NADPH oxidase of neutrophils forms superoxide anion but does not reduce cytochrome *c* and dichlorophenolindophenol

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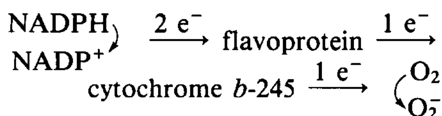
Superoxide (O_2^-) production by partially purified NADPH oxidase from guinea pig neutrophils was markedly increased when the cells were activated by exposure to phorbol-myristate acetate. On the contrary, NADPH-dependent cytochrome *c* and 2,6-dichlorophenolindophenol (DCIP) reductase activities in preparations from resting and activated neutrophils were similar. The apparent K_m values for NADH and NADPH of the reductase activities were different from those of the O_2^- producing enzyme. The electron acceptors did not inhibit the oxygen consumption by NADPH oxidase in the presence of superoxide dismutase. Even in anaerobiosis the oxidase failed to reduce cytochrome *c* and DCIP. These results suggest that NAD(P)H-dependent dye reductase activities are not involved in the electron transport system responsible for the O_2^- production by neutrophils.

NADPH oxidase *Superoxide* *2,6-Dichlorophenolindophenol reductase* *Neutrophil metabolism*

1. INTRODUCTION

Superoxide (O_2^-) production by neutrophils is catalyzed by a membrane-bound NADPH oxidase. This enzyme, or enzymatic system, is dormant in resting cells and becomes activated after the interaction of neutrophils with phagocytosable or soluble stimulants [1–6]. Much evidence has accumulated in recent years indicating that oxidase contains FAD [7–11], a cytochrome *b*-245 [12–15], and needs phospholipids for optimal activity [16]. A 'mini' electron-transport chain has been therefore postulated on the membrane of activated neutrophils [9,17]:



In this scheme, a flavoprotein dehydrogenase would transfer electrons from NADPH to cytochrome *b*-245 which would, in turn, reduce O_2 to O_2^-. However, any attempt to isolate and purify the active oxidase and the single components has been unsuccessful, so that true evidence on the precise nature of the enzyme are lacking. The possibility of measuring the activity of the dehydrogenase component by the reduction of suitable electron acceptors such as DCIP was suggested [18–20]. This could be an effective device for understanding the nature and the properties of the components of the oxidase system.

Here we have reinvestigated the matter by comparing the interaction of the NADPH oxidase with artificial electron acceptors such as DCIP or cytochrome *c* and with the physiological one, molecular oxygen. In order to reduce the interference by other reductases present in neutrophils [10,19,21] and not related to the NADPH oxidase, the study was carried out utilizing as the source of oxidase a solubilized and partially purified preparation, obtained with a procedure that preserves high oxidase activity [22].

Abbreviations: DCIP, 2,6-dichlorophenolindophenol; PMA, phorbol-12 myristate-13 acetate; SOD, superoxide dismutase; DTPA, diethyltriaminepentaacetic acid

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2. MATERIALS AND METHODS

NADPH (type III), NADH (type III), PMA, cytochrome *c* type VI, DCIP, xanthine oxidase, grade III, glucose oxidase, type VII, were obtained from Sigma Chemie GmbH (München). Human copper/zinc SOD was a gift from Dr J.V. Bannister (Inorganic Chemistry Department, Oxford). All other reagents were of the highest grade available.

Neutrophils were obtained from guinea pigs injected intraperitoneally with 1% sodium caseinate [10]. When indicated, the cells were activated by incubating 2×10^7 cells/ml in Krebs Ringer phosphate buffer (pH 7.4) containing 5 mM glucose and 0.2 μg/ml PMA for 90 s at 37°C. Cells were homogenised with a Potter-type apparatus, centrifuged at $250 \times g$ for 8 min to sediment nuclei and unbroken cells. The supernatant obtained was centrifuged at $100000 \times g$ for 30 min. NADPH oxidase was extracted from the particulate fraction with 0.4% sodium deoxycholate and a partial purification was achieved by column chromatography on Ultrogel AcA22 (LKB Products, Bromma, Sweden) as in [22]. The final enzyme preparation was dissolved in 10 mM K-phosphate buffer (pH 8.0) containing 20% glycerol, 2 mM NaN_3 and 1 mM phenylmethylsulfonylfluoride. NAD(P)H-dependent cytochrome *c* and DCIP reducing activities were measured in a Perkin-Elmer 576 double beam spectrophotometer at 22°C. Reference and sample cuvette contained 50 mM Hepes buffer (pH 7.0) containing 1 mM DTPA, 2 mM NaN_3, NADPH or NADH at the desired concentration and, as electron acceptors, 80 μM cytochrome *c* or 60 μM DCIP. When indicated, 60 μg/ml SOD were added to both cuvettes. Cytochrome *c* reduction was monitored as the increase of absorbance at 550 nm and DCIP reduction as the decrease of absorbance at 600 nm. The reaction was started by adding enzyme in the sample cuvette and an identical volume of the same buffer where the enzyme was dissolved in the reference cuvette. The reduction of cytochrome *c* and of DCIP were calculated using extinctions of 21.1 $mM^{-1} \cdot cm^{-1}$ [23] and 16.1 $mM^{-1} \cdot cm^{-1}$ [24], respectively. Values were expressed as e^- trapped/min considering that 1 cytochrome *c* is reduced by 1 e^- and 1 DCIP is reduced by 2 e^-.

Oxygen consumption was monitored using a Clark-type oxygen electrode.

Protein was determined as in [25].

3. RESULTS AND DISCUSSION

NADPH-dependent cytochrome *c* and DCIP reducing activities of enzyme preparations from resting and activated neutrophils are reported in table 1. Activated enzyme reduces the acceptors at much higher rates than resting enzyme when SOD is absent. In its presence, however, no significant differences related to the activation can be observed. This indicates that the increase of the activity induced in the stimulated cells is accounted for by the O_2^--dependent pathway of the reduction of cytochrome *c* and DCIP alone. On the contrary, the SOD insensitive reducing activity is not involved in the activation process. Data of table 1 also show that DCIP is as effective as cytochrome *c* in revealing O_2^- formation. This indicates that

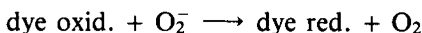
Table 1

NADPH-dependent cytochrome *c* and DCIP reduction by partially purified preparations obtained from resting and activated neutrophils

	Cytochrome *c* reduction			DCIP reduction		
	No SOD	+ SOD	$\Delta \pm$ SOD (O_2^-)	No SOD	+ SOD	$\Delta \pm$ SOD (O_2^-)
Resting enzyme	14.2 ± 2.9	11.4 ± 2.5	2.8 ± 0.3	12.6 ± 5.9	12.4 ± 6.4	0.2 ± 0.2
Activated enzyme	99.8 ± 8.5	17.3 ± 2.9	82.5 ± 9.1	91.6 ± 10.7	11.3 ± 5.8	80.3 ± 8.5

The assays were carried out as described in section 2, in the presence of 0.15 mM NADPH. $\Delta \pm$ SOD (O_2^-) represents: reduction in the absence of SOD − reduction in the presence of SOD. Values are nmol $e^- \cdot min^{-1} \cdot mg$ protein^{-1} and represent the means ± SE of 4 experiments

DCIP does not intercept the electron flux to O_2 catalyzed by the O_2^- forming oxidase. This was also confirmed by experiments where the activity of the oxidase has been measured as oxygen consumption. If exogenous electron acceptors instead of O_2 accept electrons from some reduced component of NADPH oxidase, they would compete with the physiological acceptor and decrease oxygen consumption. The results in table 2 show that this is not the case: O_2 consumption by the NADPH oxidase in the presence of SOD was unaffected by the presence of cytochrome *c* and DCIP. A different behaviour was observed with a flavoprotein oxidase, xanthine oxidase, the oxygen consumption of which was almost completely inhibited by DCIP, but not by cytochrome *c*. During the O_2 consumption assay with xanthine oxidase in the presence of DCIP the dye was rapidly reduced (not shown), indicating that the inhibition of O_2 consumption was effectively due to removal of electrons from the enzyme. The experiments reported in table 2 were performed in the presence of superoxide dismutase because in its absence an apparent inhibition of O_2 consumption is caused by O_2 regenerated in the reaction [26]:



$$\text{dye oxid.} + O_2^- \longrightarrow \text{dye red.} + O_2$$

The lack of inhibition of O_2^- formation and of O_2 consumption, could be theoretically due to the extremely high affinity of O_2 for the oxidase [27]. The possibility of trapping electrons from the activated enzyme in the absence of oxygen was tested by measuring the reduction of cytochrome *c* and of DCIP in anaerobiosis. As shown in fig.1, the O_2^- production was practically abolished by anaerobiosis and no increase of the SOD-insensitive reduction rate of DCIP and cytochrome *c* was observed, indicating that even in anaerobiosis the electron flow was not diverted to artificial acceptors.

The results so far presented show that the partially purified preparation of oxidase employed in this investigation also catalyzed a SOD-insensitive NADPH-dependent dye reduction. Two pieces of evidence suggest that this SOD-insensitive reduction is due to the activity of a contaminant

Table 2

Oxygen consumption by NADPH oxidase and by xanthine oxidase: effect of DCIP and of cytochrome *c*

Additions	O_2 consumption	
	NADPH oxidase[a]	Xanthine oxidase[b]
None	42.5 ± 5.7	28.6 ± 6.2
80 μM DCIP	47.8 ± 4.8	2.6 ± 1.7
80 μM cytochrome *c*	42.0 ± 5.8	30.2 ± 6.5

[a] nmol $O_2 \cdot min^{-1} \cdot$ mg protein^{-1}
[b] nmol $O_2 \cdot min^{-1} \cdot 40\,\mu$g xanthine oxidase^{-1}

NADPH oxidase was solubilized and partially purified from PMA-activated neutrophils. Assay medium was 50 mM Hepes (pH 7.0) containing 1 mM DTPA, 2 mM NaN_3, 60 μg/ml SOD and 0.15 mM NADPH (in a) or 1 mM xanthine (in b). $T = 22°C$. The average values of 3 experiments ± SE are reported

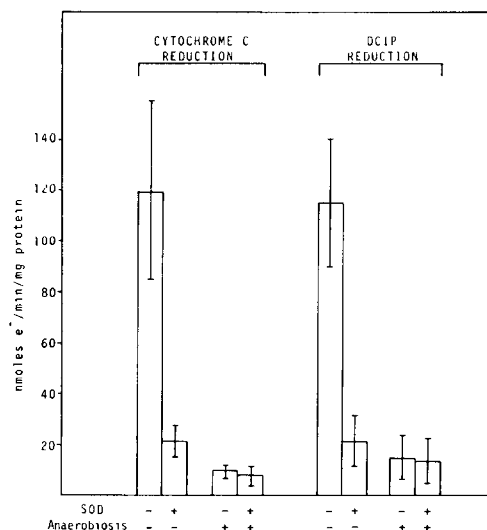


Fig.1. Effect of anaerobiosis on the NADPH-dependent cytochrome *c* and DCIP reduction by partially purified preparations of NADPH oxidase. Enzyme was obtained from PMA-activated neutrophils. The assay medium was 50 mM Hepes (pH 7.0) containing 1 mM DTPA, 10 mM glucose, 2000 units/ml catalase, 0.15 mM NADPH, 80 μM cytochrome *c* or 60 μM DCIP and, where indicated (+), 60 μg/ml of SOD. When anaerobiosis was required (+), the buffer was previously bubbled for at least 10 min with O_2-free nitrogen, 200 μg/ml glucose oxidase were added to the assay mixture and the assay was carried out with stoppered cuvettes under a flux of O_2-free nitrogen. The mean values of 3 experiments ± SD are reported.

Table 3

Kinetic properties of NAD(P)H-dependent cytochrome *c* and DCIP reducing activities of partially purified enzyme preparations from activated neutrophils

	Cytochrome *c* reduction		DCIP reduction	
	SOD-inhibited (O_2^-)	SOD-insensitive	SOD-inhibited (O_2^-)	SOD-insensitive
K_m NADPH	26.7	5.3	47.0	6.1
K_m NADH	286.0	2.2	450.0	1.3
V_{max} NADPH	103.1	22.4	88.8	31.6
V_{max} NADH	117.6	68.5	90.9	42.5

The data from a representative experiment are reported. Values of K_m represent μmol/l, and values of V_{max} represent nmol $e^- \cdot min^{-1} \cdot mg$ protein^{-1}

cytochrome *c* and DCIP reductase. The first is that the activity of cytochrome *c* and DCIP reductase is similar in enzyme preparations obtained from resting and activated granulocytes. The second is the difference in the kinetic properties with the NADPH oxidase, shown in table 3. The K_m for NADPH of the O_2^- forming reaction is one order of magnitude lower than that for NADH, while the K_m for NADPH of the SOD-insensitive reaction is higher than that for NADH. Moreover, the SOD-insensitive reductase has an affinity for pyridine nucleotides much higher than that of NADPH oxidase.

In conclusion, the results reported here suggest that NADPH oxidase does not give electrons to exogenous acceptors such as cytochrome *c* or DCIP and that the preferential and perhaps obligate acceptor is molecular oxygen. The molecular structure responsible for this inaccessibility of the reduced sites of the enzymes (or of the enzymatic chain) to electron acceptors different from oxygen, remains to be clarified. These results suggest two important considerations. Since practically all flavin-linked dehydrogenases can be oxidized by artificial electron acceptors [28], the fact that DCIP does not react with the NADPH oxidase raises some doubts on the involvement of a flavoprotein dehydrogenase as a component of the oxidase itself. The second consideration is that the NADPH oxidase is 'protected' from the withdrawal of electrons which are completely utilized for the formation of O_2^- and H_2O_2. In other words, the only function of the activity of this enzyme is the formation of strong weapons for killing bacteria and other invading organisms and cells.

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REFERENCES

[1] Rossi, F. and Zatti, M. (1964) Br. J. Exp. Pathol. 45, 548–559.
[2] Patriarca, P., Cramer, R., Moncalvo, S., Rossi, F. and Romeo, D. (1971) Arch. Biochem. Biophys. 145, 255–262.
[3] Hohn, D.C. and Lehrer, R.I. (1975) J. Clin. Invest. 55, 703–713.
[4] De Chatelet, L.R., McPhail, L.C., Mullikin, D. and McCall, C.E. (1975) J. Clin. Invest. 55, 714–721.
[5] Babior, B.M., Curnutte, J.T. and McMurrich, B.J. (1976) J. Clin. Invest. 58, 989–996.
[6] Cohen, H.J., Chovaniec, M.E. and Davies, W.A. (1980) Blood 55, 355–363.
[7] Babior, B.M. and Peters, W.A. (1981) J. Biol. Chem. 256, 2321–2323.
[8] Light, D.R., Walsh, C., O'Callaghan, A.M., Goetzl, E.J. and Tauber, A.I. (1981) Biochemistry 20, 1468–1476.
[9] Cross, A.R., Jones, O.T.G., Garcia, R. and Segal, A.W. (1982) Biochem. J. 208, 759–763.
[10] Bellavite, P., Cross, A.R., Serra, M.C., Davoli,

A., Jones, O.T.G. and Rossi, F. (1983) Biochim. Biophys. Acta 746, 40–47.

[11] Gabig, T.G. (1983) J. Biol. Chem. 258, 6352–6356.

[12] Segal, A.W. and Jones, O.T.G. (1978) Nature 276, 515–517.

[13] Cross, A.R., Jones, O.T.G., Harper, A.M. and Segal, A.W. (1981) Biochem. J. 194, 599–606.

[14] Gabig, T.G., Schevish, E.W. and Santinga, J.T. (1982) J. Biol. Chem. 257, 4114–4119.

[15] Borregaard, N., Simons, E.R. and Clark, R.A. (1982) Infect. Immun. 38, 1301–1303.

[16] Gabig, T.G. and Babior, B.M. (1979) J. Biol. Chem. 254, 9070–9074.

[17] Michell, B. (1983) Trends Biochem. Sci. 8, 117–118.

[18] Green, T.R. and Schaefer, R.E. (1981) Biochemistry 20, 7483–7487.

[19] Wakeyama, H., Takeshige, K. and Minakami, S. (1983) Biochem. J. 210, 577–581.

[20] Green, T.R., Wirtz, M.K. and Wu, D.E. (1983) Biochem. Biophys. Res. Commun. 110, 873–879.

[21] Segal, A.W. and Peters, T.J. (1977) Clin. Sci. Mol. Med. 52, 429–442.

[22] Bellavite, P., Serra, M.C., Davoli, A., Bannister, J.V. and Rossi, F. (1983) Mol. Cell. Biochem. 52, 17–25.

[23] Van Gelder, B.F. and Slater, E.C. (1962) Biochim. Biophys. Acta 58, 593–595.

[24] Dawson, R.M.C., Elliot, D.C., Elliot, W.H. and Jones, K.M. (1969) Data for Biochemical Research, 2nd edn, p.436, Oxford University Press, New York.

[25] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.

[26] Dri, P., Bellavite, P., Berton, G. and Rossi, F. (1979) Mol. Cell. Biochem. 23, 109–122.

[27] Gabig, T.G., Bearman, S.I. and Babior, B.M. (1979) Blood 53, 1133–1139.

[28] Dixon, M. and Webb, E.C. (1979) Enzymes, 3rd edn, p.483, Longman, London.