Presence of cytochrome b_{-245} in NADPH oxidase preparations from human neutrophils

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Received 3 February 1986

The composition of NADPH oxidase purified by Red Sepharose chromatography of extracts from human neutrophil membranes was investigated. In contrast to that was recently reported by others, the enzyme isolated according to this procedure contained a high concentration of cytochrome b_{-245} and little FAD. The results reinforce the belief that cytochrome b_{-245} is a major component of the NADPH oxidase and plays a fundamental role in the formation of O_2^- by neutrophils.

NADPH oxidase *Cytochrome* b_{-245} *Phagocyte metabolism* *Superoxide formation*

1. INTRODUCTION

The molecular composition of the O_2^--forming NADPH oxidase of neutrophils has been widely investigated in recent years. Although firm evidence for the precise nature of the enzyme is lacking, according to the most accepted hypothesis the oxidase is a sort of electron-transport system involving a flavoprotein dehydrogenase, a special type of cytochrome *b* with a very negative (−245 mV) redox potential and possibly quinones:

$$\text{NADPH} \rightarrow \text{NADP}^+ \xrightarrow{e^-} \text{FAD} \xrightarrow{e^-} \text{cytochrome } b \xrightarrow{e^-} O_2 \rightarrow O_2^-$$

In particular, the involvement of cytochrome b_{-245} in NADPH oxidase activity seems to be well established [1–5]. However, in a recent report on the purification of human NADPH oxidase by Red Sepharose chromatography, analysis of the enzyme preparation revealed the presence of FAD but not of cytochrome b_{-245} [6]. The finding of NADPH oxidase activity without the participation of cytochrome b_{-245} is at variance with the hypothesis above. We therefore repeated these experiments and studied the possible presence of heme chromophores using highly sensitive spectrophotometric techniques.

Abbreviations: PMA, phorbol 12-myristate 13-acetate; PMSF, phenylmethanesulphonyl fluoride

2. MATERIALS AND METHODS

2.1. *Purification of NADPH oxidase*

Human NADPH oxidase was extracted and purified from neutrophils according to Markert et al. [6]. Neutrophils were isolated by dextran sedimentation and Ficoll centrifugation of 300 ml human blood and activated with serum opsonized zymosan as described by Gabig and Babior [7]. Activated neutrophils were then suspended in ice-cold 10 mM Tris-HCl, pH 7.4, containing 0.34 M sucrose, 1 mM PMSF, 2 μM pepstatin, 2 μM leupeptin, 1 mM NaN_3 and homogenized by sonication with three 20 s pulses at 150 W (Labsonic 1510, B. Brown). The membrane fraction was isolated from the homogenate using a discontinuous sucrose density gradient as described in [6]. The membrane fraction was suspended at ~3 mg protein/ml in 0.34 M sucrose, containing 1 mM PMSF and 1 mM NaN_3. Solubilization of

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00145793/86/$3.50

NADPH oxidase was carried out by mixing 2 ml membranes with 2 ml extraction buffer, composed of 20 mM Na glycinate, pH 8.0, and containing 50% (v/v) glycerol, 2% Na deoxycholate, 1 mM NaN_3 and 1.7 μM $CaCl_2$ [6]. The mixture was incubated at 4°C for 30 min and then centrifuged at 100000 × g for 45 min. After ultracentrifugation the supernatant was subjected to chromatography through Red Sepharose CL-6B (Pharmacia) according to Markert et al. [6]. 1-ml fractions were collected at a flow rate of 10 ml/h.

2.2. *Assays*

NADPH oxidase activity was assayed as O_2^- formation according to [6]. Cytochrome b_{-245} was measured by reduced-minus-oxidized difference spectroscopy, using an absorption coefficient ($\epsilon_{559} - \epsilon_{540}$) of 21.6 $mM^{-1} \cdot cm^{-1}$ [8]. Where the concentration of the chromophore was supposed to be less than 50 nM we proceeded as follows: 5 air-oxidized and 5 dithionite-reduced spectra of the same sample were memorized by a computer-aided Beckman DU-8 spectrophotometer. The sum of the oxidized spectra was then subtracted from that of the reduced spectra. The resulting signal was therefore 5-fold amplified while the signal-to-noise ratio was greatly improved, thus allowing recording of spectra with a full-scale limit of 0.05 A and detection of cytochrome b_{-245} at concentrations as low as 5–10 nM.

FAD was measured fluorimetrically according to Faeder and Siegel [9] after extraction by heating and perchloric acid precipitation of proteins [10]. Proteins were assayed according to the method of Lowry as modified by Markwell et al. [11] in membranous material, whereas for detergent-containing samples and column fractions the procedure of Schaffner and Weissmann [12] was followed. Bovine serum albumin was used as standard for both methods. When necessary, samples were concentrated by ultrafiltration on an Amicon YM5 membrane.

3. RESULTS

Fig.1 shows the elution profile from Red Sepharose affinity chromatography of solubilized NADPH oxidase. The first peak of NADPH oxidase activity (panel A) was not retained by the resin, while a second peak containing 7% of the

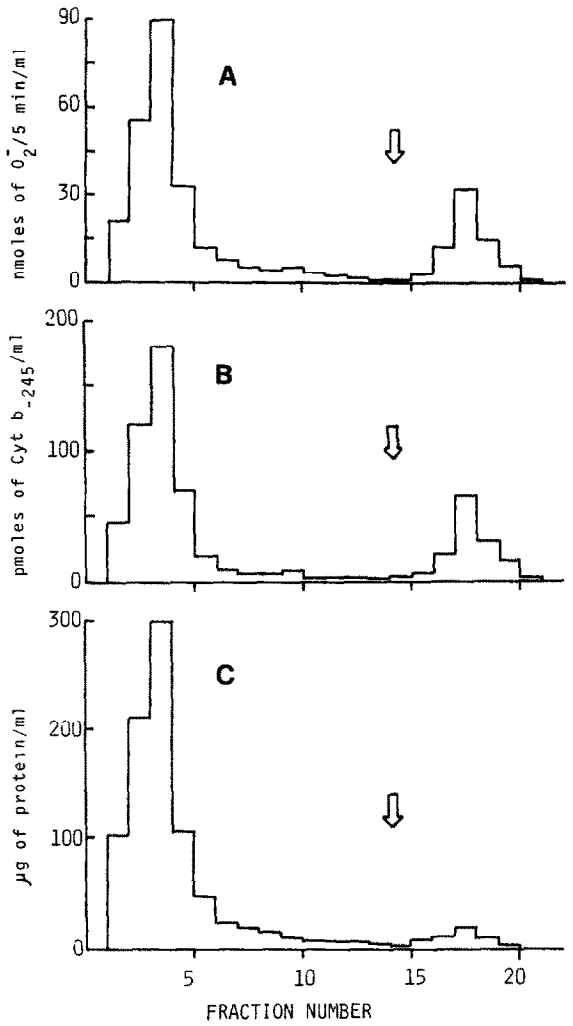

Fig.1. Red-Sepharose chromatography of solubilized membranes from zymosan-activated human neutrophils. 2 ml solubilized membranes containing 892 nmol O_2^-/5 min, 1312 pmol cytochrome b_{-245}/ml and 3.15 mg protein were loaded on a 3 ml Red Sepharose column equilibrated with 30 ml of 10 mM Na glycinate, pH 8.0, containing 25% (v/v) glycerol, 0.17 M sucrose, 0.85 μM $CaCl_2$, 1 mM NaN_3, 0.15% (v/v) Lubrol PX and 0.15% Na deoxycholate. After a wash with 12 ml of the same buffer, the elution buffer (10 mM Na glycinate, pH 8.0, containing 25% (v/v) glycerol, 0.17 M sucrose, 0.85 μM $CaCl_2$ and 1 mM NaN_3) was introduced. 1-ml fractions were collected at a flow rate of 10 ml/h. (A–C) Elution profile of NADPH oxidase activity, cytochrome b_{-245} and proteins, respectively. 28% of the enzymatic activity, 35% of cytochrome b_{-245} and 31% of proteins were not retained by the resin while the elution buffer (indicated by the arrows) was able to release 7% of the O_2^--forming activity, 7.4% of cytochrome b_{-245} and 0.9% of proteins.

loaded activity was released when elution buffer was introduced into the column. Based on the activity/protein ratio this peak contained the most purified fractions, in good agreement with the results of Markert et al. [6]. Fig. 1B shows that all fractions containing NADPH oxidase also contained cytochrome b_{-245}, the elution profile of which was perfectly superimposable on that of the enzymatic activity.

Fig.2 shows the reduced-minus-oxidized difference spectrum of the pooled fractions (17–20) of the second peak from Red Sepharose chromatography. By using highly sensitive spectrophotometry and computerized amplification a definite cytochrome *b* spectrum is visible, which allows quantitation from the characteristic α-peak at 559 nm.

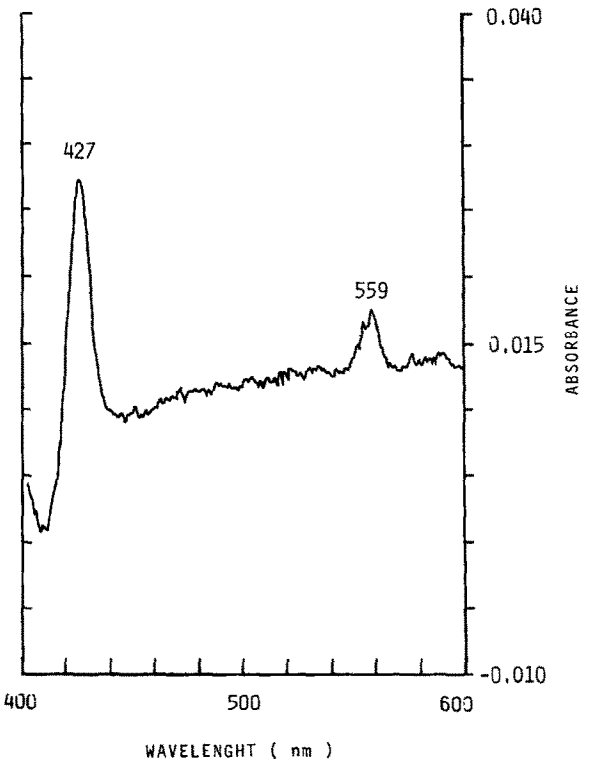

Fig.2. Reduced-minus-oxidized visible spectrum of Red Sepharose purified NADPH oxidase. Fraction 17–20 (see fig.1) were pooled and a 5-fold amplified spectrum of the sample was taken with a single-beam Beckman DU-8 spectrophotometer (see section 2). Characteristic α- (559 nm) and γ- (427 nm) peaks of cytochrome b_{-245} are well defined. The concentration of chromophore in the sample calculated from the α-peak (see section 2) was 36 nM.

Table 1 summarizes the results obtained from the purification procedure. The postnuclear supernatant contained NADPH oxidase activity of 32 nmol O_2^-/5 min per mg protein, a value lower than that reported by Markert et al. [6]. The activation procedure was repeated in 5 separate experiments and the resulting specific activities of the postnuclear supernatants ranged from 25 to 50 nmol O_2^-/5 min per mg protein. No significant improvement in activation was obtained by using PMA (1 μg/ml) instead of opsonized zymosan as stimulant. Whether the relatively low activity of the oxidase was due to the small degree of activation or some deactivation during preparation of the postnuclear supernatant could not be established. In any case the enzyme activity was sufficient to allow accurate measurement during the overall purification procedure. The specific activity of NADPH oxidase increased progressively after each experimental step and was finally purified 48-fold with respect to the starting material. This value is the same as that obtained by Markert et al. [6]. It is worth noting that purification of the oxidase was accompanied by parallel purification of the cytochrome b_{-245}, whose final specific content in the Red Sepharose eluate was 2.2–2.6 nmol/mg protein.

Besides cytochrome *b*, another possible component of the NADPH oxidase is a flavoprotein (FAD-containing) dehydrogenase [6,13,14]. We therefore investigated the FAD content of the purified fractions but found it to be below the sensitivity limit of the method (~5 pmol/ml). In a 5-fold concentrated preparation the FAD content amounted to 7 pmol/ml, while the concentration of cytochrome *b* in the same preparation was determined to be 156 pmol/ml, the FAD/cytochrome *b* ratio therefore being 1:22.

4. DISCUSSION

Considerable discrepancies are still present in the literature regarding the composition of the NADPH oxidase of phagocytes (reviews [14–16]), however one of the most widely accepted points is the involvement of cytochrome b_{-245} [1–5]. This heme protein would function as a terminal oxidase in the trans-membrane electron-transport system

Table 1

Summary of the purification procedure

	NADPH oxidase			Cytochrome b_{-245}		
	Specific activity[a]	Purification factor	Yield (%)	Specific content[b]	Purification factor	Yield (%)
Postnuclear supernatant	32	1	100	50	1	100
Membranes	153	4.8	30	241	4.8	31
Solubilized membranes	283	8.8	30	417	8.3	29
Red Sepharose purified eluate	1533	48	2	2544	51	2.1

[a] nmol O_2^-/5 min per mg protein
[b] pmol cytochrome *b*/mg protein

The results of a representative experiment are shown. The postnuclear supernatant obtained from 300 ml human blood used as starting material contained 2928 nmol O_2^-/5 min of NADPH oxidase activity, 4500 pmol cytochrome b_{-245} and 90 mg proteins

from NADPH to molecular oxygen [2,17–19]. Therefore the report of an NADPH oxidase preparation in which cytochrome b_{-245} was undetectable [6] seemed especially puzzling to us and prompted our reinvestigation of the matter. Since the authors [6] suggested the possibility that the lack of spectral peaks in their purified preparations was due to insufficient sensitivity, we used highly sensitive computerized spectrophotometry in order to analyze the column fractions. The detection limit of cytochrome b_{-245} was thus lowered by a factor of 10 compared to the sensitivity limit indicated in [6]. With this method a reduced-minus-oxidized difference spectrum showing the characteristic Soret bands of cytochrome b_{-245} was detected in all active fractions. Moreover, the distribution profile and purification factor of cytochrome b_{-245} were perfectly superimposable on those of NADPH oxidase activity.

These results are in agreement with our previous work showing that the purification of NADPH oxidase is invariably associated with that of cytochrome b_{-245} [10,20,21]. The absolute requirement of this heme protein for O_2^- formation by neutrophils is also consistent with (i) the lack of oxidase activity in cells of chronic granulomatous disease patients who are genetically deficient in this cytochrome [22] and (ii) the finding that all the electron flux from NADPH to oxygen is accounted for by the turnover of cytochrome b_{-245} oxidation/reduction [23].

It is worth noting that the FAD/cytochrome *b* ratio of 1 FAD/22 cytochromes reported here is similar to the value (1 FAD/19 cytochromes) we determined in NADPH oxidase purified from pig neutrophils by gel filtration chromatography [10]. This indicates that the low FAD/cytochrome *b* ratio is a characteristic feature of this enzymatic system irrespective of the purification procedure and animal species.

In conclusion, this report is a defence of the belief that NADPH-dependent O_2^- formation by neutrophils cannot be catalyzed without the participation of cytochrome b_{-245}. Further work is necessary to characterize the precise components of the oxidase, their role in O_2^- formation and the significance of the low FAD/cytochrome *b* ratio.

ACKNOWLEDGEMENTS

The work was supported by a grant from the Italian National Research Council, Special Project 'Oncologia' contract 85.02342.44 and by a grant from Ministero Pubblica Istruzione (fondi 40%).

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