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# Immunological studies on the respiratory burst oxidase of pig blood neutrophils

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Recently, a flavin enzyme (p*I* 5.0), that is probably responsible for superoxide ($O_2^-$)-generating oxidase activity, was separated by isoelectric focusing-polyacrylamide gel electrophoresis (IEF-PAGE) from neutrophil membranes in our laboratory [(1987) J. Biol. Chem. 262, 12316–12322]. In the present work, we performed immunological studies on this enzyme derived from pig blood neutrophils. The enzyme extract obtained on IEF-PAGE was injected into guinea pigs to raise antibodies. IgG antibody against the p*I* 5.0 protein inhibited maximally 54% of the $O_2^-$-generating activity of the membrane-solubilized oxidase, whereas the normal serum IgG was not inhibitory at all. Our results further confirmed that the enzyme (p*I* 5.0) is one of the component(s) of the $O_2^-$-generating system. The enzyme gave rise to a band corresponding to a major protein of $72 \pm 4$ kDa on both non-denaturing and SDS-PAGE. Immunoblotting after SDS-PAGE demonstrated labelling of peptides of 70–72, 28–32 and 16–18 kDa.

Neutrophil; Respiratory burst; NADPH oxidase; Superoxide generation; FAD enzyme; Antibody

## 1. INTRODUCTION

When bacteria are ingested, neutrophils exhibit a burst of cyanide-insensitive respiration [2] for the production of superoxide anion ($O_2^-$) [3], which then is converted to other reactive oxygen intermediates functioning in the host defense system against bacterial infections [4]. Neutrophils possess a membrane-bound NADPH oxidase responsible for the activity of the respiratory burst oxidase [5]. This activity also appears when neutrophils are stimulated with membrane-perturbating agents [6,7]. While the nature of the NADPH oxidase has not yet been elucidated, recent studies have shown that it may consist of multiple components, a *b*-type cytochrome [8,9] and a flavoprotein [1,10]. Recently, an FAD enzyme, which is possibly part of the respiratory burst oxidase, was separated by IEF-PAGE in our laboratory [1]. The enzyme, which focused at p*I* 5.0, showed characteristics similar to those of the oxidase and a high specificity for NADPH. In the course of IEF on gels, we also observed weak activity at p*I* 4.6, but only with fatty acid-stimulated cells, under some conditions.

Here, we separated the p*I* 5.0 and 4.6 proteins from the solubilized NADPH oxidase by IEF-PAGE, which were then injected into guinea pigs to raise antibodies against the proteins. We found that the antibody against the p*I* 5.0 protein inhibited the $O_2^-$-generating activity of the NADPH oxidase markedly, whereas the p*I* 4.6 protein antibody was far less inhibitory. We further analyzed the interactions between these antibodies and the oxidase using immunoblotting [11].

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*Abbreviations:* IEF-PAGE, isoelectric focusing-polyacrylamide gel electrophoresis; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; NBT, nitroblue tetrazolium

## 2. MATERIALS AND METHODS

### 2.1. *Materials*

Materials were obtained from the following sources: $\beta$-octyl glucoside, Calbiochem (San Diego, CA) and Dojin



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(Kumamoto); ampholine, LKB (Bromma); phenylmethylsulfonyl fluoride (PMSF), Nakarai (Kyoto); nitroblue tetrazolium (NBT), cytochrome *c*, leupeptin, chymostatin, pepstatin and superoxide dismutase (SOD), Sigma (St. Louis, MO); protein A-Sepharose CL-4B and electrophoresis calibration kits, Pharmacia (Japan); nitrocellulose membrane filters (type BA 85, 0.45 μm), Sleicher & Schull (Dassel); X-Omat film (type XAR-5), Eastman Kodak (Rochester, NY); Freund's complete and incomplete adjuvant, Iatron (Tokyo); $^{125}$I-protein A, Amersham (Bucks, England).

2.2. *Neutrophils and membrane vesicles*

Neutrophils isolated from pig blood [12] were briefly incubated at 37°C with and without myristate (110 nmol/$10^7$ cells) for preparation of stimulated and resting cells, respectively [6]. Stimulated and resting neutrophils were disrupted by sonication to isolated membrane vesicles as reported in [1].

2.3. *Solubilization of NADPH oxidase*

Membrane-bound NADPH oxidase was solubilized using 0.75% octyl glucoside, 30% glycerol and 0.19 M sucrose, in 50 mM K-Na phosphate buffer, pH 7.4 [1], in the presence or absence of the following protease inhibitors: leupeptin (50 μg/ml), chymostatin (50 μg/ml), pepstatin (50 μg/ml), PMSF (1 mM) and EGTA (0.1 mM) at 0°C for 30 min. Each mixture was centrifuged at $10^5 \times g$ for 60 min in a Beckman 60 Ti rotor. An aliquot of the solubilized fraction was loaded onto an IEF-PAGE gel as described later. Protein was determined by the method of Lowry et al. [13].

2.4. *IEF*

The flavin enzyme was separated by means of disc IEF-PAGE (5.0 × 60 mm; diameter × length) as in [1,14]. IEF was carried out for a total of 1700 V·h in a refrigerator at 2°C. After focusing, the enzyme activity was detected with NBT-NADPH [1,15].

2.5. *Elution of proteins from IEF-PAGE*

Proteins were eluted from gel slices with an electrophoretic concentrator (Max yield-GP; Atto, Tokyo) by electrophoresis at 4°C and 2 W for 3 h [16], as in [1]. The concentrated protein in the collection chamber was frozen at −80°C before use.

2.6. *Immunization and antiserum preparations*

Proteins extracted from gel slices for stimulated cells were adjusted to isotonicity with NaCl, subsequently emulsified with an equal volume of Freund's complete adjuvant and then injected intra-subcutaneously (i.s.) into guinea pigs (0.1 mg protein/guinea pig) as antigens. Preimmunized sera were obtained before immunization. 1 month after immunization, animals were reinoculated i.s. with the same antigenic dose using Freund's incomplete adjuvant, 1 month later immunized sera being examined by the passive hemagglutination (PHA) test with antigen-conjugated erythrocytes [17]. IgG fractions were isolated from antisera by protein A affinity chromatography [18].

2.7. *Effects of antibodies on the oxidase*

Reaction mixtures contained an aliquot of the solubilized oxidase (31–75 μg/ml), 50 μM cytochrome *c*, 6 μg/ml catalase, 0.17 M sucrose. 65 mM Na-K phosphate buffer, pH 7.4, and various amounts of the IgG fractions. After 4 min incubation at room temperature, the reaction was initiated by the addition of 0.1 mM NADPH to measure $O_2^-$-generating activity in terms of SOD-inhibitable cytochrome *c* reduction [1,3].

2.8 *Determination of molecular masses*

Molecular masses were determined by electrophoresis on two kinds of gels: (i) SDS-PAGE was carried out according to Laemmli [19]; (ii) non-denaturing PAGE was performed with a 4–20% gradient polyacrylamide gel containing 0.75% octyl glucoside at 4°C and 5 mA for 16 h [20].

2.9. *Immunoblotting*

Membrane vesicles were solubilized in the presence of the protease inhibitors mentioned above, the resultant supernatant (10–20 μg protein) being subjected to SDS-PAGE via two procedures; directly and after precipitation with trichloroacetic acid, respectively. After electrophoresis, proteins were transferred to a nitrocellulose membrane at a constant current of 80 mA for 90 min at 8°C, using a blotting unit obtained from Marisol (Tokyo), according to Towbin et al. [11]. Blotted membranes were treated with 10% bovine serum albumin and then incubated with the IgG antibodies at 4°C for 16 h. After rinsing several times, membranes were incubated with $^{125}$I-protein A for 30 min, rinsed again, dried, and then exposed to film at −80°C for 2 weeks to 1 month.

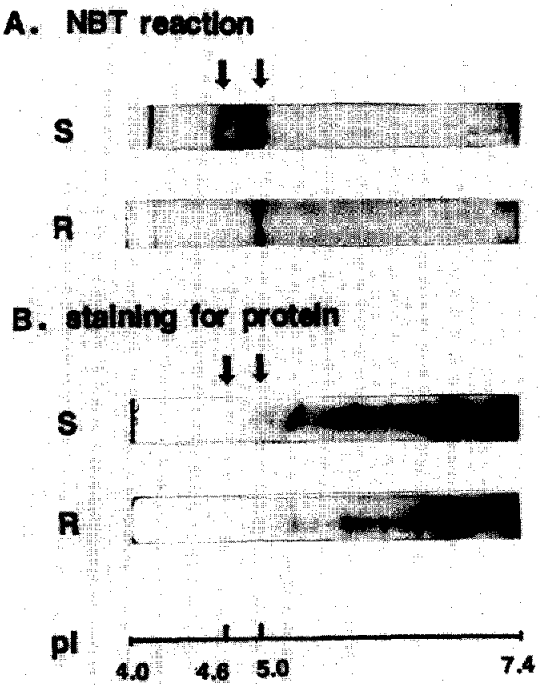


Fig.1. NADPH-dependent NBT-reducing activity in IEF gels (A) and Coomassie blue staining of proteins (B). Solubilized fractions from stimulated (S) and resting cells (R) were loaded onto gels and then subjected to IEF. Each gel was stained for NADPH-dependent NBT-reducing activity and protein.

## 3. RESULTS

### 3.1. *NADPH-dependent NBT reduction on IEF-PAGE*

Neutrophils were briefly incubated with and without myristate as a stimulator, and then fractionated to obtain membrane fractions of stimulated (S) and resting (R) cells, respectively. Solubilization of these membranes with octyl glucoside resulted in a high yield of NADPH oxidase (70–80%), the yield (4–5%) of granule enzymes being low, as reported in [1]. Solubilized fractions from stimulated and resting cells were loaded on gels and then subjected to IEF. Fig.1A shows the gels stained for NBT reduction in the presence of NADPH. NADPH-dependent NBT-reducing activity appeared markedly at p*I* 5.0 and weakly at p*I* 4.6 for stimulated cells, but only at p*I* 5 for resting cells. In the presence of NADH, NBT-reducing activity appeared at p*I* 6.2 and 4.6 for activated cells, but was only evident at p*I* 6.2 for resting cells (not shown). The p*I* 4.6 band observed for stimulated cells was specific for fatty acid activation, as discussed later. The profiles of protein migration on IEF-PAGE with Coomassie blue staining showed no marked differences between stimulated and resting cells, as shown in fig.1B.

### 3.2. *SDS-PAGE of extracted proteins*

After IEF, proteins were extracted from gel slices corresponding to p*I* 4.6 and 5.0. Extracts from stimulated and resting cells contained similar concentrations of the p*I* 5.0 protein. The extracted fractions were concentrated electrophoretically, subjected to SDS-PAGE and then stained with Coomassie blue. Fig.2a,b shows the peptide bands

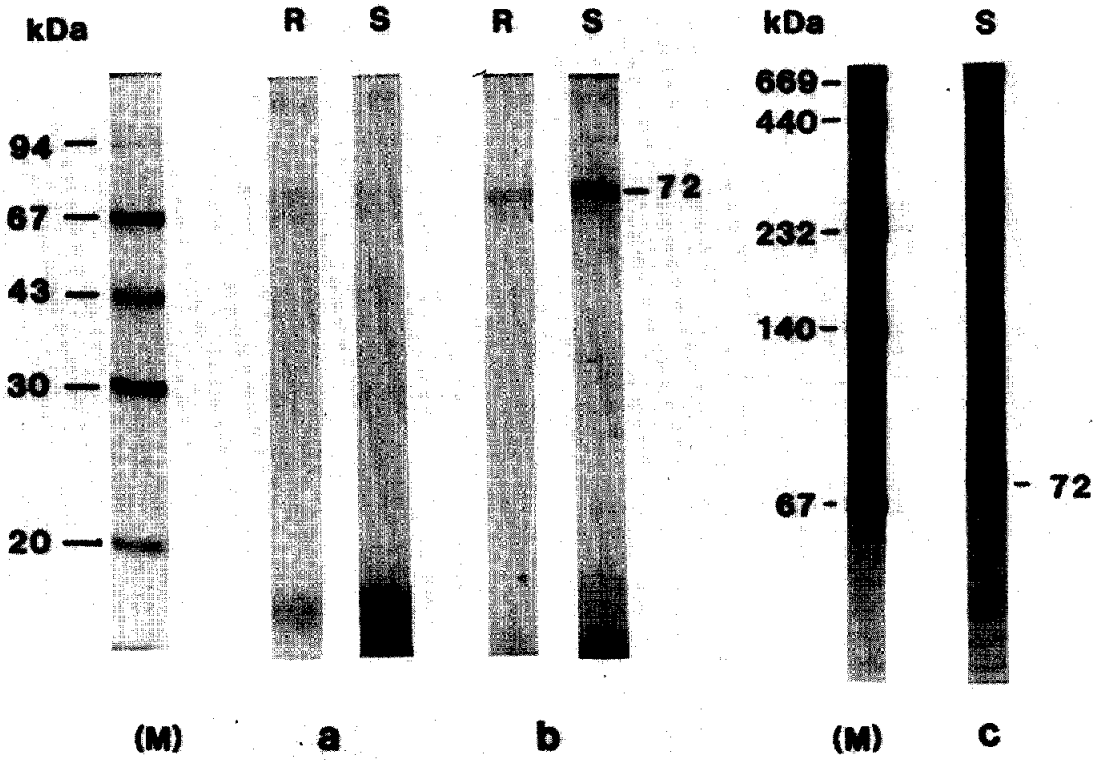


Fig. 2. SDS-PAGE (12.5%) and non-denaturing PAGE (4–20%) of enzyme extracts using staining with Coomassie blue. SDS-PAGE of the p*I* 4.6 (a) and p*I* 5.0 (b) proteins from stimulated (S) and resting (R) cells. Aliquots (75 μl) of p*I* 4.6 and 5.0 extracts (13.5 and 12.5 μg proteins of S and R extracts, respectively) were introduced onto the SDS-PAGE gels. Note the single band (72 kDa) of the p*I* 5.0 extract on a non-denaturing gel (c).

for the p*I* 4.6 and 5.0 proteins from resting (R) and stimulated (S) cells, respectively. In all cases, a distinct band was observed at 72 ± 4 kDa. Small proteins (16–18 kDa) were seen in greater amounts in stimulated cells (S) than in resting cells (R), and more so in the p*I* 4.6 extract than in that of p*I* 5.0. Since non-denaturing PAGE gave a clear 72 kDa band (fig.2c), the small molecules appear to be degraded proteins or unknown components, as discussed later.

### 3.3. *Effects of antibodies on $O_2^-$-generating activity*

The solubilized oxidase from stimulated cells was applied to IEF-PAGE gels which had been sectioned at positions corresponding to p*I* 4.6 and 5.0. The proteins were extracted from both sections and then injected into guinea pigs as antigens. Antibody activities were assayed by the PHA test [17]. As shown in fig.3, antibodies against the p*I* 5.0 and 4.6 proteins caused positive hemagglutination which occurs on immune reaction of an antigen-antibody complex with erythrocytes. In contrast, preimmune (control) sera gave negative results (fig.3, nor.).

The effect of each IgG fraction on $O_2^-$-generating activity of the solubilized oxidase was investigated with various amounts of IgG. No precipitation occurred in any of the mixtures. The addition of IgG antibody against the p*I* 5.0 protein markedly inhibited $O_2^-$-generating activity in all three preparations; maximum inhibition of oxidase activity amounted to 54%, with 3 μg protein IgG and 1 μg solubilized enzyme (fig.4, three continuous lines; ▲,■,●). In contrast, the p*I* 4.6 protein IgG inhibited activity by about 15% only, even at a high IgG/protein ratio (fig.4, broken line; ▼). Normal serum IgG had no effect on the enzyme activity (fig.4, dotted line; ○). Enhancement of enzyme activity occurred with increasing ratios of p*I*

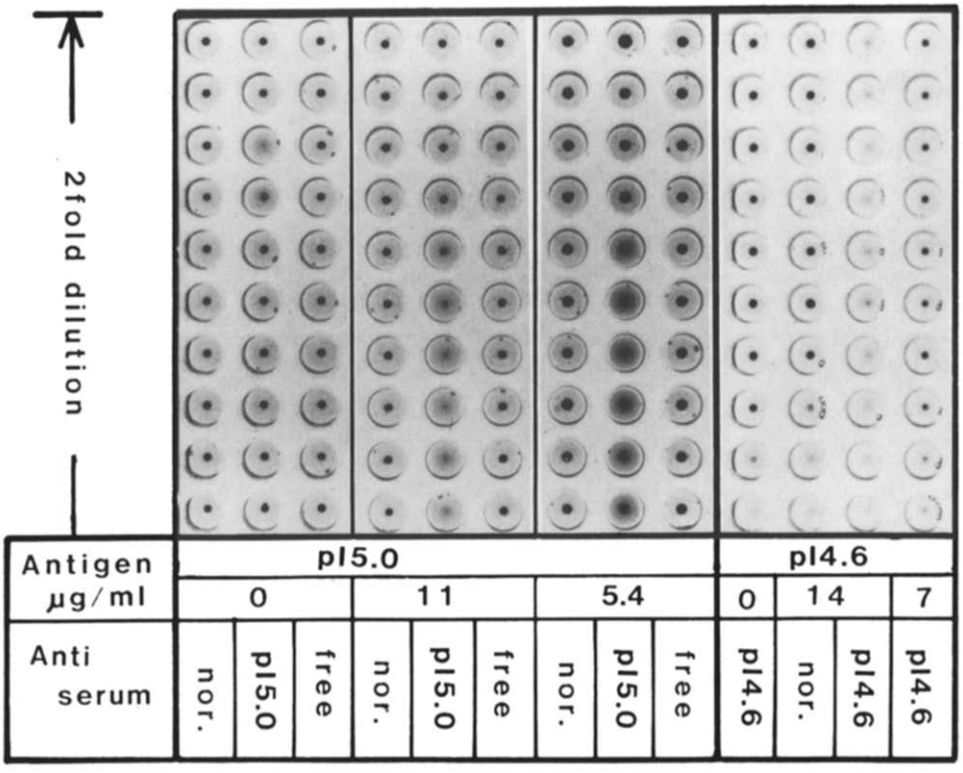


Fig. 3. Passive hemagglutination (PHA) test for antibodies against the p*I* 5.0 and 4.6 proteins. Human type O Rh-positive erythrocytes were sensitized, for each concentration of antigen, as indicator cells. Each antiserum was serially diluted 2-fold with phosphate-buffered saline containing 0.1% normal rabbit serum, and the resulting antibody showed hemagglutination.

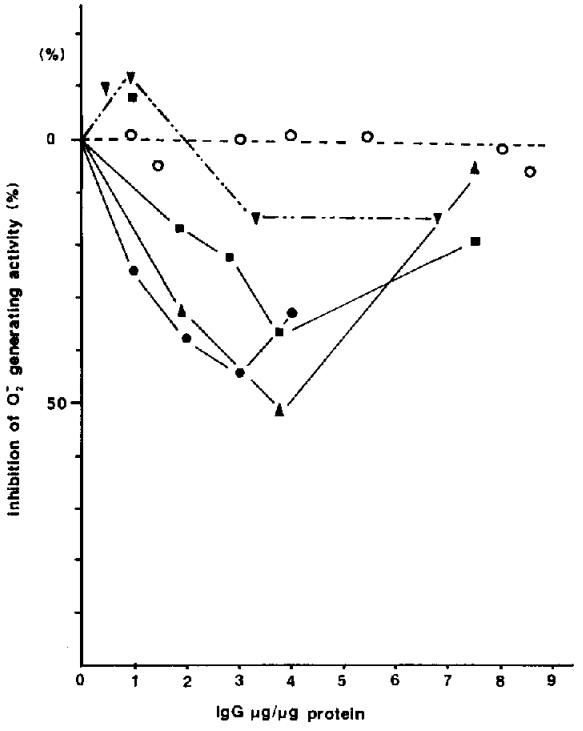


Fig.4. Effects of antibodies on $O_2^-$-generating activity of solubilized NADPH oxidase from stimulated cell membranes. IgG fractions against the p*I* 5.0 protein (three solid lines; (▲,■,●), p*I* 4.6 protein (broken line, ▼) and normal IgG (dotted line, (○) were used.

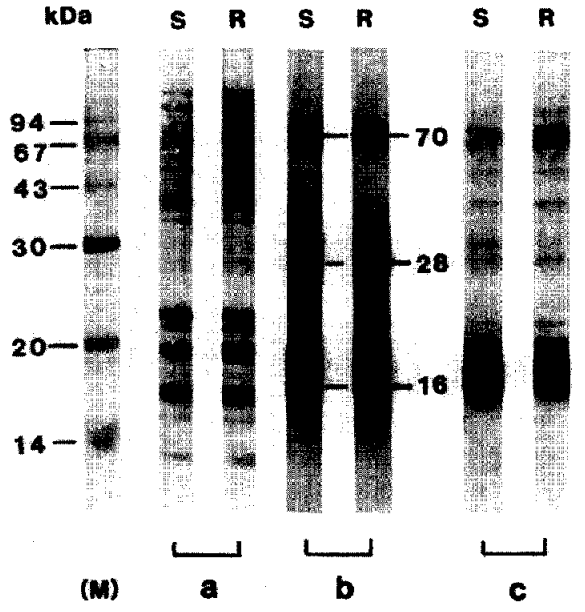


Fig.5. SDS-PAGE (a) and immunoblotting (b,c). Membrane NADPH oxidase of stimulated (S) and resting (R) cells was respectively solubilized with octyl glucoside in the presence of protease inhibitors, and then subjected to SDS-PAGE and blotting. Blots stained with amido black-10 (a) and $^{125}$I-labelled immunoblots with the p*I* 5.0 protein antibody (b) are shown.

5.0 protein-IgG concentration over 10 μg IgG/μg protein of the soluble fraction. The formation of immune complexes probably resulted in clustering of the NADPH oxidase components, as discussed later.

### 3.4. *Immunoblotting analysis of the* p*I 5.0 proteins*

Fig.5a illustrates SDS-PAGE of the supernatants containing solubilized oxidase from membranes after staining with amido black-10. Fig.5b shows immunoblotting profiles of the solubilized proteins that reacted with antibody against the p*I* 5.0 extract. Three groups of proteins, i.e. 70–72, 28–32 and 16–18 kDa, were labelled in the soluble fractions of both resting and stimulated cells. We observed that the immunblotting profiles (fig.5b) differ markedly from those in fig.5c, in which the solubilized fractions were treated with trichloroacetic acid and then loaded on SDS-PAGE gels.

## 4. DISCUSSION

Recent work in our laboratory demonstrated that the NADPH-dependent enzyme focusing at p*I* 5.0 was a flavoprotein [1]. The enzyme (p*I* 5.0) showed greater activity in stimulated than in resting cells (fig.1), and the distinct reducing activity of stimulated cells was split into two bands corresponding to p*I* 5.0 and 4.6 on IEF (p*I* 4.0–6.5) on a thick gel (fig.1). During the course of IEF on gels, we occasionally found an NBT-stained band tailing from pH 5.0 towards pH 4.6, suggesting that a portion of the enzyme migrates from pH 5.0 to 4.6. The p*I* 4.6 band was seen only with fatty acid activation, i.e. it did not appear with other stimulators such as phorbol myristate and opsonized zymosan. The p*I* 4.6 band was not observed on a long thin gel and showed NBT-reducing activity with either NADH or NADPH [1]. From the data, this band appears to be due to partial modification of the enzyme, with a change in $p_I$ value and loss of substrate specificity, which probably occurred during the drastic stimulation of cells by myristic

acid [6]. This speculation was further confirmed by the results of immunological experiments, as shown here.

In the present study, we attempted immunization with the two proteins corresponding to the NADPH-dependent NBT-reducing bands, namely the p*I* 5.0 and 4.6 proteins from stimulated pig neutrophils. The antibody against the p*I* 5.0 protein inhibited maximally 54% of the $O_2^-$-generating activity of the solubilized oxidase, whereas the p*I* 4.6 protein IgG was far less inhibitory (fig.4). The inhibitory effect of the p*I* 5.0 enzyme antibody on the $O_2^-$-generating activity was specific, since non-immunized serum IgG had no effect at all on enzyme activity, as shown in fig.4. The apparent molecular mass of the antibody-reacted protein was estimated by immunoblotting after SDS-PAGE. Immunoblotting of the p*I* 5.0 enzyme antibody demonstrated $^{125}$I-labelled bands at 70–72, 28–32 and 16–18 kDa when protease inhibitors were added to the solubilized enzyme (fig.5b). We failed to detect bands at 70–72 and 28–32 kDa on the immunoblot sheet when the solubilized oxidase was treated with trichloroacetic acid (fig.5c) or without protease inhibitors (not shown). This suggests that the p*I* 5.0 enzyme is readily cleaved by proteases or acid treatment to yield small fragments. In the present experiment, we used the extract of gel slices corresponding to the p*I* 5.0 band of stimulated cells as an antigen for immunization. The p*I* 5.0 enzyme extract gave a major protein of 72 ± 4 kDa and minor bands (16–18 kDa) on SDS-PAGE (fig.2). Non-denaturing PAGE showed only a single band, estimated to correspond to 70–72 kDa (fig.2c). Thus, the immune-reacted 28–32 kDa bands might be due to some cleaved peptides derived from the oxidase or unknown components in the p*I* 5.0 extract.

Immunological studies on enzymes of electron-transport systems have been reported by many laboratories, for example concerning antibodies against P-450 reductase [21] in liver microsomes, heme-protein oxidoreductase [22] in chromatophores and cytochrome $b_5$ [23], all of which showed remarkable inhibition of their activities. A recent study reported that the antibody to homologous liver P-450 reductase inhibited about 30% of the $O_2^-$-generating activity in the membrane fraction [24]. Here, with regard to the p*I* 5.0 enzyme, we observed remarkable inhibition of oxidase activity by its antibody IgG (fig.4). The results provide further evidence that the flavin enzyme focusing at p*I* 5.0 is a component of the NADPH oxidase system in the plasma membrane [1]. Enhancement of the oxidase activity was observed with increasing IgG concentration over 10 μg IgG/μg protein of the solubilized oxidase (not shown). Formation of an immune complex at higher IgG concentration probably resulted in clustering of the oxidase components, leading to an increase in the electron-transferring efficiency [25,26].

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