrophils is strongly suggestive but there is not demonstration that the missing flavoprotein is actually NADPH oxidase. In fact CGD is not a single disease, but a complex syndrome where besides NADPH oxidase deficiency (38), several other defects such as deficiency of cytochrome b (39) of NADH-NBT reductase (40), of NADPH-DCIP reductase (32), of NADH oxidase (41), of α-naphtyl butyrate esterase (42), of glutathione peroxidase (43) and abnormal erythrocyte and leukocyte antigens (44, 45) have been shown.

Table I. Evidences in favour of the participation of FAD in O_2^- formation.

	References
Postulated theoretically to link two-electron donor (NADPH) with formation of O_2^-	30
The O_2^- forming activity by membranes solubilized with Triton X-100 is enhanced by the addition of FAD in the assay system	20, 31
FAD analogues inhibit O_2^- forming activity	8
2,6 dichlorophenol indophenol (DCIP) is reduced by NADPH oxidase and inhibits O_2^- formation	32, 33
FAD is absent or decreases in membrane extract and in specific granules of patients affected by CGD	24, 27, 34

Cytochrome b

The evidences suggesting the involvement of a b-type cytochrome in the electron transport system responsible for O_2^- production by phagocytes are summarized in Table II. This particular cytochrome is present in neutrophils, eosinophils, monocytes and macrophages, but not in lymphocytes and red cells (36). Its most striking features are the mid point oxido-reduction potential of −245 mV, much lower than that of most b-type cytochromes, and the ability of bind CO and of being rapidly oxidized by O_2. These properties are characteristic of a terminal oxidase. However, in spite of this large body of data, some experimental data raise doubts on the role of cytochrome b: the CO-binding by cytochrome b_{-245} has been recently questioned (53), a marked discrepancy between the rate of O_2^- formation and the rate of cytochrome b reduction by NADPH in anaerobiosis has been reported by many laboratories (10, 30, 47, 49).

Quinones

The involvement of ubiquinone in O_2^- forming activity has been suggested mainly on the basis of the effect of some inhibitors (54, 55) and of the finding of quinones in membranes and phagocytic vesicles from neutrophils (55, 56). On the contrary, Cross, et al. (57) have recently demonstrated that the content of quinone in neutrophils is very low and that it is exclusively associated to the mitochondrial fraction. Marked differences in the quinone content of granulocytes measured by different groups have been attributed to the different cell preparations containing different amount of contaminating lymphocytes and monocytes (57).

Other cofactors

The chromophore 450–455 described by Green, et al. (26) cannot be part of the oxidase system because its reduction is inhibited by KCN and it is known that O_2^- formation is insensitive to this compound. It is conceivable that the chromophore 450–455 corresponds to compound II of myelo-peroxidase, which absorbs at 456 nm (58).

The involvement of thiol groups in the NADPH oxidase activity has been proposed mainly on the basis of the inhibitory effect exerted by p-chloromercuribenzoate (PCMB). We also observed that PCMB raises the $Em_{7.0}$ of cytochrome b from −245 to −175 (47). This would suggest that −SH groups are important in the function of cytochrome b, either by direct involvement in the electron transfer, or by maintaining the right conformation of the protein.

The importance of phospholipids in the O_2^- forming activity has been firstly pointed out by Gabig and Babior (7) and confirmed in our laboratory (52).

Studies on partially purified enzyme preparations

The lack of true informations on the components of

Table II. Evidences in favour of the participation of cytochrome b_{-245} in O_2^- formation.

	References
The low mid point potential ($Em_{7.0} = -245$)	46
The $Em_{7.0}$ is modified by parachloromercuribenzoate and by Triton X-100, inhibitors of the oxidase	36, 47
It is reduced in phagocytes activated under anaerobic conditions	48
It is rapidly reoxidized by O_2 ($t^1/_2 = 4.7$ ms)	49
It binds CO, although with low affinity	47, 50
During cell differentiation, it appears in parallel with the development of the O_2^- producing capacity	51
It is absent in all the patients with the X-linked form of chronic granulomatous disease (CGD). When present in CGD, it is not reduced in phagocytes activated under anaerobic conditions	28
It copurifies with NADPH oxidase activity	15, 52

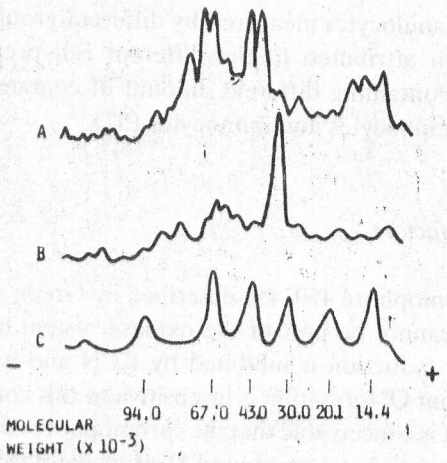

Fig. 2. Densitometric recording traces of SDS-polyacrylamide gel electrophoresis of solubilized and partially purified preparations from guinea pig neutrophils. Trace A is the crude deoxycholate extract; trace B is the most purified preparation obtained after gel filtration on Ultrogel AcA22, centrifugation on glycerol density gradient and treatment of the active fractions with 0.4 M NaCl; trace C are protein standards.

NADPH oxidase, prompted us to try the purification of the enzyme. In common with other groups which attempted the solubilization and purification of the oxidase (7, 8, 20, 59, 60), we experienced many difficulties because the enzyme has proven to be extremely unstable. Nonetheless, we were able to solubilize, stabilize and partially purify the oxidase from guinea pig, human and pig neutrophils and from guinea pig elicited macrophages.

The procedure consisted in the solubilization of subcellular particles with deoxycholate (guinea pig neutrophils), or with deoxycholate plus Lubrol PX (human and pig neutrophils and macrophages), in the presence of glycerol as stabilizing agent. The solubilized extract was then rapidly chromatographed on Ultrogel AcA22 (LKB Products) equilibrated with buffer containing glycerol but not detergents. With this method the NADPH oxidase was eluted as major peak in the void volume and was purified 3–5 fold with respect to the solubilized extract. Further purification was obtained by centrifugation of the peak eluted from chromatography on a glycerol density gradient and by treatment of the active enzyme with 0.4 M NaCl (52). The electrophoresis pattern of the most purified preparation (Fig. 2) showed the presence of many protein bands, indicating that complete purification was not achieved. Nonetheless, a protein band of 32 000 daltons, hardly visible in the first extract, emerged as the major electrophoretic component in the last preparation. Only this band progressively increased as the purification proceeded, suggesting that it could represent an important component of the oxidase.

The study of these partially purified and active preparations has given new insights on the properties and on the nature of the NADPH oxidase.

Separation of O_2^- forming activity from other oxidoreductases

It has been suggested (26, 32, 33) that the first step of the oxidase reactions consists in the oxidation of NADPH catalyzed by a flavoprotein dehydrogenase that can be measured as SOD-insensitive cytochrome c and DCIP reductase. This suggestion is questionable for several reasons. First of all, gel filtration chromatography allowed us to clearly distinguish the O_2^- forming activity (NADPH oxidase) from NAD(P)H dependent oxido-reductases (NAD(P)H dehydrogenase). NADPH oxidase was measured as superoxide (SOD)-inhibitable reduction of cytochrome c, whereas the other oxidoreductases were measured as SOD-insensitive reduction of cytochrome c and of DCIP. Fig. 3 shows a typical chromatographic experiment carried out with pig neutrophils. The first peak eluted from Ultrogel AcA22 column contained most of NADPH oxidase, whereas NAD(P)H-cytochrome c reductase and NAD(P)H-DCIP reductase were eluted in a second peak in the included volume of the column. NAD(P)H-cytochrome c and DCIP-reductase activities were much higher with NADH than with NADPH as substrate. Similar results have been obtained with guinea pig neutrophils and guinea pig peritoneal elicited macrophages.

A second evidence against the participation of cytochrome c and DCIP-reductase activities in the O_2^- forming reactions comes from the analysis of the kinetic properties of the various enzyme activities. The Km of the oxidase for NADPH is one order of magnitude lower than that for NADH (26.7 and

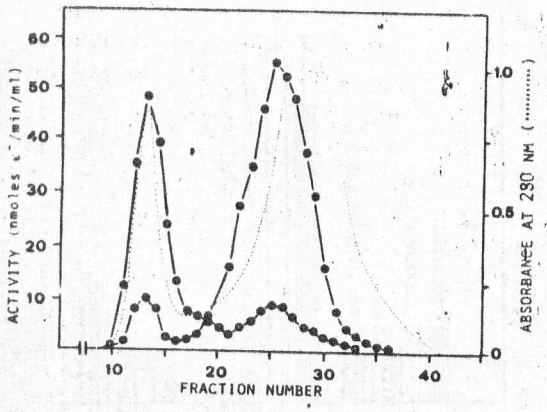

Fig. 3. Gel filtration chromatography of the solubilized extract from pig neutrophils. Subcellular particles from PMA-activated pig neutrophils were treated with 0.4 per cent sodium deoxycholate and 0.4 per cent Lubrol PX and were centrifuged at 100000 g for 1 hr. Ten ml of the supernatant (solubilized extract) were applied to an Ultrogel AcA22 column (3.8 × 16 cm) and fractions of 4 ml were collected at a flow rate of 1 ml/min. NADPH oxidase activity (○—○) was measured as superoxide dismutase inhibitable cytochrome c reduction (O_2^-) in the presence of 0.15 mM NADPH. NADH-DCIP reductase (●——●) was measured as superoxide dismutase insensitive DCIP reduction in the presence of 0.15 mM NADH. The distribution of NADH-cytochrome c reductase was similar to that of NADH-DCIP reductase (not shown).

286.0 μM respectively), whereas that of SOD-insensitive oxidoreductase activity for NADH lower than that for NADPH (cytochrome c reductase: Km for NADH 2.2 μM, Km for NADPH 5.3 μM; DCIP reductase: Km for NADH 1.3 μM, Km for NADPH 5.3 μM). Moreover, only the rate of O_2^- formation was markedly enhanced in the enzyme isolated from activated cells, whereas the rate of SOD-insensitive dye reduction was similar in resting and in activated enzyme preparations (61).

The involvement of a flavoprotein dehydrogenase in the oxidation of NADPH is questionable also on the basis of another experimental finding. If this enzyme were able to give electrons directly to cytochrome c and DCIP as most of oxido reductase do, the addition of an exogenous electron acceptor in the presence of SOD should block the normal electron flux from NADPH to O_2 and therefore should inhibit oxygen consumption and O_2^- production, as schematically represented in Figure 4. The results demonstrate that this is not the case. In fact both oxygen consumption in the presence of SOD

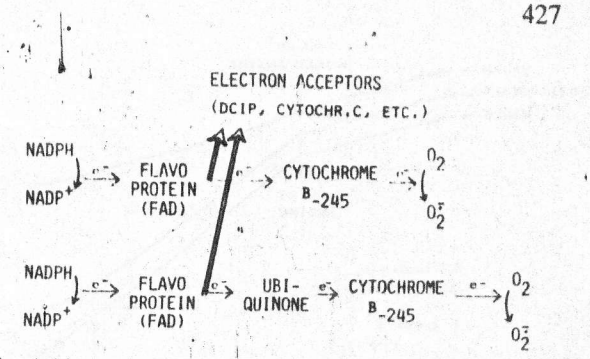

Fig. 4. Mechanism by which electron acceptors should inhibit superoxide production ad oxygen consumption by NADPH oxidase.

(Fig. 5) and O_2^- production by NADPH oxidase were unaffected by electron acceptors.

Since the ability of reducing exogenous electron acceptors is a general property of flavoprotein dehydrogenases, the above presented observations lead to two possible conclusions, that is either the NADPH oxidase does not involve a flavoprotein, or the structural organization of the electron transport chain is such as to isolate the flavin mojety so that the transfer of reducing equivalents to acceptors different from molecular oxygen is hindered.

FAD and cytochrome b content of partially purified preparations

The functional studies presented above raised some doubts about the flavoprotein nature of NADPH oxidase. In an attempt to clarify this point we have looked at the flavin content of the solubilized and partially purified enzyme preparations.

Figure 6 shows the FAD and cytochrome b content in the solubilized extract and in the two main peaks obtained from the AcA22 chromatography (see Fig. 3). FAD specific content in the first peak from gel filtration decreased with respect to the solubilized extract. On the contrary, cytochrome b specific content in this peak increased about 5 fold. As a consequence, the fractions enriched in NADPH oxidase were characterized by a ratio cytochrome b to FAD of 19 : 1. The distribution of flavins in the gel filtration experiment indicated that most of flavoproteins were retained in the

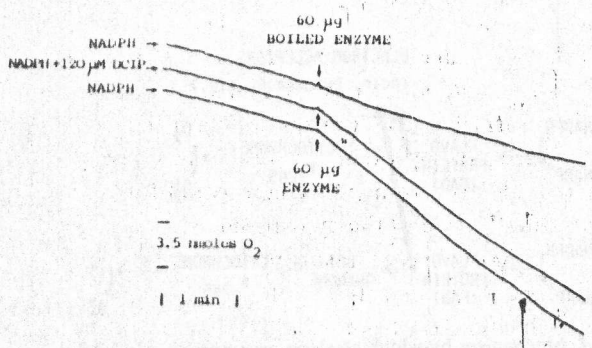

Fig. 5. Polarographic recording traces of oxygen consumption by NADPH oxidase in the absence and in the presence of 2.6 dichlorophenol indophenol (DCIP). The NADPH oxidase from guinea pig PMA-activated neutrophils was partially purified through chromatography on Ultrogel AcA22 and the oxygen consumption was measured with a Clark-type oxygen electrode in the presence of 0.3 mM NADPH and of 80 μg/ml superoxide dismutase.

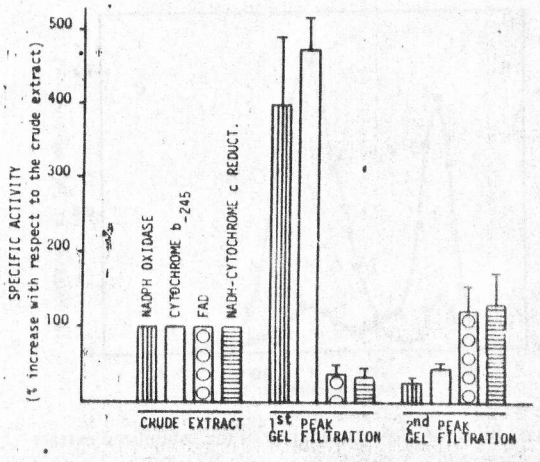

Fig. 6. Enzymatic activities, cytochrome b and FAD content in the two main peaks obtained after gel filtration on Ultrogel AcA22 of the crude solubilized extract from PMA activated pig neutrophils.

second peak and were therefore separated from NADPH oxidase and cytochrome b. The similarity with the distribution of SOD-insensitive oxidoreductases suggests that most of FAD is associated with these enzymes. With regard to the meaning of the small amount of FAD remaining associated with the first peak of gel filtration, two explanations can be advanced. One explanation might be that the small amount of FAD represents a flavoprotein related to NADPH oxidase and forming a complex with many cytochrome b molecules. Such a complex would be composed by one flavoprotein surrounded by about twenty cytochromes. A similar model was proposed for explaining the non-equimolar concentration of flavoprotein and of cytochrome p−450 in liver microsomes (62). Alternatively, the small content of FAD of the first peak represents a contamination by different oxidoreductases due to the non complete purification of the preparation. If this is the case one can conclude that NADPH oxidase is not a flavoprotein. Further purification and other experimental approaches are needed for such a conclusion. It is worthy pointing out, however, that this possibility is not in disagreement with the finding that the oxidase does not give electrons to artificial acceptors.

With regard to the possible role of cytochrome b, the evidence of a co-purification with oxidase activity (Fig. 6) is in favor of its participation in O_2^- formation. Also after the centrifugation of the preparation obtained from AcA22 chromatography on a glycerol density gradient (25–35 per cent) the oxidase activity and the cytochrome b co-distributed (52). During many attempts at purifing the NADPH oxidase with various procedures of extractions, of chromatographic separations, of separation by ultracentrifugation, we never measured O_2^- forming activity in the fractions devoid of cytochrome b. Solubilization of subcellular particles from guinea pig neutrophils with deoxycholate did not affect the $Em_{7.0}$ of the cytochrome b, which was found to be −245 mV (47), in accordance with the potential reported by others for the membrane bound cytochrome b (46). Since fractions enriched with oxidase activity and with cytochrome b were also enriched with phospholipid content (52), it is conceivable that both the normal $Em_{7.0}$ and the high O_2^- forming activity were due to the phospholipid milieu which preserved the normal structure of the enzyme. Also in macrophages the low-potential cytochrome b represented more than 80 per cent of the b-type cytochromes present in these cells. In fact, partially purified fractions obtained from guinea pig peritoneal elicited macrophages (Fig. 7) contained less than 10 per cent con-

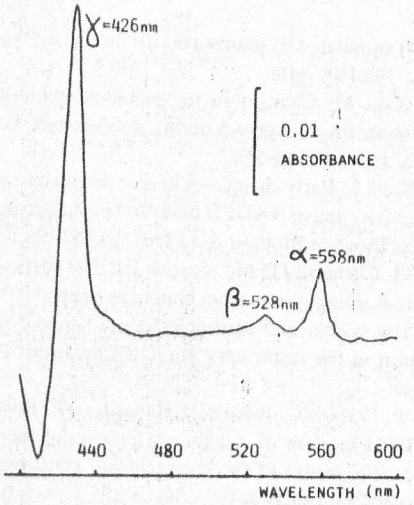

Fig. 1. Reduced-oxidized difference spectrum of partially purified cytochrome b from guinea pig peritoneal elicited macrophages. Subcellular particles from PMA-activated macrophages were treated with 0.3 per cent deoxycholate and 0.3 per cent Lubrol PX and the solubilized extract was chromatographed through an Ultrogel AcA22 column. The dithionite reduced-oxidized spectrum of the fractions eluted in the first peak and containing NADPH oxidase and cytochrome b is reported. Protein concentration: 0.35 mg/ml.

taminating microsomal and mitochondrial cytochromes. Experiments carried out with enzyme extracted from guinea pig neutrophils confirmed that cytochrome b_{-245} is able of bind CO, although with low affinity (47). The most controversial problem concerns the reduction of cytochrome b by NADPH. These experiments must be carried out in the absence of O_2, because in its presence cytochrome b is rapidly reoxidized (49). Many authors reported that the reduction of cytochrome b by NADPH under anaerobic conditions is slow (30, 47, 49, 53) and does not account for the production of O_2^- by phagocytes (63). In collaboration with Dr. O.T.G. Jones and Dr. A.R. Cross of the University of Bristol and using a partially purified enzyme preparation from activated pig neutrophils we have shown that cytochrome b was not reduced by NADPH in anaerobiosis even after long time of incubation. This result is incompatible with a scheme of the O_2^- forming reactions such as that shown in Figure 1. Therefore, alternative hypotheses should be considered, for example: 1) cytochrome b is not a component of NADPH oxidase, as recently suggested by Babior (63); 2) cytochrome b is reduced by a flavin semiquinone, which in turn derives from the reaction between fully reduced flavoprotein and O_2. In this case, a branched electron transport chain would form O_2^- both at the flavin and the cytochrome sites; 3) the presence of oxygen is necessary in order to keep the cytochrome b in an active state that allows the normal electron flux from NADPH; 4) other reactions involving O_2 are intermediate between NADPH oxidation and cytochrome b reduction.

The non reliability of the scheme reported in Figure 1 is also inferred by the observation that quinones are not associated with cytochrome b-containing membranes (57) and with the partially purified NADPH oxidase. In fact, analysis of the active fractions obtained after AcA22 chromatography failed to demonstrate even minimal amount of quinone, thus excluding the participation of this cofactor in the reaction mechanism.

In conclusion, the nature of NADPH oxidase is far from to be clarified. Our results indicated that cytochrome b has much more chances than the flavoprotein for to be actually part of the oxidase. If flavoprotein is not involved, the concept of electron-transport chain as it has been postulated should be reconsidered. Efforts to achieve complete purification of the enzyme become therefore more and more necessary.

Summary

NADPH oxidase, the O_2^- forming enzyme of phagocytes, is considered an electron transport system in whom a flavoprotein, a b-type cytochrome and possibly quinones are involved. However, direct evidence on the participation of single components is lacking in the absence of complete purification of the enzyme. Studies on partially purified preparations from activated pig neutrophils and guinea pig macrophages demonstrated copurification of cytochrome b_{-245} with O_2^- forming activity, but no quinones and only traces of flavoproteins were found. Partially purified enzyme of neutrophils contained cytochrome b and FAD in a 19:1 ratio.

The oxidase did not reduce artificial electron acceptors in the presence of NADPH. The data are discussed with regard to the more widely accepted hypotheses of the chemical composition of the oxidase and to the conventional models of electron transport systems.

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