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Immunological properties of $O_2^{\cdot -}$ generating oxidase from bovine neutrophils

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Two antisera have been prepared against the $O_2^{\cdot -}$ generating oxidase purified from bovine polymorphonuclear neutrophils (PMNs). The first antiserum was directed against the enzymatically active fraction obtained after isoelectric focusing (p*I* oxidase), which consisted of a major protein of M_r 65 000 [(1985) Biochemistry 24, 7231–7239]. The second antiserum was directed against the 65 kDa band excised from an SDS-polyacrylamide gel after electrophoresis of the p*I* oxidase preparation. The p*I* oxidase antiserum inhibited $O_2^{\cdot -}$ generation by PMN cells, PMN membranes and detergent-solubilized membranes. The 65 kDa band antiserum was virtually non-inhibitory against PMN cells; in contrast, it was nearly as potent as the p*I* oxidase antiserum on PMN membranes and detergent-solubilized membranes. Inhibition of $O_2^{\cdot -}$ generation by the p*I* oxidase antiserum was correlated with the immunoreactivity of four membrane-bound proteins of 65, 54, 18 and 16 kDa; the 65 kDa band antiserum reacted only with the two proteins of 65 and 54 kDa. It is concluded that the 18 and 16 kDa proteins, present in trace amounts in the p*I* oxidase preparation, are probably potent catalysts of the respiratory burst.

Neutrophil; Respiratory burst; Superoxide generating oxidase

1. INTRODUCTION

Polymorphonuclear neutrophils (PMNs), in the presence of a variety of stimuli, react by a marked enhancement of O_2 uptake (respiratory burst) and the concomitant NADPH-dependent formation of the superoxide anion $O_2^{\cdot -}$. The oxidase responsible for the respiratory burst is thought to consist of a flavoprotein and a *b*-type cytochrome of low potential, absorbing at 559 nm (reviews [1–3]). Three groups have reported the purification of an $O_2^{\cdot -}$ generating oxidase of M_r 65000–67000 from human, bovine and pig blood PMNs [4–6], containing bound FAD [4,6], and characterized by an isoelectric point of 5.0 [5,6] and the presence of a specific NADPH binding site identified by photolabeling with an azido derivative of NADP [7] and by covalent binding of the 2′,3′-dialdehyde derivative of NADPH [8]. On the other hand, recent genetic data have revealed that the X-linked form of granulomatous disease is associated with the absence of a membrane protein of M_r 54000–56000 which is characterized by the presence of sites of glycosylation and might be one of the two subunits of cytochrome *b* (review [9]). In fact, purified cytochrome *b* from human PMNs [10,11] and bovine PMNs [12,13] appears to be a heterodimer. As shown for human PMN cytochrome *b*, the larger subunit is a 76–92 kDa glycoprotein, whereas the smaller 22 kDa subunit probably contains the heme prosthetic group [10,11]. The molecular masses of the subunits of bovine PMN cytochrome *c* are slightly lower, ≃20 kDa for the smaller subunit [12,13].

The present report describes the characterization of antibodies generated in rabbits against the $O_2^{\cdot -}$

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Abbreviations: PMN, polymorphonuclear neutrophil; SOD, superoxide dismutase; PMA, 4-phorbol 12-myristate 13-acetate; PBS, phosphate buffer saline consisting of 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 and 1.5 mM KH_2PO_4, pH 7.4; EMP, 20% ethylene glycol, 0.1 M mannitol, 10 mM Na phosphate, pH 7.4; BSA, bovine serum albumin

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generating oxidase prepared from bovine PMNs [5] in terms of inhibition of the respiratory burst and immunoreaction against protein components of bovine PMNs. The effects of two antisera were tested: the first antiserum was directed against the enzymatic fraction obtained after isoelectric focusing (p*I* oxidase antiserum) and the second against the 65 kDa band obtained after SDS-polyacrylamide gel electrophoresis of the p*I* oxidase preparation (65 kDa band antiserum).

2. MATERIALS AND METHODS

2.1. *Materials*

Chemicals used included ferricytochrome *c* (horse heart grade VI), superoxide dismutase and phorbol myristate acetate from Sigma; NADPH from Boehringer; sucrose from Merck; DE-52 cellulose from Whatman; Percoll and Sephadex G200 from Pharmacia and ampholines from LKB.

2.2. *Biological preparations*

Bovine PMNs were prepared as described [5,14]. A plasma membrane fraction enriched in $O_2^{\cdot-}$-generating oxidase was prepared from PMA-activated PMNs by differential centrifugation [5]. The membrane fraction collected at $100000 \times g$ (fraction C100) was solubilized in 0.2% Triton X-100, and the oxidase was isolated from this membrane extract by a purification procedure described in [5]. This procedure included chromatography on DE-52 cellulose, gel filtration on Sephadex G-200 and finally isoelectric focusing.

2.3. *Measurement of $O_2^{\cdot-}$ formation and O_2 uptake*

The rate of generation of $O_2^{\cdot-}$ by NADPH oxidase was calculated from the difference between the rate of reduction of ferricytochrome *c* at 550 nm (ϵ_{550} = 21 $mM^{-1} \cdot cm^{-1}$) in the absence and presence of SOD [5]. The assay was carried out routinely at 25°C in 2 ml PBS medium supplemented with 100 μM ferricytochrome *c*, 1 mM $MgCl_2$ and about 100 μg of membrane protein. The reaction was initiated by addition of 250 μM NADPH (or NADH). After a linear phase of 1–4 min, 50 μg SOD was added and recording continued for a further 2 min. O_2 uptake was measured by polarography [14].

2.4. *Preparation of antisera*

Before immunization, a sample of preimmune serum was collected. Rabbits were given multiple injections of the oxidase preparation. For each injection, 100–200 μg protein in 0.5 ml EMP supplemented with 0.1% Triton X-100 was mixed with an equal volume of complete Freund's adjuvant by repeated passage through a needle. The emulsion was injected by multiple intradermic injections into the back of the rabbits. The injections were repeated 3 times, every 2 weeks. 1 month after the last intradermic injection, a booster injection was administered intramuscularly, and 2 weeks afterwards, bleeding was performed. Two types of antisera were prepared. The first antiserum was generated against the oxidase preparation obtained at the final step of the purification, namely the isoelectric focusing (p*I* oxidase). This antiserum will be referred to as the p*I* oxidase antiserum. In the second case, antiserum was generated against the 65 kDa protein band excised from the SDS-polyacrylamide gel after electrophoresis of the p*I* oxidase. The latter antiserum will be designated the 65 kDa band antiserum.

2.5. *Separation and immunocharacterization of PMN membrane proteins*

Proteins were precipitated by 0.1 N perchloric acid. The precipitate was washed with cold acetone, and redissolved in an SDS-Tris buffer, pH 6.8 [15]. After 2 min of treatment at 100°C in the presence of β-mercaptoethanol, proteins were subjected to gel electrophoresis with a 5% stacking gel and a 10% resolving gel [15]. Immunoreactive proteins were immunocharacterized on nitrocellulose sheets after electrophoretic transfer from the gel under 24 V for 150 min in 25 mM Tris, 33 mM glycine, 0.05% SDS and 20% methanol, final pH 8.3 [16]. The blots were soaked for 16 h at 4°C in PBS, pH 7.4, supplemented with 3% BSA and 1% Tween 20 to saturate non-specific protein binding sites on nitrocellulose. Filters were then incubated for 2 h at 20°C with the specific antisera diluted 50-fold in PBS supplemented with 3% BSA and 0.05% Tween 20. After six washes in the same medium, the nitrocellulose sheets were incubated for 2 h at room temperature with goat anti-rabbit antibodies conjugated with peroxidase (Institut Pasteur Production). The peroxidase activity on the nitrocellulose sheets was revealed by staining with diaminobenzidine in the presence of nickel and cobalt salts [17].

3. RESULTS AND DISCUSSION

3.1. *Inhibitory effect of antisera on the respiratory burst of bovine PMNs*

The $O_2^{\cdot-}$ generating oxidase elicited in PMN cells by PMA was inhibited by the p*I* oxidase antiserum, whereas the preimmune serum was virtually inactive. It could therefore be concluded that the plasma membrane-embedded oxidase has immunoreactive epitopes exposed to the external medium. The double reciprocal plots of the rate of O_2 uptake as a function of the O_2 concentration showed that the p*I* oxidase antiserum affects both the K_m of the oxidase for O_2 and the maximum velocity, suggesting direct interaction of antibodies with specific epitope(s) at or near to the O_2 reacting site of the oxidase (fig.1). At a fixed concentration of antiserum which was about half saturating, the K_m of the membrane-bound oxidase for O_2 was 25 μM, a value about 3-fold higher than the K_m of the control cells for O_2 in the presence of the preimmune serum. The V_{max} values for O_2 uptake were 6 and 9 nmol $O_2 \cdot min^{-1} \cdot mg\ protein^{-1}$ in the presence and absence of antiserum respectively. A comparison of V_{max}/K_m values indicated a 4.5-fold greater catalytic efficiency of the oxidase in control

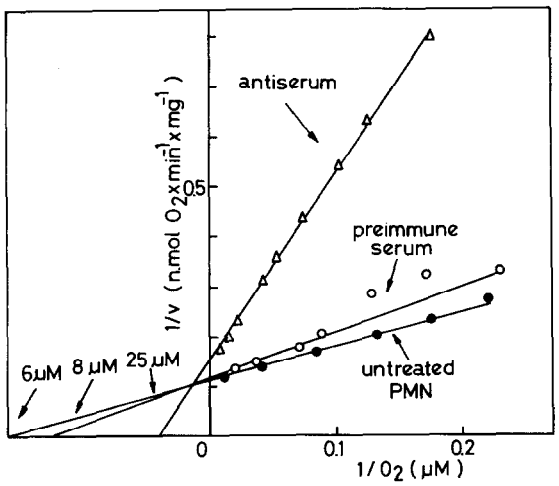

Fig.1. Double reciprocal plots of the rate of O_2 uptake by bovine PMNs treated by PMA, after preincubation with a preimmune serum or with p*I* oxidase antiserum. PMNs (4 mg protein) in 1.5 ml PBS supplemented with 25 mM glucose were preincubated either with the preimmune serum (100 μl) (○), or with the p*I* oxidase antiserum (100 μl) (△) for 20 min at 37°C. Stimulation of O_2 uptake was elicited by addition of 1 μg PMA in 2 μl DMSO. A control was performed in the absence of rabbit serum (●).

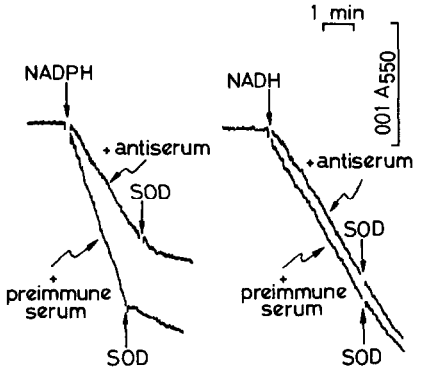

Fig.2. Effect of the p*I* oxidase antiserum on the rate of $O_2^{\cdot-}$ formation (SOD-sensitive cytochrome *c* reduction) in the presence of NADPH or NADH by the C100 membrane fraction from bovine neutrophils. 100 μl samples of the C100 membrane fraction of PMA activated PMN containing 0.3 mg protein were incubated either with 400 μl preimmune serum or with 400 μl p*I* oxidase antiserum for 5 min at 37°C. Then the whole preincubation medium was transferred to the photometric cuvette containing the assay medium. Cytochrome *c* reduction in the presence of NADPH or NADH and the effect of SOD were assayed as detailed in section 2.

cells compared to cells treated with p*I* oxidase antiserum.

The specificity of inhibition was evidenced not only by the absence of effect of the preimmune serum (see above), but also by the demonstration that the p*I* oxidase antiserum added to PMN membranes inhibited the SOD-sensitive cytochrome *c*-reductase dependent on NADPH, and not the SOD-insensitive NADH cytochrome *c*-reductase (fig.2). Inhibition of $O_2^{\cdot-}$ formation by the membrane fraction incubated with the p*I* oxidase antiserum increased with the amount of added antiserum to reach a plateau of about 30% for volumes of antiserum higher than 50 μl (fig.3A). After solubilization of the PMN membranes by Triton X-100, the plateau of inhibition disappeared and inhibition increased to values up to 70–80% (fig.3B). These results indicate that

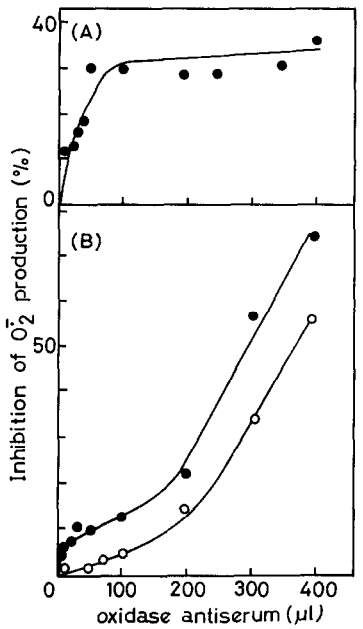

Fig.3. Effect of increasing concentrations of anti-oxidase antisera on the rate of $O_2^{\cdot-}$ formation by the C100 membrane fraction of bovine PMNs prior and after solubilization by detergent. (A) PMN membranes incubated with p*I* oxidase antiserum. Conditions were similar to those of fig.2. (B) Triton extract of PMN membranes. The C100 membrane fraction was solubilized by 0.2% Triton X-100 and 0.2 mg protein incubated for 5 min at 37°C with the p*I* oxidase antiserum (●) or the 65 kDa band antiserum (○). The percentage of inhibition of $O_2^{\cdot-}$ formation (cf. section 2) was calculated with respect to a control assay where antiserum was replaced by preimmune serum.

epitopes present in the membrane-bound oxidase which were masked to the p*I* oxidase antiserum became accessible after treatment of membranes by detergent.

The 65 kDa band antiserum was much less efficient than the p*I* oxidase antiserum on the respiratory burst of bovine PMN cells. Only for O_2 concentrations well below the K_m value was inhibition significant. However, when solubilized PMN membranes were used instead of PMN cells, the $O_2^{\cdot-}$ generating activity was efficiently inhibited by the 65 kDa band antiserum (fig.3B). There are two possible explanations for these results. (i) The epitopes of the oxidase complex reacting with the 65 kDa band antiserum are located at the inner surface of the plasma membrane or are present in the portion of the protein embedded in the lipid bilayer of the PMN membrane; they became accessible only after disruption of the membrane by detergent. (ii) As the 65 kDa protein contains the NADPH binding site [7,8], this protein might be the NADPH dehydrogenase component of the oxidase complex, the second component being an O_2 reacting protein, possibly cytochrome *b*-559. The 65 kDa band antiserum would react with the NADPH deshydrogenase component, but not with the O_2 binding protein.

In this first series of experiments carried out on the respiratory burst, the demonstration of inhibition of $O_2^{\cdot-}$ production required undiluted antisera. This was due (i) to the low titer of the antisera, and (ii) to the large amounts of PMN cells utilized in the oxygraphic assay (1 mg protein is equivalent to 1.6×10^7 cells).

3.2. *Immunoenzymatic characterization of membrane components of bovine PMN oxidase*

The immunoblot shown in fig.4 illustrates the reactivity of the p*I* oxidase antiserum and the 65 kDa band antiserum against protein components contained in a Triton extract of bovine PMN plasma membranes after separation of these components by SDS-polyacrylamide gel electrophoresis. After treatment with the 65 kDa band antiserum, only bands of 65 and 54 kDa were revealed on the immunoblot (fig.4A). The immunoreactivity of the 54 kDa protein cannot be readily explained. It is possible that this protein was derived by cleavage from the 65 kDa protein.

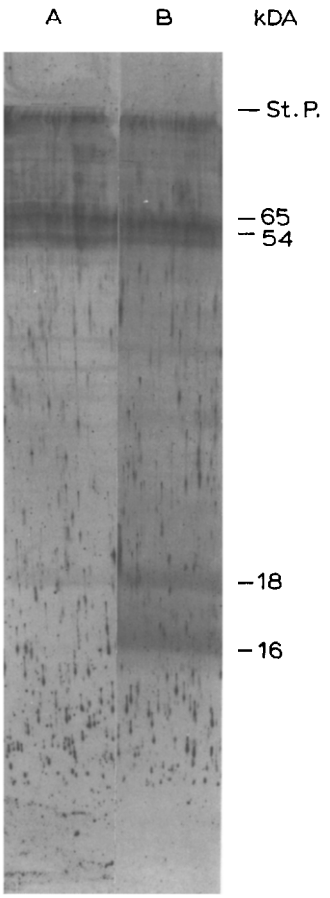

Fig.4. Immunocharacterization of protein components of bovine PMN membranes reacting with the p*I* oxidase antiserum and the 65 kDa band antiserum. The C100 membrane fraction of bovine PMN was lysed by 1% Triton X-100 and 300 μg protein of the extract was subjected to SDS-polyacrylamide gel electrophoresis. Blotting and immunodetection are described in section 2. Lanes: A, reaction with the 65 kDa band antiserum; B, reaction with the p*I* oxidase antiserum; St.P., starting point.

It cannot be excluded, however, that the 54 kDa protein is the same as that which is absent in human PMN chronic granulomatous disease [9].

After treatment with the p*I* oxidase antiserum, not only protein bands of 65 and 54 kDa were revealed, but also bands of 18 and 16 kDa (fig.4B). The presence of these additional proteins of small molecular mass provided evidence that the p*I* oxidase preparation contained minute amounts of these small molecular mass proteins which escaped detection on the SDS-polyacrylamide gel by staining with Coomassie blue [5]. The immunoreactive

species of 16–18 kDa might be implicated in the O_2 reactivity of the p*I* oxidase in PMN cells, since the p*I* oxidase antiserum competed efficiently with O_2 in the assay of the respiratory burst of intact PMNs. It should be recalled that the 65 kDa protein can be labeled by photoactivatable NADPH [7] and dial NADPH [8]; it therefore contains the NADPH binding site and might correspond to the NADPH-dependent reductase moiety of the oxidase complex. This is fully consistent with the minor effect of the 65 kDa bound antiserum on the respiratory burst of PMN cells, contrasting with the significant potency of this antiserum on detergent treated membranes, and the fact that the NADPH binding site of the oxidase is exposed to the interior of the cells, reacting with cytosolic NADPH. In contrast, epitopes of the oxidase complex contained in the 16–18 kDa proteins are most likely exposed to the outside, as the p*I* oxidase antiserum which reacts with them also inhibits the respiratory burst in PMN cells.

Specific neutralization of the 16–18 kDa proteins by the p*I* oxidase antiserum, but not by the 65 kDa band antiserum, could provide a simple explanation for the difference of action of the two antisera with respect to the respiratory burst in PMN cells. Discussion about the identity of the 18 and 16 kDa proteins is at present speculative. The 16 kDa protein might be derived from the 18 kDa protein by cleavage. It might be hypothesized that the immunoreactive 18 kDa protein is the heme-bearing subunit of cytochrome *b* that was recently reported (see section 1). In a previous paper on the purification of an $O_2^{\cdot -}$ generating protein of 65 kDa from bovine PMNs [5], it was concluded that the 65 kDa protein could be the NADPH binding component of an oligomeric complex with possible additional redox components, for example cytochrome *b*-559 which would be the O_2 reacting component of the oxidase complex. The data reported in the present paper reinforce this idea.

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