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The NADPH oxidase of guinea pig polymorphonuclear leucocytes

Properties of the deoxycholate extracted enzyme

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Summary

NADPH oxidase from stimulated guinea pig granulocytes was extracted with deoxycholate. The solubilized enzyme was stable in 20% glycerol. Solubilized enzyme was free of myeloperoxidase activity. The properties of the deoxycholate solubilized enzyme indicated that it is a high molecular weight complex with a flavoprotein, calmodulin and cytochrome b possibly forming part of the complex. Maximum activity was between pH 7.0 and 7.5. The K_m value was 15.8 μM for NADPH and 434 μM for NADH indicating that NADPH is the preferential substrate.

Introduction

Leucocytes undergo a dramatic stimulation of oxygen consumption with resultant production of superoxide and hydroxyl radicals and hydrogen peroxide during phagocytosis and when stimulated with membrane perturbing agents. Glucose oxidation through the hexosemonophosphate shunt is also observed (1–4). The key event of this respiratory bust appears to be the activation of a plasma membrane-bound NAD(P)H oxidase, which is inactive in the resting state (5–9). The enzyme has been characterized as a flavoprotein (10, 11) utilizing NADPH as substrate (6, 12–15) and catalyzes the reaction: $NADPH + 2O_2 \rightarrow 2O_2^- + NADP^+ + H^+$. Recently a b-type cytochrome associated with the phagocytic plasma membrane has been implicated in the NADPH oxidase activity (9, 16).

Various attempts have been carried out on the solubilization and isolation of the NADPH oxidase from various phagocytic cells (10, 11, 17–21). Very little progress has, however, been achieved. This is due to the fact that all the procedures utilized resulted in an extremely unstable enzyme. A procedure for the extraction and isolation of a highly active and stable NADPH oxidase from stimulated guinea pig granulocytes (PMNL) is here presented. The properties of the enzyme are also reported.

Materials and methods

1. Materials

Cytochrome c type VI, NADPH type III and phorbol-12 myristate-13 acetate (PMA) were obtained from Sigma Chemical Co. (St. Louis, Mo, U.S.A.). Cyano [^{57}Co] cobalamin was purchased from The Radiochemical Centre, Amersham, England. Trifluoperazine was a gift from Smith, Kline and French Laboratories, Herts, England. Human copper/zinc superoxide dismutase was prepared from outdated blood (22). All other reagents were the highest grade available.

2. Cell preparation, activation and homogenization

Guinea pigs were injected intraperitoneally with 50 ml of sterile 0.9% NaCl containing 1% sodium caseinate and PMNL were collected after 12 hours

with two peritoneal lavages. The cell suspension was filtered through gauze, centrifuged for 10 min at 500 **g** and after separation contaminating erythrocytes were removed by hypotonic lysis, suspended in Krebs Ringer phosphate buffer (KRP), pH 7.4, containing 5 mM glucose and 0.5 mM $CaCl_2$. Maximum activation of PMNL was achieved by incubating 2×10^7 cells/ml in buffer also containing 0.2 μg/ml PMA for 90 seconds at 37 °C. The incubation was terminated by the addition of ice cold buffer and the cells were immediately centrifuged at 1000 **g** for 8 min, suspended in 0.34 M sucrose (4×10^8/ml), buffered with 10 mM Tris-HCl, pH 7.0, containing 2 mM phenylmethyl sulfonylfluoride as protease inhibitor. Cells were homogenized with a Potter-type homogenizer at 4 °C until 90% disruption had occurred.

3. Preparation of subcellular particles and extraction of NADPH oxidase

The homogenate was centrifuged at 250 **g** for 8 min to sediment nuclei and unbroken cells. The supernatant obtained was centrifuged at 100 000 **g** for 30 min. The resulting pellet ('cell-free particles') was suspended in 5 mM phosphate buffer, pH 8.0, at a protein concentration of 5 mg/ml. Sodium deoxycholate (DOC), recrystallized from ethanol (23), was added to the cell-free particles at various concentrations and the mixture was kept at 0 °C for 20 min. The cell-free particles were also treated with deoxycholate in the presence of 20% glycerol and the suspension was then diluted six-fold with ice cold phosphate buffer, pH 8.0, containing 20% glycerol. The DOC-treated particles were centrifuged at 100 000 **g** for 60 min. The resulting supernatant ('deoxycholate extract') was utilized for kinetic and chromatographic investigations.

4. Assays

NADPH oxidase activity was quantitated as superoxide production by measuring the superoxide dismutase inhibition of the reduction of cytochrome c (14). Assays were carried out in a Perkin Elmer 576 double beam spectrophotometer at 22 °C. Reference and sample cuvette contained 50 mM Hepes buffer, pH 7.0, containing 110 mM KCl, 25 mM NaCl, 1 mM diethyltriaminepentaacetic acid, 2 mM NaN_3, 0.1 mM cytochrome c and enzyme. The final volume was 1 ml. The reference cuvette also contained 30 μg of superoxide dismutase. The reaction was started by the addition of 75 nmoles of NADPH to both cuvettes. The absorption change at 550 nm was monitored. The reduction of cytochrome c was calculated using an extinction of 21.1 mM^{-1} cm^{-1} (24). Where indicated, NADPH oxidase was also measured as NADPH consumption by following the decrease in absorbance at 340 nm. The oxidation of NADPH was calculated utilizing a coefficient of 6.2 mM^{-1} cm^{-1}.

Myeloperoxidase was measured by following the oxidation of guaiacol in the presence of H_2O_2 (25), β-glucuronidase by monitoring phenophtalein released from phenophtalein β-glucuronide (26) and 5'-nucleotidase by following adenosine released from AMP (27). Unsaturated vitamin B_{12}-binding capacity was determined by the charcoal radioassay (28). Protein concentration was measured after trichloroacetic acid precipitation (29).

5. Chromatography

The deoxycholate extract was concentrated by ultrafiltration using an Amicon XM-300 membrane (Amicon, N.V., Amsterdam, Holland) and was chromatographed on Bio-Gel HTP hydroxylapatite (Bio-Rad Laboratories, Richmond, U.S.A.) and on Ultrogel AcA22 (LKB Products, Bromma, Sweden). Hydroxylapatite columns were developed by stepwise increases in the phosphate buffer concentration up to 600 mM. All operations were carried out at 4 °C.

Results

1. Preparation of NADPH oxidase

Treatment of cell-free particles from PMA activated PMNL with various concentrations of DOC in 5 mM phosphate buffer resulted in extraction of the NADPH dependent superoxide forming activity. Optimal extraction was at 0.3% detergent concentration (Fig. 1). The activity of the extracted enzyme in phosphate buffer rapidly decreased at 4 °C; more than 90% was lost after 18 hours. The superoxide forming activity was found to be more stable when after extraction, the DOC concentra-

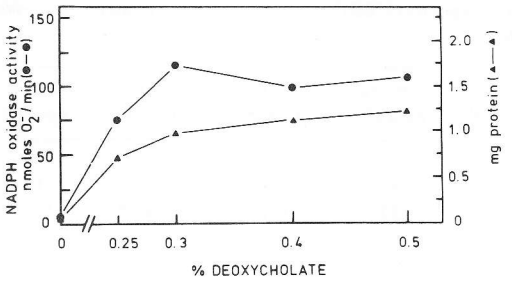

Fig. 1. Effect of various concentrations of deoxycholate on the solubilization of NADPH oxidase and of proteins from cell-free particles of PMA activated guinea pig PMNL. Values represent the oxidase activity and the proteins of the supernatants derived from 2.5×10^8 PMNL.

tion was brought to 0.05% by dilution with buffer and by adding different concentrations of glycerol (Fig. 2). In phosphate buffer containing 0.05% DOC and 20% glycerol about 60% of the NADPH oxidase activity was maintained for 40 hours. At −70 °C practically all the activity was preserved. Other stabilizing agents such as dimethylsulfoxide were not as effective as glycerol. The procedure adopted for all extraction experiments was: 5 mg protein from cell-free particles extracted for 20 min at 0 °C in 1 ml of 5 mM phosphate buffer containing 0.3% DOC and 20% glycerol and diluted 5-fold with buffer containing 20% glycerol and centrifuged at 100 000 **g** for 60 min. The preparation was either used immediately or stored at −70 °C.

The activity of NADPH oxidase of extracts from PMA activated PMNL ranged from 60.9 to 172.9 nmoles superoxide/min/mg protein in a series of eight experiments. The NADPH oxidase activity of extracts from two cell-free particle preparations from resting granulocytes was 1.9 and 2.0 nmoles superoxide/min/mg protein.

2. Characterization and properties of NADPH oxidase

Table 1 reports the total protein concentration, NADPH oxidase activity, cytochrome b concentration and the activity of the known markers of the plasma membrane azurophilic and specific granules of cell-free particles and in the deoxycholate extract. The deoxycholate extract contained 32.3% of total proteins, 28.7% of NADPH oxidase and 25.2% of 5′-nucleotidase, a typical marker of plasma membrane with respect to the cell-free particles.

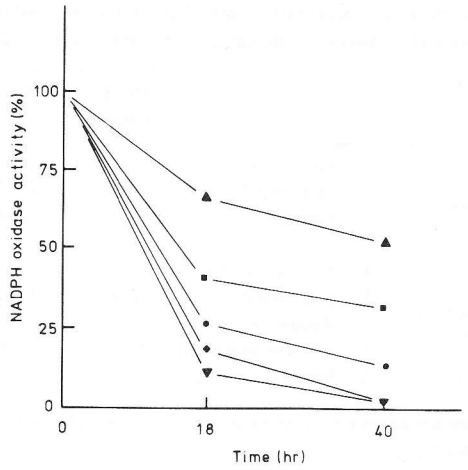

Fig. 2. Stability of the solubilized NADPH dependent superoxide generating activity. The deoxycholate extract was maintained at 4 °C in 5 mM K-phosphate buffer, pH 8.0, containing 0.3% DOC (▼), 0.3% DOC plus 20% glycerol (◆), 0.05% DOC (●), 0.05% DOC plus 10% DMSO (■), 0.05% DOC plus 20% glycerol (▲).

The specific activity of both the enzymes did not appreciably vary in the extract. A different behaviour was observed with regards to the two enzymes contained in the azurophilic granules. Myeloperoxidase activity in the deoxycholate extract was only 7.6% of that of cell-free particles, whereas β-glucuronidase activity indicated 70.1% solubilization. Unsaturated B_{12}-binding capacity (UBBC), a property of transcobalamins contained in the specific granules (28) was almost completely extracted by deoxycholate.

Spectral analysis of the cell-free particles and of the deoxycholate extract showed the characteristic features of a cytochrome b (reduced, λ_α 558 nm, λ_γ 426 nm) and also a peak around 473 nm, indicating the presence of myeloperoxidase (Fig. 3). Assuming a $\Delta\epsilon_{425-410}$ of 200 000 M^{-1} cm^{-1} for cytochrome b (11) the amount of the chromophore present is 127.6 pmoles/mg protein and 148.9 pmoles/mg protein in cell-free particles and in deoxycholate extract respectively, and the amount recovered in the extract was 37.8% of total (Table 1). The ratio between the height of the peaks of myeloperoxidase and of cytochrome b decreases in the deoxycholate extract as compared to the ratio measured in the cell-free particles (Fig. 3). This is in agreement with the data presented in Table 1 which indicates a

Table 1. Protein concentration, enzymatic activities, unsaturated B_{12}-binding capacity (UBBC) and cytochrome b content of cell-free particles and of deoxycholate extract from PMA activated guinea pig PMNL.

		Protein (mg)	NADPH oxidase[b]	Myelo-peroxidase[c]	β-glucuro-nidase[d]	5'-nucleo-tidase[e]	UBBC ($\mu g\ B_{12}$)	Cyt. b (pmoles)
Cell-free particles	Total protein or activity[a]	21.3	4149	64.1	32.4	2268	1034	2718
	Specific activity	–	194.8	3.01	1.52	101.6	47.7	127.6
Deoxycholate extract	Total protein or activity[a]	6.9	1193	4.9	22.7	573	968	1027
	Recovery (% of cell-free particles)	32.3	28.7	7.6	70.1	25.2	93.6	37.8
	Specific activity	–	172.9	0.71	3.3	81.6	137.8	148.9

[a] Values are given for proteins of activity obtained from 10^9 PMNL.
[b] nmoles O_2^-/ min.
[c] μmoles tetraguaiacol/ min.
[d] μmoles phenophtalein/ 4 hours.
[e] μmoles adenosine/ min.

lower solubilization of myeloperoxidase than of cytochrome b.

The maximum activity of NADPH oxidase was found to occur between pH 7.0 and 7.5 and the ratio between NADPH consumed and superoxide produced at all pH values was always found to be 2 (Fig. 4). This is in agreement with the stoichiometry of the reaction $2O_2 + NADPH \rightarrow 2O_2^- + NADPH^+ + H^+$. The K_m values of the reaction between the oxidase and NADPH and NADH are 15.8 μM and 434 μM respectively (Fig. 5). These data indicate that NADPH is the preferential substrate for the solubilized oxidase, as previously shown for the particle-associated enzyme (5, 6, 12–15).

The effect of inhibitors of metabolic pathways on the NADPH oxidase activity is reported in Table 2. The activity was found to be practically insensitive

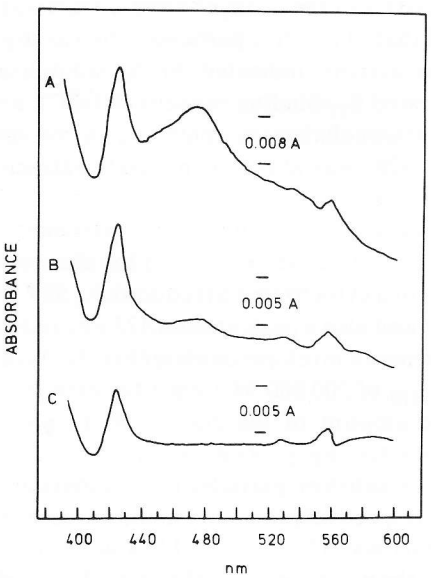

Fig. 3. Dithionite difference spectra (reduced minus untreated) of various preparations from PMA activated guinea pig PMNL. (A) Cell-free particles, 0.731 mg protein/ ml; (B) Deoxycholate extract 0.503 mg of protein/ ml; (C) Peak of activity eluted from Ultrogel AcA22 column, 0.130 mg of protein/ ml.

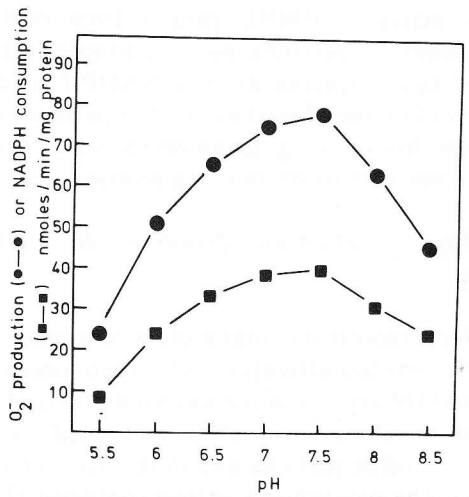

Fig. 4. pH dependence of superoxide O_2^- formation and of the NADPH oxidizing activity of the deoxycholate extract from PMA activated PMNL. Results are the mean of three different preparations.

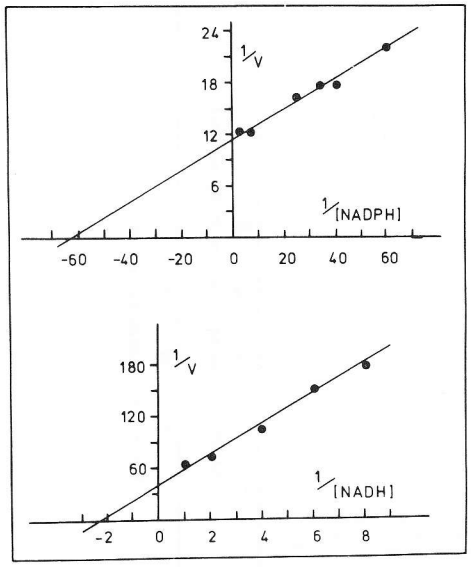

Fig. 5. Lineweaver-Burke plots of NADPH and NADH oxidase activities of the solubilized preparation obtained from PMA activated PMNL.

to cyanide, rotenone, antimycin A, to thenoyltrifluoroacetone, a quinone inhibitor, to EGTA and to EDTA. Almost complete inhibition was obtained in the presence of p-chloromercurybenzoic acid, an inhibitor of thiol groups. Tetracaine, a possible calmodulin inhibitor, was found to inhibit

Table 2. Effect of various compounds on the deoxycholate solubilized NADPH oxidase activity.

		Superoxide production % of control
1 mM	Potassium cyanide	87.0
20 μM	Rotenone	96.6
5 μg/ml	Antimycin A	114.0
1 mM	Thenoyltrifluoroacetone	86.3
0.1 mM	p-Chloromercurybenzoic acid	5.0
1 mM	EGTA	102.0
1 mM	EDTA	91.8
1 mM	Tetracaine	42.0
40 μM	Trifluoperazine	18.0
1 mM	Quinacrine	32.4
1 mM	Batophenanthroline sulfonate	16.0
1 mM	Salicylhydroxamic acid	95.0

Superoxide production was measured as described in text, except that the assay mixtures were supplemented with the indicated compounds and the enzyme was preincubated for 5 min before the addition of NADPH.

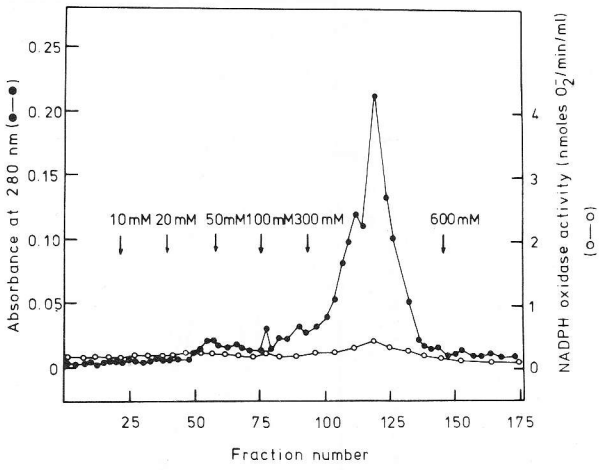

Fig. 6. Hydroxylapatite chromatography of the deoxycholate extract from PMA-activated PMNL. 9.5 ml of sample containing 0.426 mg/ml of protein and a specific activity of 70 nmoles superoxide/min/mg protein were adsorbed on a column of hydroxylapatite (20 × 3 cm) equilibrated with 5 mM K-phosphate buffer pH 7.0 containing 20% glycerol. Elution conditions were performed as described in the text. Fractions of 1.5 ml were collected.

the enzyme activity by about 60%. Trifluoperazine, a specific calmodulin inhibitor, was found to inhibit 80% of the activity at 40 μM concentration. The inhibition by trifluoperazine was observed both when the enzyme was preincubated with the compound and when it was added during enzymatic reaction. Quinacrine, a flavin inhibitor exerted 68% inhibition at 1 mM concentration. This is in agreement with the observation that the oxidase is a flavoprotein (11). The rate of superoxide formation was not modified by the addition of FAD to the assay mixture. An inhibitory effect was also exerted by the iron-chelating compound sodium batophenanthroline sulfonate, whereas another metal chelator, the salicylhydroxamic acid, was ineffective.

3. Chromatographic behaviour of NADPH oxidase

The deoxycholate extract was adsorbed on hydroxylapatite at 5 mM phosphate buffer, pH 7.0 containing 20% glycerol. The column was eluted with a stepwise gradient of 5, 10, 20, 50, 100, 300 and 600 mM phosphate. Virtually all the protein adsorbed was eluted with 300 mM phosphate (Fig. 6). Eluted protein was found to have no NADPH oxidase activity.

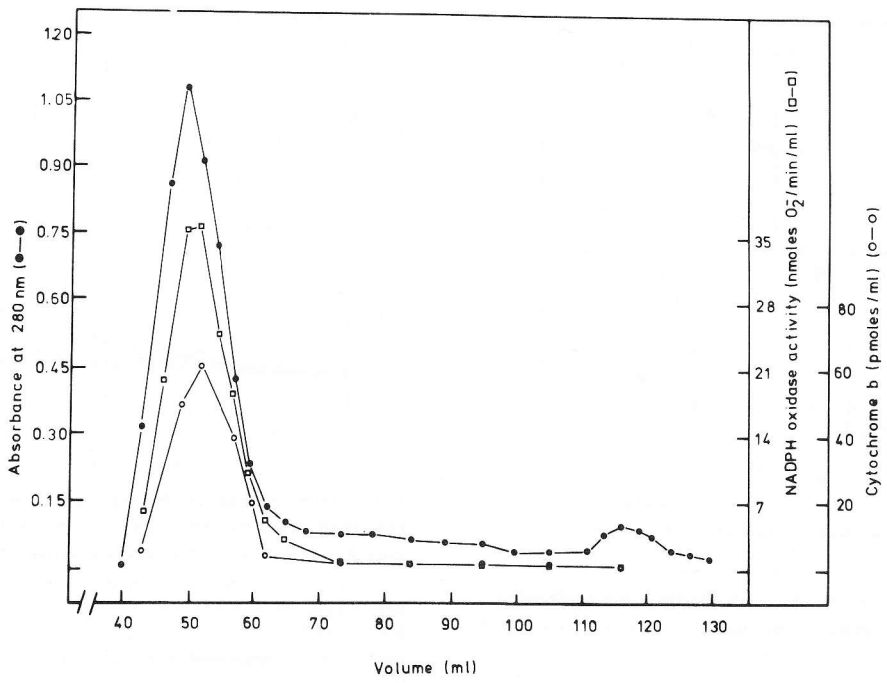

Fig. 7. Gel filtration of Ultrogel AcA22 of deoxycholate extract from PMA activated PMNL. 7 ml of sample containing 0.05% deoxycholate (total) protein 6.1 mg, NADPH oxidase activity 153.9 nmoles superoxide/min/mg protein, cytochrome b content 100.8 pmoles/mg protein were loaded on the column (57 × 1.6 cm) equilibrated with 5 mM K-phosphate buffer, pH 8.0 containing 20% glycerol. Elution was performed with the same buffer and fractions of 2.7 ml were collected.

The extracted enzyme was also concentrated and loaded on a column of Ultrogel AcA22. The elution pattern is shown in Fig. 7. The oxidase activity coincided with the main protein peak which eluted in the void volume. The pooled fractions of the main peak ('pool Ultrogel AcA22' in Table 3) contained 38% of the proteins loaded on the column and 31.4% of the NADPH oxidase activity. The low recovery of NADPH oxidase was probably due to the spontaneous decay in the activity during the time necessary for the chromatography. This is shown by the fact that the specific activity of a sample of the preparation loaded on the column decreased from 153.9 (Table 3) to 64.4 nmoles super-

Table 3. Protein concentration, enzymatic activities, unsaturated B_{12}-binding capacity (UBBC) and cytochrome b content of the edoxycholate extract and of the pool of fractions of the main protein peak eluted from Ultrogel AcA22 chromatography.

		Proteins (mg)	NADPH oxidase[b]	Myelo-peroxidase[c]	β-glucuro-nidase[d]	5'-nucleo-tidase[e]	UBBC ($\mu g\ B_{12}$)	Cyt. b (pmoles)
Deoxycholate extract	Total proteins or activity[a]	6.05	931.4	3.4	23.0	552.0	422.8	610
	Specific activity	–	153.9	0.56	3.8	91.2	69.9	100.8
Pool AcA22	Total proteins or activity[a]	2.3	292.6	0.27	0.25	324.0	8.6	566.0
	Recovery (% of deoxycholate extract)	38.0	31.4	7.9	1.1	58.7	2.0	92.7
	Specific activity	–	127.2	0.12	0.11	138.4	3.7	246.1

a, b, c, d, e See notes of Table 1.

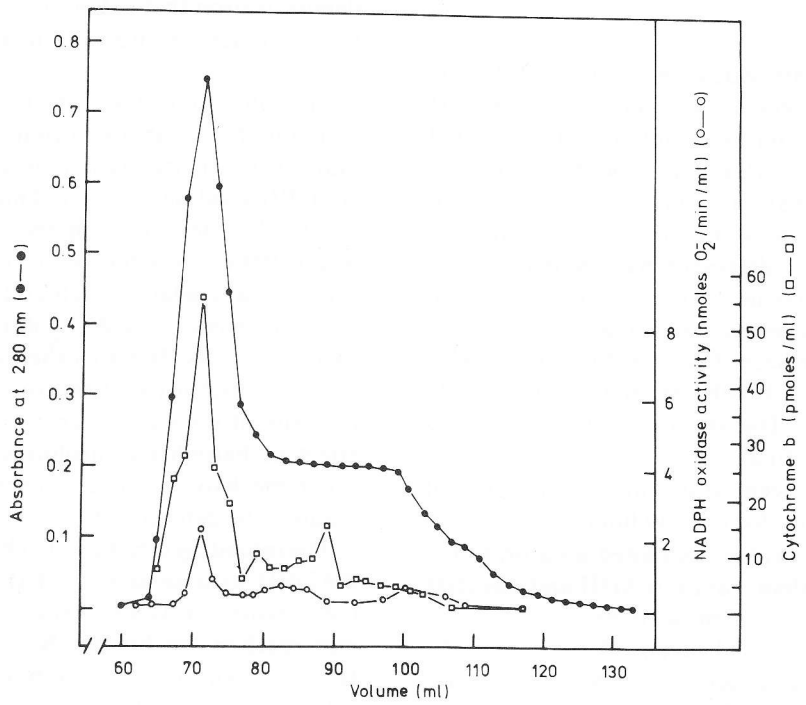

Fig. 8. Gel filtration on Ultrogel AcA22 of deoxycholate extract from PMA activated PMNL. 3 ml of sample containing 0.3% deoxycholate (total protein 7.7 mg, NADPH oxidase activity, 33.7 nmoles superoxide/ min/ mg protein, cytochrome b content 132 pmoles/ mg protein) were loaded on a column (85 × 1.6 cm) equilibrated in 5 mM K-phosphate buffer at pH 8.0 containing 20% glycerol and 0.15% deoxycholate. Elution was performed with the same buffer and fractions of 1.83 ml were collected.

oxide/ min/ mg protein when maintained 30 hours at 4 °C, which is the duration of the chromatographic experiment. If the activity of the NADPH oxidase eluted from the column (31.4%) is corrected for spontaneous decay in activity, the recovery of oxidase activity approximates to 75%.

Dithionite difference spectra of the protein eluted from the Ultrogel AcA22 column showed that the only chromophore present is cytochrome b (Fig. 3a). The recovery of the cytochrome was 92.7% of the amount loaded on the column (Table 3). The fractions eluted in the void volume of Ultrogel AcA22 column were practically devoid of myeloperoxidase and of β-glucuronidase activity and of unsaturated B_{12} binding capacity (Table 3). However 58.7% of 5′-nucleotidase activity was present in the main protein peak. Practically no other protein was eluted in the included volume of the column. The same gel filtration pattern was observed with four different preparations from PMA activated PMNL and with a preparation from unstimulated PMNL. In this latter case the specific activity of NADPH oxidase eluted in the main protein peak was only 2.2 nmoles superoxide/ min/ mg protein.

In an attempt to define if the elution pattern obtained following Ultrogel AcA22 chromatography was caused by enzyme aggregation on removal of detergent, gel filtration was repeated in the presence of 0.15% deoxycholate. A similar procedure was adopted by others (34) in order to prevent protein aggregation and to resolve adenylate cyclase from other membrane bound proteins. The elution profile obtained in presence of deoxycholate is shown in Fig. 8. NADPH oxidase activity appeared as a major peak in the void volume. The elution pattern of cytochrome b again coincided with the oxidase activity. This procedure, however, markedly inactivated the NADPH oxidase, the specific activity of the major peak was 7.9 nmoles superoxide/ min/ mg protein and the overall recovery of activity with respect to that loaded on the column was only 2.8%. Protein and cytochrome b recovery in these conditions were, however, almost 100%.

Discussion

The results obtained describe a procedure for the extraction of a very active and stable NADPH oxidase activity from the membrane of stimulated guinea pig granulocytes. The procedure accounts for the fact that a high concentration of deoxycholate is necessary for optimal extraction but also that this detergent has an inactivating effect on the enzyme. With this procedure an enzyme (or an enzymatic system) can be solubilized from cell-free particles of activated leucocytes with a high specific NADPH dependent superoxide forming activity. The storage of 0–4 °C of the extract in the final mixture containing low deoxycholate concentration and 20% glycerol maintained activity for over 40 hours.

The extracted oxidase exhibited an affinity for NADPH higher than that for NADH and a neutral pH optimum, in agreement with the characteristics of the particle-associated enzyme (5, 6, 12–15). The ratio of superoxide generation to NADPH oxidation by the enzyme is 2 at every pH investigated (Fig. 4). This indicates that the electron transfer from reduced pyridine nucleotide to oxygen follows a one-electron pathway. The fact that the stoichiometry is unaffected by pH is a feature that distinguishes the NADPH oxidase of PMNL from other superoxide forming enzymes, such as xanthine oxidase, whose reaction (univalent or divalent reduction of oxygen) is strongly influenced by pH conditions (30).

The catalytic properties of the extracted oxidase, were investigated in the presence of known inhibitors of metabolism (Table 2). Activity was unaffected by mitochondrial inhibitors but was markedly inhibited by the sulphydryl reagent p-chloromercurybenzoic acid. These findings are in agreement with previous observations on cells and on the oxidase activity that thiol groups are involved in activity.

The involvement of calmodulin in the mechanism of activation of the respiratory burst of PMNL has been proposed mainly on the basis of the inhibitory effect exerted by trifluoperazine (31, 32). Trifluoperazine also inhibits the NADPH oxidase activity of subcellular particles (32). Trifluoperazine and tetracine inhibited the deoxycholate-extracted oxidase activity both if preincubated with the enzyme and if added after the reaction has been initiated by NADPH. This represents a further demonstration that calmodulin plays a significant role in the activation and in the activity of NADPH oxidase.

The inhibition of activity by the iron-chelating compound batophenanthroline sulfonate might indicate the participation of a metal ion in the NADPH oxidase reaction. This inhibition is similar to that described on the cyanide insensitive NAD(P)H oxidoreductase of yeast (33). However, other metal chelators such as EGTA, EDTA and salicylhydroxamic acid, did not have any effect on oxidase activity. Whether these differences are due to the accessibility of these compounds to the catalytic site of the enzyme or whether the inhibitory effect of batophenanthroline sulfonate is due to other mechanisms independent on chelation remains to be determined.

The inhibiting effect exerted by quinacrine on the NADPH oxidase activity (Table 2) indicates that the enzyme is a flavoprotein. A similar conclusion was reached for human NADPH oxidase (10). However, whilst Gabig and Babior (10) had to add FAD to regain oxidase activity following extraction with Triton X-100 our enzyme activity was not increased following addition of FAD to the assay mixture. This suggests that treatment of cell-free particles with deoxycholate does not remove FAD from the enzyme as can possibly happen in the presence of Triton X-100. Spectroscopic evidence from the presence of FAD in the deoxycholate extracted enzyme is currently under investigation.

Attempts to purify the NADPH oxidase by adsorption chromatography on hydroxylapatite were unsuccessful. The main protein peak eluted with 300 mM phosphate was devoid of an oxidase activity. This inactivation could possibly have resulted because of the high phosphate concentration required for desorbing the bound protein. High salt concentrations have been reported to inactivate the NADPH oxidase (20) although the possibility of some co-factor required for activity being irreversibly bound to the hydroxylapatite cannot be discounted. Gel filtration of deoxycholate extract performed on Ultrogel AcA22 equilibrated in the 5 mM phosphate pH 8.0 and 20% glycerol, resulted in the oxidase activity appearing as a single peak in the excluded volume. The activity recovered was 31.4% of that loaded and the specific activity of the pooled fractions was 127.2 nmoles superoxide/min/mg protein. The specific activity of oxidase did

not increase after chromatography, however, the eluted protein was devoid of myeloperoxidase and β-glucuronidase activity and unsaturated B_{12} binding capacity.

Since the exclusion limit of Ultrogel AcA22 resin is of about 1.2×10^6 daltons, the gel filtration performed in 5 mM phosphate and 20% glycerol indicated that oxidase activity is eluted as high molecular weight complex. The question arises whether this complex forms as a result of solubilization or whether it represents a 'physiological' arrangement of various moieties (flavoprotein, phospholipids, cytochrome b, metal ions, quinones). Removal of detergent after solubilization has been shown to cause aggregation of membrane-bound protein (34). In order to verify whether the absence of deoxycholate caused the formation of the aggregate reported in Fig. 7, the gel filtration was repeated in the presence of 0.15% deoxycholate. The result obtained (Fig. 8) indicated the main activity still eluted as a high molecular weight complex in the presence of detergent.

It is worth pointing out that the cytochrome b chromophore noted in the extract (Fig. 3) eluted with the NADPH oxidase activity on gel filtration in the presence and absence of deoxycholate. Also a constant ratio has been obtained between the NADPH oxidase activity and the amount of cytochrome b in cell-free particles and in the deoxycholate extract. This fact might be in agreement with the proposal that this cytochrome is a component of the superoxide generating system (9) and that its presence in the protein peaks containing NADPH oxidase activity is not necessarily dependent on the formation of aggregates.

Acknowledgements

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