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THE NADPH OXIDASE ACTIVITY OF GUINEA PIG NEUTROPHILS

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1. INTRODUCTION

Neutrophils undergo a cyanide insensitive increase in oxygen consumption during stimulation. This is due to the activation of a membrane-bound NADPH oxidase catalyzing the one electron reduction of oxygen forming superoxide [$2O_2$+NADPH $\rightarrow 2O_2^-$+$NADP^+$+H^+][1,2]. An electron transport chain comprising a flavoprotein[3] and b-type cytochrome[4,5] have been proposed to be responsible for the electron flow to oxygen. Ubiquinone-50 has been suggested to be responsible for carrying electrons between components of the electron transport chain[6]. The activity also seems to require phospholipid[7]. The instability of the NADPH oxidase has been a major problem during the purification procedure[3,8,9]. We have previously reported a method for the extraction with deoxycholate and partial purification of a very active NADPH oxidase from membranes of guinea pig neutrophils[10]. In the present paper a further improvement to the reported purification procedure is reported and also an attempt was made at identifying the components of the NADPH oxidase by sodium dodecylsulphate (SDS) polyacrylamide gel electrophoresis.

2. MATERIALS AND METHODS

Reagents, assays, cell preparation and activation, preparation of subcellular particles and solubilisation of NADPH oxidase activity and gel filtration on Ultrogel AcA22 were essentially the same as previously described[10]. Pooled fractions from the Ultrogel AcA22 containing NADPH oxidase and cytochrome b were concentrated and layered on top of a linear density gradient (25% to 35% glycerol (v/v) in 10mM phosphate buffer, pH 8.0 containing 1mM EDTA. The gradient was centrifuged for 14 hr at 131,000g. One ml fractions were collected by puncturing the bottom of the tube. The portion of the gradient containing the bulk of the NADPH oxidase activity and cytochrome b was pooled and concentrated by centrifugation at 100,000g.

Protein samples were subjected to SDS-polyacrylamide gel electrophoresis using slab gels (8x8cm) consisting of a linearly increasing polyacrylamide concentration (4 to 30%) purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). The running buffer was 40mM Tris-HCl, pH 8.0 containing 20mM sodium acetate, 2mM EDTA and 1% SDS. Samples were incubated with 1% SDS for 4-5 hr at room temperature. Electrophoresis was conducted at a constant voltage of 150 volts for 2 hr. Gels were stained according to Fairbanks et al[11] and scanned with an Ultrascan Laser Densitometer 2202 (LKB, Bromma, Sweden).

3. RESULTS

The complex eluted from Ultrogel AcA22 chromatography was previously shown to contain both NADPH oxidase activity and cytochrome b. When sedimented through a linear glycerol density gradient the NADPH oxidase activity together with

cytochrome b moved to the centre of the gradient and represented about 50% of the initial amount loaded, whilst 50% sedimented to the bottom of the tube. A low density protein peak without NADPH oxidase activity and cytochrome b was also obtained. The specific activity of the most active pooled glycerol gradient fractions increased 3.5 fold with respect to that of the solubilised particles. However, if the specific activity of the glycerol gradient fractions is corrected for the decay observed during the experimental procedure a purification of 5.0 instead of 3.5 is obtained. The purification ratio obtained for cytochrome b is 5.7. This is similar to that obtained for NADPH oxidase activity. In a further attempt to purify the oxidase activity the enzyme preparations obtained from glycerol gradient was treated with dissociating agents such as 8M urea, 1% Lubrol, 1% Trition X-100 and 1M KCl. All these procedures, however, resulted in complete loss of activity. Treatment with 0.4M NaCl resulted in only 20-30% loss of activity. During the course of the purification procedure, protein samples were analyzed by SDS-polyacrylamide gel electrophoresis. A progressive decrease of the number of polypeptide bands in the sample was obtained during the various purification steps. The intensity of most of the bands does not increase in the various purification fractions whilst a band with a molecular weight of around 32,000 daltons (Fig. 1) shows a progressive enrichment. A comparison between the enrichment of the band and that of NADPH oxidase specific activity and cytochrome b specific content in the corresponding fractions showed that its enrichment strictly paralleled that of NADPH oxidase and cytochrome b.

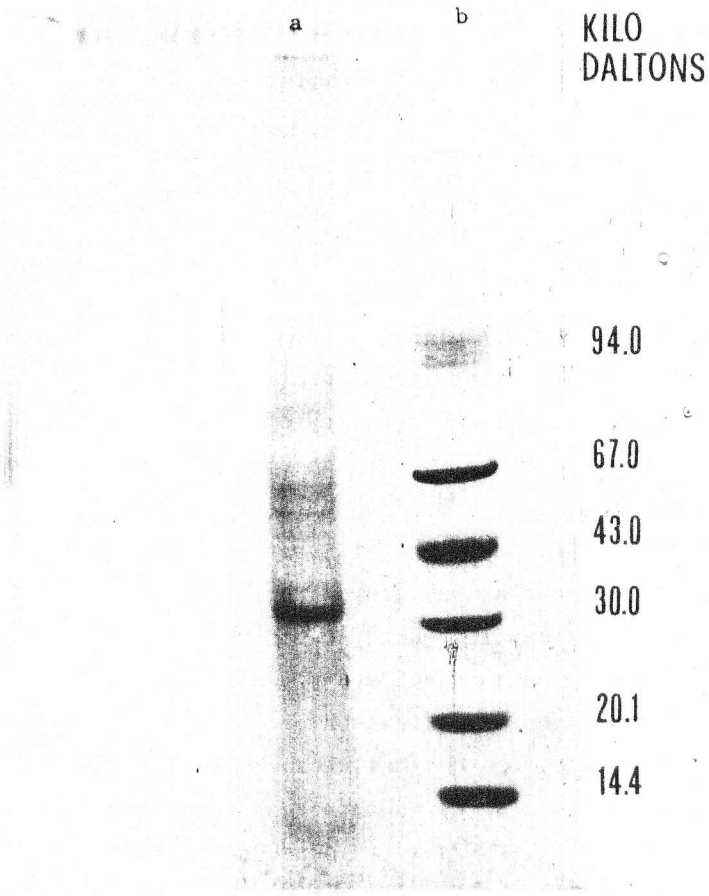

FIGURE 1 SDS-polyacrylamide gel electrophoresis of protein (25µg) obtained following treatment with 0.4M NaCl (a) and (b) standards

4. DISCUSSION

An improved procedure is described for the purification of NADPH oxidase activity from phorbol myristate acetate activated guinea pig neutrophils. The procedure consists of gel filtration chromatography on Ultrogel AcA22 of deoxycholate-extracted subcellular particles, followed by

isopycnic glycerol density gradient centrifugation and treatment with 0.4M NaCl. The final preparation is a high molecular weight complex containing the original phospholipid content of cell membrane and has a 5.0 fold increase in the specific NADPH oxidase activity. This procedure is the first report on the purification of solubilized NADPH oxidase activity from neutrophils, since previous attempts have been hampered by extremely rapid loss in enzymatic activity following detergent solubilization[3,8,9]. The detergents are probably causing the loss of phospholipids which appear to be essential for activity of many membrane-bound enzymes[12] and also of NADPH oxidase[7]. In our conditions the enzyme activity was found to be more stable since the detergent was removed during gel filtration resulting in the preservation of the phospholipid content. The increase of NADPH oxidase specific activity in the various purification steps was found to accompany a parallel increase in the specific content of cytochrome b. It is worth pointing out that this cytochrome has characteristics similar to those reported for cytochrome b of human neutrophils[13]. It has an $Em_{7.0}$ of -245mV, binds CO to about 30% and its reduced form is reoxidized by air[14].

The analysis of SDS-polyacrylamide gel electrophoresis of various fractions obtained during the purification procedure shows that only one protein band with apparent molecular weight of 32,000 was greatly enriched in the purified enzyme preparation, thus suggesting that this band is a component of the oxidase system. This conclusion is also supported by the fact that this band was found to be absent in the low density protein peak of the glycerol gradient which is devoid of NADPH oxidase activity.

The component(s) of the NADPH oxidase corresponding to this enriched band remain(s) to be identified. At first sight, this band seems to be cytochrome b_{-245}, because its percent increase relative to total protein parallels the purification ratio of the cytochrome. However, since the enrichment of the 32,000 daltons band parallels also the purification ratio of the NADPH oxidase activity, the identification of the band only with cytochrome b requires the assumption that this hemoprotein be, as a matter of fact, the oxidase itself. On the other hand, other components, such as a flavoprotein, have been proposed as participants in the transfer of electrons from NADPH to oxygen[6,9,14,15]. If this is the case, the enriched band could correspond to both flavoprotein and cytochrome b, with a molecular weight so similar that they cannot be separated by the electrophoretic system used in the present study. Further investigations are in progress in our laboratory to verify these two alternative hypotheses.

The SDS polyacrylamide gel electrophoresis, even of the most purified active complex, showed also the presence of other light protein staining bands. The fact that none of these bands is enriched during the various purification steps, but on the contrary progressively decrease suggests that they are contaminants still linked to the phospholipid protein complexes and not components of the NADPH oxidase system.

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