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The $O_2^{\cdot-}$ generating oxidase activation of bovine neutrophils

Evidence for synergism of multiple cytosolic factors in a cell-free system

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Activation of the $O_2^{\cdot-}$ generating oxidase in neutrophils can be achieved with a cell-free oxidase-activating system, which consists of a high speed supernatant (cytosol), a particulate fraction enriched in plasma membrane, GTP-γ-S, arachidonic acid and Mg ions. Cytosolic proteins from bovine neutrophils were fractionated by chromatography on Mono Q and Mono S colums into two fractions, neither of which was able to activate efficiently the membrane-bound oxidase. However, when combined and added to the cell-free system under optimized conditions, they acted synergistically, activating the oxidase to virtually the same extent as crude cytosol. This synergism demonstrates that more than one cytosolic factor is required for oxidase activation, and can be used to trace the cytosolic factors during the course of their purification.

Respiratory burst; Oxidase activation; Neutrophil; Superoxide ion

1. INTRODUCTION

Activation of the NADPH oxidase of neutrophils during phagocytosis by a number of soluble or particulate stimuli [1] results in the formation of large amounts of the superoxide anion $O_2^{\cdot-}$. Since the demonstration that the neutrophil oxidase can be activated in a cell-free system [2,3], a number of studies have been undertaken to analyze the minimal requirement of this sytem [4–10]. In the case of bovine neutrophils, an efficient cell-free system was set up, which consists of a particulate fraction enriched in plasma membrane and in dormant oxidase, a soluble fraction termed cytosol, GTP-γ-S, Mg^{2+} and arachidonic acid [9]. The conditions of oxidase activation have been optimized [11]. In the present paper, we report studies on the chromatographic resolution of crude cytosol into two protein fractions, which, when combined, act synergistically to activate the oxidase.

2. MATERIALS AND METHODS

2.1. *Materials*

The following materials were supplied by the companies indicated. Boehringer: GTP-γ-S, NADPH and superoxide dismutase; Pharmacia: Percoll, Mono Q and Mono S columns; Sigma: arachidonic acid, and ferricytochrome *c*. Arachidonic acid was dissolved in absolute ethanol and stored at −80°C under N_2 until used.

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Abbreviations: NBT, nitro blue tetrazolium; SOD, superoxide dismutase; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethane sulfonic acid); $O_2^{\cdot-}$, superoxide anion

2.2. *Biological preparations*

Neutrophils were obtained from bovine blood as described in [12,13] and suspended in PBS 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. They were then disrupted by ultrasonic treatment for 4 × 15 s at 2–4°C with a Branson sonifier, at 40 W output. Non-disrupted cells, nuclei and granular material were discarded after centrifugation at 10000 × *g* for 10 min at 4°C in a Sorvall rotor. The resulting supernatant was centrifuged at 130000 × *g* for 1 h at 4°C in a Beckman Rotor 40, yielding a pellet that was dispersed by sonication in PBS. This pellet was enriched in plasma membrane which presumably contains the resting form of the $O_2^{\cdot-}$ generating oxidase [13]; for convenience it was referred to as membrane fraction. The supernatant was further centrifuged at 300000 × *g* for 1 h in a Beckman SW65 Rotor at 4°C to eliminate small membraneous particles. This high-speed supernatant was termed cytosol. Both membrane and cytosol fractions could be stored at −80°C without loss of activity for several weeks.

2.3. *Oxidase assay*

Reduction of O_2 into $O_2^{\cdot-}$ by the neutrophil oxidase was measured spectrophotometrically as the superoxide dismutase (SOD) sensitive portion of ferricytochrome *c* reduction. The assay medium was placed in a photometric cuvette at 25°C. It consisted of 2 ml of PBS supplemented with 100 μM ferricytochrome *c*, 2 mM $MgSO_4$ and the reconstituted oxidase activating system (see below). The reaction was initiated by addition of 200 μM NADPH. Cytochrome *c* reduction was recorded at 550 nm for 3–4 min. Then, 70 μg of SOD was added, and the recording continued for 2–4 min. The difference between the slopes before and after addition of SOD was regarded as the rate of $O_2^{\cdot-}$ production. Alternatively a rapid screening assay was used, based on reduction of NBT and estimation of absorbance at 570 nm (fig.1).

2.4. *Optimization of oxidase activation in a cell free system*

The cell-free system used for the activation of bovine neutrophil

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oxidase was similar to that described in [9]. In brief, the membrane fraction (60 μg protein), the cytosolic fraction (100–150 μg protein), 10 μM GTP-γ-S and 10 mM $MgSO_4$ in either PBS or a mixture of PBS and 10 mM Tris, pH 7.5, were mixed in the volume of 150–180 μl, the final concentration of NaCl being adjusted to 0.2 M. An optimal amount of arachidonic acid in ethanol, determined by a preliminary assay, was added. The maximal amount of ethanol used was not higher than 5% of the total volume. There was no detectable effect of ethanol at this concentration on oxidase activation. After a 10 min incubation at room temperature, the cell-free suspension was then transferred to the photometric cuvette containing cytochrome *c* and 2 mM $MgSO_4$ in PBS, and the oxidase reaction was initiated by addition of NADPH as described above. As previously reported [11], oxidase activation depends on the precise adjustment of the amount of added arachidonic acid relative to that of membrane and cytosol proteins. In fact, a variation of only 15% in the concentration of arachidonic acid with respect to the optimal concentration may result in a 50% decrease of oxidase activation. Assays carried out with NaCl under our experimental conditions showed that the optimal salt concentration was between 0.1 and 0.3 M NaCl. Higher concentrations were inhibitory, 50% inhibition being attained with 0.6 M NaCl. When optimal conditions of oxidase activation were fulfilled, the kinetics of oxidase activation vs cytosol protein were typically Michaelian [11]. Consequently, when the amount of cytosol used was kept relatively low with respect to the membrane fraction, the elicited oxidase activity was virtually linearly related to the amount of added cytosol. In the experiments to be presented in this paper, which were aimed at the titration of the oxidase activating potency of cytosolic fractions, the amount of added cytosol was always less (3–5-fold) than that required for maximal oxidase activation.

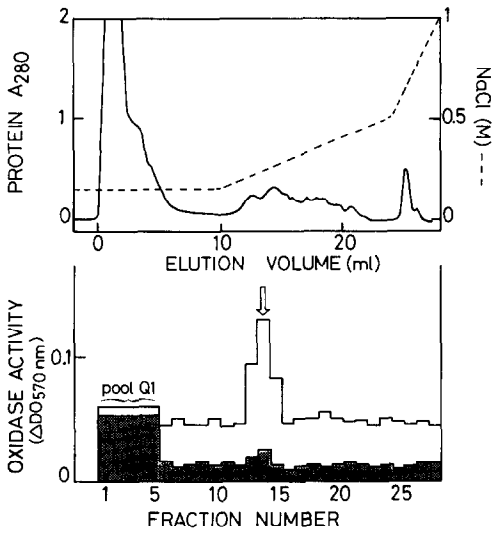

Fig.1. Detection of cytosolic protein species endowed with an oxidase activating potency by means of synergistic activation. 10 mg of bovine neutrophil cytosol in 1 ml PBS was diluted twice with 10 mM Tris, pH 7.5, and injected into a Mono Q column (1 ml bed vol.) equilibrated with 10 mM Tris, pH 7.5, plus 150 mM NaCl. Washing with the equilibration buffer yielded a fraction Q1, as described in table 1. Then, a linear 150–500 mM NaCl gradient programmed in an FPLC system was applied. 1-ml fractions were collected. For oxidase activation 30-μl aliquots were added in the absence of Q1 protein (hatched bars) and the presence of 70 μg of Q1 protein (open bars) to the membrane fraction, GTP-γ-S, arachidonic acid and $MgSO_4$ as in table 1. The elicited oxidase was revealed by a rapid screening assay, using the reduction of NBT (200 μM) in the presence of 2 mM $MgSO_4$ and 200 μM NADPH in 1 ml of PBS. The reaction was allowed to proceed for 5 min at room temperature, and was stopped by 100 μl of 10% SDS. $O_2^{\cdot-}$ production was estimated from absorbance at 570 nm. The active peak eluted between 200 and 280 mM NaCl is indicated by an arrow.

3. RESULTS

3.1. *Resolution of neutrophil cytosol into distinct fractions capable of enhancing oxidase activation synergistically*

The activation of the membrane-bound oxidase in a cell-free system, prepared from a neutrophil homogenate, requires the presence of soluble protein(s) present in the cytosol fraction (cf. [9]). The experiments to be presented here show that more than one cytosolic protein is needed for oxidase activation.

In the first experiment (table 1), 10 mg of cytosol protein in 1 ml PBS was applied to a Mono Q column equilibrated in 10 mM Tris-HCl, pH 7.5, and 150 mM NaCl. Sequential elution of the column first with the equilibration buffer, then with 250 mM NaCl buffered with 10 mM Tris-HCl, pH 7.5, and finally with 1 M NaCl/10 mM Tris, pH 7.5, yielded fractions which were assembled in 3 pools of 5 ml each, termed Q1, Q2 and Q3, containing 6.8, 1.5 and 1.2 mg protein, respectively. The protein recovery was 95%. 50 μl of each pool was assayed for oxidase activation under the op-

Table 1

Fractionation of cytosolic proteins from bovine neutrophils by Mono Q chromatography. Synergistic effects of fractions recovered by stepwise elution of the Mono Q column

Fractions (μg protein)	$O_2^{\cdot-}$ formed (nmol·min^{-1})	Recovery (%)	Factor of enhancement
Q1 (68 μg)	2.9	35	–
Q2 (15 μg)	0.8	9	–
Q3 (12 μg)	0.5	6	–
Q1 (68 μg) + Q2 (15 μg)	7.0	84	1.9
Q1 (68 μg) + Q2 (15 μg) + Q3 (12 μg)	7.9	95	1.9
Cytosol (100 μg)	8.3	100	–

1 ml of cytosol (10 mg protein) from bovine neutrophil homogenate was applied to a Mono Q HR 5/5 column (1 ml bed vol.) equilibrated in 10 mM Tris-HCl, pH 7.5, and 150 mM NaCl. The column was eluted with the equilibration buffer at a rate of 1 ml/min. Five fractions of 1 ml were collected and assembled in a pool, Q1, containing 6.8 mg protein. This was followed by elution with 250 mM NaCl and 10 mM Tris, pH 7.5. Five fractions of 1 ml were again collected and assembled in a pool, Q2, containing 1.5 mg protein. A final wash with 1 ml of 1 M NaCl and 10 mM Tris, pH 7.5, followed by 4 ml of 10 mM Tris, pH 7.5, yielded 1.2 mg protein (pool Q3). 50 μl aliquots of pools Q1, Q2 and Q3 and 10 μl of crude cytosol were assayed for their ability to elicit oxidase activity in a cell-free system consisting of 60 μg membrane protein, 10 μM GTP-γ-S, 10 mM $MgSO_4$, and an optimal amount of arachidonic acid in a final volume of 180 μl. The final NaCl concentration of the medium was adjusted to 0.2 M. The optimal amount of arachidonic acid was determined for each type of combination; it ranged between 60 and 80 nmol. After 10 min of incubation at room temperature the suspension was transferred to a photometric cuvette to assay the elicited oxidase activity as described in section 2. The percentage of recovery refers to the relative oxidase activity of the cytosolic fractions compared to that of crude cytosol. The factor of enhancement is the ratio of the elicited oxidase activity obtained after mixing aliquots of the Q pools to the arithmetic sum of the elicited oxidase activities obtained with the separate Q pools

timal conditions detailed in the legend of table 1. The oxidase activating potency of the corresponding amount of cytosol, namely 100 μg protein in 10 μl, was also tested as a control under the same conditions.

When assayed separately, the three Q pools exhibited a moderate oxidase activating potency compared to cytosol (table 1). However, when mixed together, the resulting oxidase activity was markedly enhanced (line 5, table 1). It was roughly twice as high as the arithmetic sum of the oxidase activities elicited by the separate fractions and close to that elicited by crude cytosol, namely 7.9 vs 8.3 nmol $O_2^{\cdot -}$ formed/min. This synergistic effect indicated that each pool contained specific proteins essential for oxidase activation and that more than one cytosolic protein was needed for full activation to occur. The factor of enhancement was virtually the same, using either fractions Q1 plus Q2 (line 4, table 1), or the three Q fractions (line 5, table 1). This suggests that the Q3 fraction contains residual active proteins common to Q1 or Q2. It must be kept in mind that the amount of cytosolic fractions used was less than the amount needed for maximal elicitation of oxidase activity (cf. section 2). This is in line with the aim of the experiment which is to titrate the activating potency of cytosolic fractions.

The experiment described in fig.1 is similar in its principle to the chromatographic fractionation of cytosolic proteins described in table 1, except that the stepwise NaCl gradient used to collect fraction Q2 was replaced by a linear 150–500 mM NaCl gradient. 1-ml fractions were collected. 30 μl aliquots were used for assay of the oxidase activating potency in the cell-free system described in the legend of table 1, in the presence and absence of 70 μg of the Q1 fraction. The oxidase activity was revealed by a screening assay with NBT as electron acceptor. Only in the presence of Q1 was an active peak evidenced due to the synergistic effect of Q1. This peak was eluted between 200 and 280 mM NaCl (fig.1). The experiment demonstrates that synergism is a means of tracing, during the course of purification, activating proteins which are not efficient per se on oxidase activation.

3.2. *Complementation studies with protein fractions recovered from crude cytosol by chromatography on Mono Q and Mono S*

The experiments described in section 3.1 indicated that oxidase activation required the combination of at least two protein fractions which could be separated by Mono Q chromatography. Due to the specific ion exchange properties of the Mono Q and Mono S columns, active proteins not retained on Mono Q are expected to be retained on the Mono S column at the same pH. On the basis of preceding experiments (cf. table 1 and fig.1), it can be predicted that synergistic oxidase activation would occur upon combination of proteins which bind to the Mono Q and the Mono S columns. The MQ fraction used was that collected by salt elution of the Mono Q column as described in fig.1. The MS fraction was obtained as described in the legend of table 2. In brief, 1 ml of crude cytosol containing 10 mg protein was diluted with 1 ml of 50 mM Hepes, pH 7.5, and was applied to a Mono S column. The column was first washed with 5 ml of 50 mM Hepes buffer, pH 7.5. Then a 0–500 mM NaCl linear gradient was applied, and 1-ml fractions were collected and assayed for oxidase activating potency in the absence and presence of the MQ fraction. An active MS peak whose activity was markedly enhanced by the MQ fraction was eluted between 260 and 340 mM NaCl (not shown). The synergistic effect of the MQ and MS fractions on oxidase activation is illustrated in table 2. 30 μl-aliquots from fractions MQ and MS, containing 9 and 3.6 μg protein, respectively, and corresponding to 10 μl of crude cytosol, i.e. 100 μg cytosol protein, were assayed for oxidase activating potency, as described in table 1. The oxidase activities elicited by the MS and MQ fractions tested separately were moderate. However, when the two fractions were combined, the elicited oxidase activity was 2.4 times as high as the arithmetic sum of the oxidase activities elicited by the MS and MQ fractions tested separately; the value attained (10.7 nmol $O_2^{\cdot -} \cdot min^{-1}$) was close to that obtained with the corresponding amount of cytosol (12.7 nmol $O_2^{\cdot -} \cdot min^{-1}$).

Oxidase activating factors present in the MS and MQ fractions were titrated by keeping the amount of the

Table 2

Synergistic oxidase activation by combination of two protein fractions obtained by chromatography on Mono Q and Mono S

Fractions (μg protein)	$O_2^{\cdot -}$ formed (nmol · min^{-1})	Recovery (%)	Factor of enhancement
MS (3.6 μg)	2.1	16	–
MQ (9 μg)	2.3	18	–
MS (3.6 μg) + MQ (9 μg)	10.7	84	2.4
Cytosol (100 μg)	12.7	100	–

The MQ fraction (0.3 mg/ml) was obtained by chromatography on Mono Q as described in fig.1. The MS fraction was obtained as follows. 10 mg of bovine neutrophil cytosol in 1 ml PBS was diluted twice with 50 mM Hepes, pH 7.5, and injected into a Mono S column (HR 5/5, 1 ml bed vol.) equilibrated with the same buffer. After recovery of the pass-through fraction, a linear 0–500 mM NaCl gradient was applied. 1-ml fractions were collected, and 30-μl aliquots were screened in the absence or presence of 30 μl of the MQ fraction (9 μg protein) for oxidase activating potency, using the same cell-free system as in table 1 (cf. also section 2) and NBT as electron acceptor. Fractions 12–14 eluted by the linear NaCl gradient (not shown) were found to be efficient in oxidase activation in the presence of the MQ fraction. These fractions were pooled. This pool was referred to as fraction MS and corresponded to 360 μg protein in 3 ml. For a more accurate assessment of the oxidase activating potency, 30 μl of fractions MQ and MS (corresponding to 10 μl of crude cytosol, i.e. 100 μg protein) were assayed under optimal conditions either separately or combined, in the cell-free system as described in table 1

MS fraction at the fixed value of 3.6 μg protein in 30 μl and varying the MQ fraction from 0 to 21 μg protein in 0 to 70 μl (fig.2). The plots of the oxidase activity vs the added MQ fraction showed a saturation plateau for volumes of the MQ fraction higher than 40 μl. As both the MQ and MS fractions were derived from 1 ml of cytosol, and were diluted to the same extent, it can be calculated from the endpoint of the titration curve (arrow in fig.2) that the activating factors of the two fractions are present in cytosol in a quasi-optimal stoichiometry.

4. DISCUSSION

Up to now, the multiplicity of the cytosolic factors involved in the activation of the membrane-bound oxidase of neutrophils has been documented by the finding of multiple active peaks recovered by chromatography of crude cytosol [8,14–17], and by complementation studies using cytosol from neutrophils of patients suffering from an autosomal recessive form of chronic granulomatous disease [17–21]. Yet the number of these factors and their mechanism of action is far from being clear.

Results reported in the literature are sometimes difficult to assess because the conditions of oxidase activation are not optimized, in particular with respect to the concentration of arachidonic acid used. In fact, under non-optimized conditions, enhancement of oxidase activation by a combination of different cytosolic fractions can arise from underestimation of the activation potencies of the fractions used. On the other hand, the propensity of cytosolic proteins to form aggregates might explain the multiplicity of active peaks reported in literature.

Using optimal conditions for assay, we have resolved bovine neutrophil cytosol by ion-exchange chromatography into two distinct active fractions. These fractions assayed separately were moderately efficient in promoting oxidase activation. However, when combined, their activating potency was synergistically enhanced. Synergism reported here was always observed under optimal conditions of oxidase activation. The protocols described in this paper to detect active cytosolic fractions can be used for studies aimed at the purification of the cytosolic factors required for oxidase activation.

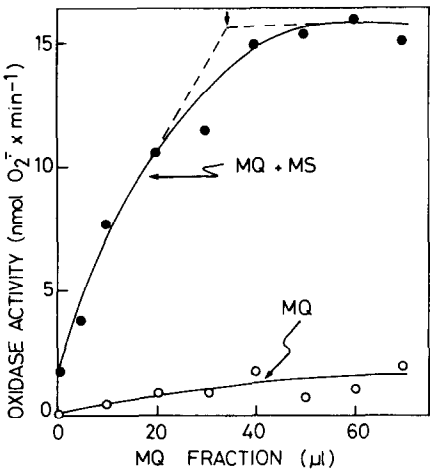

Fig.2. Titration of the oxidase activating potency of the MQ fraction by complementation with the MS fraction. The MS fraction was recovered as detailed in table 2. A fixed amount of this MS fraction (3.6 μg in 30 μl) was incubated for 10 min with increasing concentrations of the MQ fraction (corresponding to vols of 0–70 μl and protein amounts of 0–21 μg), in the cell-free system described in table 1. The elicited oxidase activity was further assayed by the SOD-inhibitable reduction of cytochrome *c*. In fig.2, the oxidase activity expressed as nmol $O_2^{\cdot-}$ formed·min^{-1}, resulting from the combination of MQ and MS fractions, is plotted against the volume of the MQ fraction used. As a control, the oxidase activity elicited by the MQ fraction alone is given as a function of the amount of the MQ fraction used. The endpoint of the titration is indicated by an arrow; it corresponds to 35 μl of the MQ fraction, which is close to the fixed volume of the MS fraction, namely 30 μl.

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