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Activation of $O_2^{\cdot -}$ Generating Oxidase of Bovine Neutrophils in a Cell-Free System. Interaction of a Cytosolic Factor with the Plasma Membrane and Control by G Nucleotides†

Erzsebet Ligeti,‡ Marianne Tardif, and Pierre V. Vignais*
Laboratoire de Biochimie, Département de Recherche Fondamentale, Centre d'Etudes Nucléaires, 85X, 38041 Grenoble Cedex, France

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ABSTRACT: Activation of the $O_2^{\cdot -}$-generating oxidase of bovine neutrophils was studied in a cell-free system, consisting of a particulate fraction enriched in plasma membrane, cytosol, arachidonic acid, and the nonhydrolyzable nucleotide GTP-γ-S. Activation of the membrane-bound oxidase was accompanied by the disappearance of the activating factor from the cytosol. Above a cytosol to membrane ratio of 25, the excess of added cytosolic factor remained in active state in the soluble fraction. The process could be partially reversed by serum albumin. Disappearance of the cytosolic factor was promoted by unsaturated long-chain fatty acids, but not by saturated ones, and occurred not only in the presence of GTP-γ-S but also in the presence of GDP-β-S or in the absence of Mg ions, although in the latter cases activation of $O_2^{\cdot -}$ production was seriously impaired. This suggests that the disappearance of the activating factor from the cytosol and the triggering effect of GTP-γ-S are related, but distinct, events in the oxidase activation process. The disappearance of the activating factor from cytosol can be explained by translocation of the cytosolic factor to the membrane fraction. Yet under some conditions, including the presence of GDP-β-S or EDTA, inactivation was prevailing and could be an alternative explanation for the results. Specific binding of radiolabeled GTP-γ-S could be demonstrated both in the membrane and in the cytosolic fractions. Although a substantial amount of GTP-γ-S was able to bind to the membrane proteins, its effect on oxidase activation was moderate compared to that of GTP-γ-S bound to cytosolic proteins. Oxidase activation was correlated with the binding of GTP-γ-S to cytosolic proteins in a range of concentrations from approximately 0.3 to 2.5 μM GTP-γ-S, with a GTP-γ-S concentration of $\simeq 1$ μM corresponding to the half-maximal oxidase activation. A GTP-binding protein of $M_r = 23\,000$ was detected in cytosol; its function in oxidase activation remains to be assessed.

The enzyme responsible for the respiratory burst in phagocytosing cells is a NADPH-specific oxidase located in plasma membrane. It catalyzes the one-electron transfer from NADPH to O_2 [for review see Rossi (1986) and Bellavite (1988)]. It is dormant in resting cells and is activated by a number of extracellular stimuli. A breakthrough in the study of the mechanism of oxidase activation was the development of a cell-free system of activation. This technique has been applied to macrophages and neutrophils of various species (Bromberg & Pick, 1984, 1985; McPhail et al., 1985; Curnutte, 1985; Gabig et al., 1987; Ligeti et al., 1988; Tanaka et al., 1988). It consists of incubation of a membrane fraction enriched in plasma membrane with cytosol in the presence of long-chain unsaturated fatty acids (Seifert & Schultz, 1987; Cox et al., 1987; Curnutte, 1985; Gabig et al., 1987; Ligeti et al., 1988) or SDS[1] (Bromberg & Pick, 1985; Pick et al., 1987; Cox et al., 1987; Babior et al., 1988). Both the rate of activation and the maximal oxidase activity achieved were found to depend on the amount of cytosol present during the incubation (Ligeti et al., 1988). This behavior suggested that there is a stoichiometric reaction between membranous and cytosolic components. Furthermore, the observation that the $O_2^{\cdot -}$-producing capacity of the membrane fraction persists after its separation from the cytosol (Gabig et al., 1987; Doussière et al., 1988) implies that the oxidase undergoes a quasi-irreversible modification after the transition from the resting state to the activated state. The possibility of the formation of a complex between cytosolic and membrane proteins was raised (Sha'ag & Pick, 1988; Doussière et al., 1988; Tanaka et al., 1988), but neither the conditions of the translocation of the activating factor from the cytosol to the membrane nor its eventual dissociation from the membrane and reappearance in cytosol were rigorously tested.

Activation in the cell-free system is stimulated by GTP-γ-S and fluoride and inhibited by GDP or GDP-β-S and depletion

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‡ Permanent address: Department of Physiology, Semmelweis Medical University, P.O. Box 259, 1444 Budapest, Hungary.

[1] Abbreviations: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PBS-Mg, PBS supplemented with 2.5 mM $MgCl_2$.

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of Mg^{2+} (Gabig et al., 1987; Seifert et al., 1986; Ligeti et al., 1988). These properties point to the participation of G protein(s) in the activation process. However, the insensitivity of the in vitro activation process to both cholera and pertussis toxins (Gabig et al., 1987; Seifert et al., 1986) suggests the involvement of G protein(s) other than G_s or G_i. Preliminary experiments have located the target sites of G nucleotides in the membrane fraction (Ligeti et al., 1988). Other experiments where guanine nucleotides were bound separately to membrane and cytosol argued for a predominant interaction with cytosolic proteins (Doussière et al., 1988), and binding of G nucleotides to a cytosolic activating factor followed by translocation of this complex to the membrane was considered.

The aims of the present work conducted with a cell-free system from bovine neutrophils were (1) to study the conditions that govern the translocation of the soluble activating factor from cytosol to the membrane fraction, (2) to explore in parallel the binding of G nucleotides to cytosol and membrane proteins and the subsequent modifications of the activation process, and (3) to clarify the relationship between the soluble activating factor and the G proteins of the cytosol.

Materials and Methods

Materials. Fatty acids and ferricytochrome *c* (horse heart, grade VI) were purchased from Sigma; NADPH, superoxide dismutase, and guanine nucleotides from Boehringer; and Percoll and Sephadex G-50 fine from Pharmacia. Ready value liquid scintillation cocktail was purchased from Beckman and fatty acid free albumin from Miles. [^{35}S]GTP-γ-S was obtained from New England Nuclear, and Centrikon P10 membrane filters were from Amicon. Fatty acids were dissolved in absolute ethanol and stored at −80 °C under N_2 until used.

Preparation of Cytosol and Membrane Fractions. Polymorphonuclear neutrophils were prepared from calf blood according to the procedure described by Morel et al. (1985). The cells obtained from 5 L of blood were resuspended in 20 mL of phosphate-buffered saline (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. Disruption of the cells was achieved by ultrasonic treatment for 4 × 15 s at 2–4 °C with a Branson sonifier at 40-W output. Nondisrupted cells, as well as nuclei and granular material, were eliminated by centrifugation at 6600*g* for 10 min in a Sorvall rotor at 4 °C. The resulting supernatant was spun for 60 min at 130000*g* in a Beckman 40 rotor. The 130000*g* pellet was resuspended in 9 mL of PBS plus 1 mM EGTA by sonication for 4 × 15 s at 0 °C at 40-W output. A previous analysis (Doussière et al., 1985) showed that this particulate fraction was enriched in plasma membrane and in the $O_2^{\cdot-}$-generating oxidase in its resting form. The protein concentration of this membrane fraction was adjusted to 8–10 mg/mL. To eliminate any membranous particles, the supernatant of this centrifugation was spun again for 60 min at 300000*g* in a Beckman SW65 rotor. The supernatant of the second high-speed centrifugation, containing on average 20 mg of protein/mL, was termed "cytosol" and stored at −80 °C. Under Results, the term cytosolic factor is used in singular to designate soluble proteins required for activation of the membrane-bound oxidase, although it is possible that more than one cytosolic factor is involved in activation.

Enzyme Assays. The reconstituted system for oxidase activation consisted of a membrane fraction (30–40 μg of protein), cytosol (0.4–0.8 mg of protein), and arachidonic acid (50–60 nmol) in a final volume of 100 μL of PBS supplemented with 2.5 mM $MgCl_2$ and 10 μM GTP-γ-S. This mixture was preincubated for 5 min at 25 °C before being added to the assay medium. When in vitro activation of the oxidase was carried out in batch, the above quantities were multiplied by a factor of 30–60.

The rate of $O_2^{\cdot-}$ production was determined as the superoxide dismutase sensitive portion of ferricytochrome *c* reduction. The assay was carried out at 22–25 °C in 2 mL of PBS supplemented with 2.5 mM $MgCl_2$, 0.1 mM ferricytochrome *c*, and 0.25 mM NADPH. The reaction was initiated by addition of 100 μL of the preincubation mixture. The rate of absorbance change was recorded at 550-nm wavelength in a Hitachi U-2000 spectrophotometer. In control assays, it was checked that reduction of cytochrome *c* was linear for at least 6–8 min. After a linear phase of 2–4 min, 50 μg of superoxide dismutase was added, and the recording continued for a further 2–4 min. The difference between the rates before and after addition of SOD was regarded as the rate of $O_2^{\cdot-}$ production. In all the reported experiments the SOD-insensitive fraction of cytochrome *c* reduction was lower than 5%. Specific activities are referred to as $O_2^{\cdot-}$ formed min^{-1} (mg of membrane protein)$^{-1}$.

Binding of [^{35}S]GTP-γ-S to Membrane and Cytosolic Fractions. The method described by Northup et al. (1982) was used with minor modifications. Membrane or cytosolic fractions were incubated in a final volume of 100 μL of PBS supplemented with 25 mM $MgCl_2$, 1.7 mM ATP, [^{35}S]-GTP-γ-S (600 000–800 000 cpm/100 μL), and varying concentrations of unlabeled GTP-γ-S. Incubation was carried out for 45 min at 30 °C, which are conditions of full equilibrium between bound and free [^{35}S]-GTP-γ-S. At the end of the incubation period, separation of the protein-bound [^{35}S]-GTP-γ-S from the free nucleotide was obtained by centrifugation–filtration of 100 μL of incubation mixture through Sephadex G-50 (fine) contained in tuberculin syringes of 1-mL volume inserted in conical centrifuge tubes according to the method of Penefsky et al. (1977). The protein content of the effluent from each column was determined separately; approximately 80% of the deposited protein was recovered. Radioactivity was measured by scintillation counting. Counts were corrected for unspecific binding determined in the presence of 1 mM GTP-γ-S.

Binding of [^{35}S]GTP-γ-S to Separated Proteins after Electrophoretic Transfer to Nitrocellulose. The technique described by Bokoch and Parkos (1988a,b) was applied with slight modifications. Membrane or cytosolic proteins were dissolved in sample buffer and separated by SDS–PAGE in 10% gels following the method of Laemmli (1970). After electrophoretic separation, the gel was washed for 15 min in 25 mM Tris and 192 mM glycine, pH 8.5. Transfer to 0.2-μm pore size nitrocellulose sheets was performed by electrophoresis for 3 h at 12 V, 1 A at 4 °C, using the above buffer without SDS. After transfer, the blots were rinsed for 15 min in PBS and incubated for 1 h at 22 °C in 10 mL of PBS containing 5 mM $MgCl_2$, 1.7 mM ATP, 1 μM GTP-γ-S, and 50 μCi [^{35}S]GTP-γ-S. Subsequently, blots were washed twice in distilled water, dried, and exposed on Fuji RX film. Autoradiograms were routinely developed after 5 days.

Micellaneous. Protein content was determined by the Lowry method, using bovine serum albumin as standard. Data represented in tables and figures are results of a typical experiment of at least five similar experiments that gave similar results.

Results

Activation of the Membrane-Bound Oxidase in the Reconstituted System Is Accompanied by Disappearance of the Oxidase Activating Potency from the Cytosolic Fraction. In

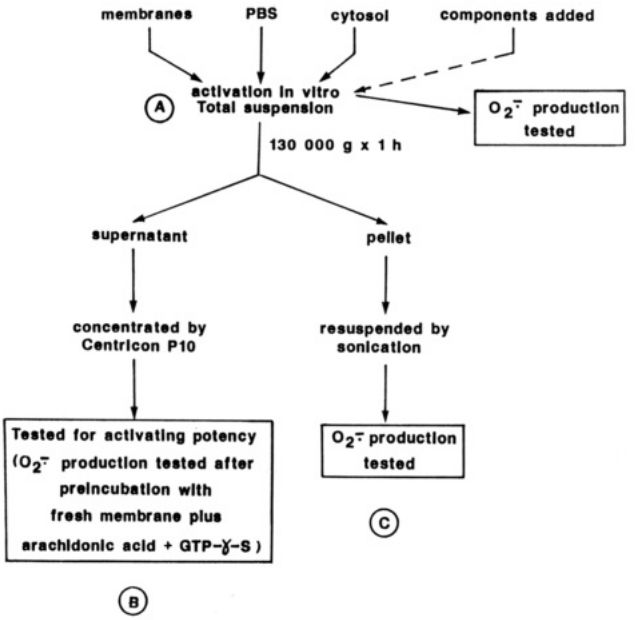

FIGURE 1: Experimental scheme of the assay of redistribution of the cytosolic factor.

a previous paper dealing with a cell-free oxidase activation system consisting of membranes and cytosol of bovine neutrophils, it was found that cytosol contains an activating factor (Ligeti et al., 1988) which was able to promote under appropriate conditions a quasi-irreversible activation of the $O_2^{\bullet-}$-generating oxidase (Doussière et al., 1988). To explore in more detail the parameters that were involved in the control of the membrane-bound oxidase activation by the soluble activating factor, the experimental procedure schematized in Figure 1 was routinely used. Membranes (1 mg) and cytosol (20 mg), prepared as described under Materials and Methods, were incubated in a final volume of 8 mL of PBS-Mg at room temperature. Different substances (fatty acids, G nucleotides), whose action was to be investigated, were added to the incubation mixture. After 5 min, the oxidase activity of the suspension was tested by transferring a small aliquot (100 μL) to a spectrophotometric cuvette containing cytochrome *c* and NADPH (values A). After 6 min, the remaining mixture was centrifuged at 130000*g* for 1 h. The sedimented membranes were resuspended in PBS by ultrasonic irradiation for 3 × 10s, and their $O_2^{\bullet-}$-producing capacity was tested immediately without further addition in the spectrophotometric cuvette (values C). The supernatant consisting of diluted cytosol was concentrated on a Centricon P10 membrane to the original volume of the cytosol fraction used for the in vitro activation. Concentration lasted for 3–4 h at 4 °C. The activating capacity of the cytosolic concentrate was assessed after incubation with fresh membranes, arachidonic acid, and GTP-γ-S according to the routine procedure described under Materials and Methods (values B).

Figure 2 illustrates the results of a typical experiment. Provided that arachidonic acid was included in the incubation step, activation of the membrane-bound NADPH-specific oxidase was achieved (bars in Figure 2A), and the membranes retained part of their elicited oxidase activity after separation from the cytosol (data not shown); however, the reconcentrated cytosolic fraction was unable to activate fresh membranes (curve in Figure 2A). When incubation was carried out in the absence of arachidonic acid, the membrane-bound oxidase was not activated (bar in Figure 2B), and the soluble supernatant fraction retained a strong activating capacity (curve in Figure 2B). Thus, the cytosolic factor necessary for the in

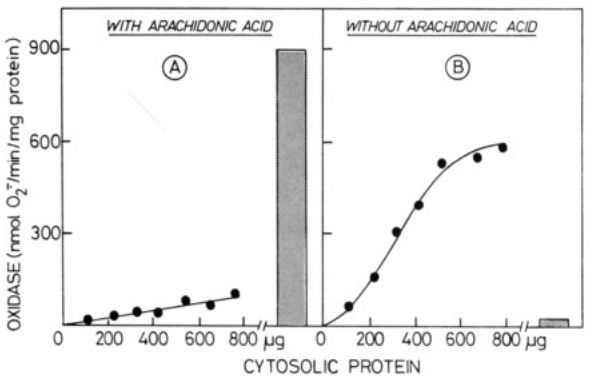

FIGURE 2: Disappearance of the cytosolic activating factor after incubation of cytosol with the membrane fraction in the presence (A) or absence (B) of archidonic acid. Membranes (890 μg of protein) and cytosol (15 mg of protein) were mixed and incubated at room temperature in a final volume of 2.5 mL of PBS containing 2.5 mM $MgCl_2$ and 20 μM GTP-γ-S. The medium was supplemented with 2 μmol of arachidonic acid in (A) but not in (B). Production of $O_2^{\bullet-}$ by a 100-μL aliquot of the suspension was tested after 5 min (shaded bars). After 6 min, centrifugation was started. Supernatants were concentrated to approximately 750 μL, and their activating capacity was tested after incubation with fresh membranes, as described under Materials and Methods, by using 30 μg of membrane protein and increasing amounts of cytosolic protein up to 800 μg (curves ●). The $O_2^{\bullet-}$-producing activity of the oxidase is given in nmol of $O_2^{\bullet-}$ min^{-1} (mg of membrane protein)$^{-1}$.

vitro activation of the $O_2^{\bullet-}$-producing oxidase resisted all the manipulations detailed above and remained active as shown in the experiment conducted in the absence of arachidonic acid (Figure 2B). This first set of results indicates that the process of activation is related to the disappearance of the cytosolic factor from the supernatant.

In all experiments described hereafter, the dependence of oxidase activation of fresh membranes on the amount of added cytosolic concentrates was studied by the procedure detailed in Figure 1. For the sake of simplicity, only the maximal values of oxidase activity are represented in tables and figures. The results assembled in each table were obtained with the same membrane fraction, and the intactness of the test membranes was routinely verified with the original cytosol.

In a first series of experiments we tested whether the disappearance of the oxidase activating factor from the cytosol could be evoked with any fatty acid or whether it was specific for arachidonic acid and other long-chain unsaturated fatty acids. The incubation was therefore carried out in the presence of different saturated and unsaturated fatty acids, which—according to our previous experience (Ligeti et al., 1988)—were either inefficient or nearly as efficient as arachidonic acid in the activation of the NADPH-specific oxidase. As summarized in Table I, the $O_2^{\bullet-}$-producing activity of the membranes and activating capacity retained in the supernatant fraction showed almost perfect mirror symmetry and the activation of the membrane-bound oxidase was strictly correlated with the disappearance of the activating factor from the cytosol.

In the next experiment, a fixed amount of membrane was incubated with increasing amounts of cytosol (Figure 3). When the ratio of cytosolic protein to membrane protein was below 25—a ratio that gives nearly optimal activation—the cytosolic factor disappeared almost completely from the soluble fraction. In contrast, with a cytosol to membrane ratio higher than 25, an increasing activating capacity was revealed in the cytosolic concentrate (up to 330 nmol of $O_2^{\bullet-}$ min^{-1} mg^{-1}) after the elicited oxidase activity in the reconstituted system has reached the maximal plateau value of 750 nmol of $O_2^{\bullet-}$ formed min^{-1} mg^{-1}. Thus, the extent of activation of the oxidase seems

Table I: Effect of Various Fatty Acids on the Distribution of the Cytosolic Factor between Membranes and Cytosolic Fractions[a]

fatty acids (concn)[b]	activity of reconstituted system (A),[c] $O_2^{\cdot-}$ formed[d]	activating potency of concd supernatant (B),[c] $O_2^{\cdot-}$ formed[d]	activity of separated membranes (C),[c] $O_2^{\cdot-}$ formed[d]
		Experiment 1	
arachidonic acid (0.8 mM)	970	42	160
stearic acid (3 mM)	36	295	3
palmitic acid (2 mM)	29	830	8
		Experiment 2	
palmitoleic acid (4 mM)	883	37	60
elaidic acid (4 mM)	231	406	18
palmitic acid (2 mM)	56	790	6

[a] Experimental conditions were the same as those detailed in the legend of Figure 2 and under Materials and Methods. [b] The concentrations of fatty acids reported in this table are those corresponding to maximal oxidase activation. [c] A, B, and C refer to the scheme of Figure 1. [d] $O_2^{\cdot-}$ production is expressed as nmol min^{-1} (mg of membrane protein)$^{-1}$.

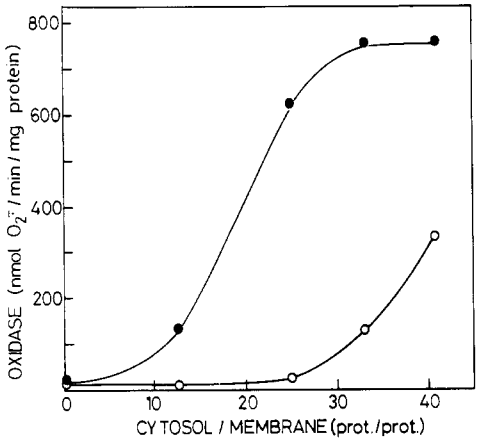

FIGURE 3: Demonstration of saturable binding sites in the neutrophil membrane fraction for the cytosolic factor. Membranes (0.36 mg of protein) were incubated with increasing amounts (4.5–14.8 mg of protein) of cytosol and 800 nmol of arachidonic acid in a final volume of 1.1 mL of PBS containing 2.5 mM Mg^{2+} and 20 μM GTP-γ-S. The oxidase activity [nmol of $O_2^{\cdot-}$ min^{-1} (mg of membrane protein)$^{-1}$] of the activated membranes (●) and the residual activating capacity of the supernatant (tested with a fresh membrane preparation) (○) are represented as a function of cytosolic protein to membrane protein ratio.

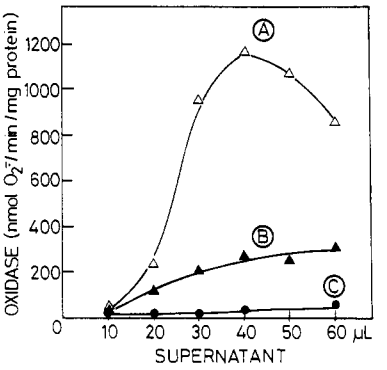

FIGURE 4: Effect of bovine serum albumin on the redistribution of the cytosolic factor. Membranes (620 μg of protein) and cytosol (10.3 mg of protein) were incubated for 6 min in a final volume of 3.2 mL of PBS containing 2.5 mM of $MgCl_2$ and 20 μM GTP-γ-S. Arachidonic acid (1.6 μmol) was present in the three samples. Fatty acid free bovine serum albumin at a final concentration of 0.1 mM was added either before (△) or 5 min after (▲) arachidonic acid. Control samples (●) did not contain albumin. Membranes were sedimented by centrifugation. The supernatants were concentrated to 850 μL, and their oxidase activating capacity was tested after incubation with fresh membranes, as described under Materials and Methods.

to be limited by the availability of the cytosolic factor. On the other hand, the disappearance of the cytosolic activating factor is a function of the quantity of membrane present, suggesting a stoichiometric relationship between the two components. In a complementary experiment in which a constant cytosolic protein to membrane protein ratio of 20 was used and the final volume of the incubation medium was varied by a factor of 20, the disappearance of the cytosolic factor from the soluble fraction remained almost complete up to a dilution of 5 times, and the incubation mixture had to be diluted 20 times to detect a significant activating capacity in the supernatant (data not shown).

Disappearance of the Cytosolic Factor from the Soluble Fraction Is Reversible. The reversible nature of the disappearance of the activating factor from the cytosol was revealed by including fatty acid free bovine serum albumin in the incubation mixture. Figure 4 summarizes the results of a typical experiment. When albumin was added to the incubation mixture prior to arachidonic acid, oxidase activation was prevented, and the separated supernatant was able to elicit $O_2^{\cdot-}$ production in fresh membranes at a maximal rate of 1150 nmol min^{-1} (mg of protein)$^{-1}$ (curve A of Figure 4). In a control experiment performed in the absence of albumin, the oxidase of the reconstituted system was activated to a maximal value, whereas the activating potency of the supernatant was very low (curve C, Figure 4). When albumin was added 5 min after arachidonic acid (in which case activation of the membrane-bound oxidase has already occurred) and oxidase activity was assayed after addition of albumin, a rapid fall of $O_2^{\cdot-}$ production by the activated membranes was observed. Two minutes after the addition of albumin, only 10% of the original oxidase activity could be detected, and the separated supernatnant was able to activate fresh membranes to produce $O_2^{\cdot-}$ ions at a maximal rate of 300 nmol min^{-1} (mg of protein)$^{-1}$. This result indicates that part of the activity of the cytosolic factor can be recovered in the soluble medium, provided that arachidonic acid is trapped by serum albumin.

Does the Disappearance of the Activating Factor from the Cytosol Depend on G Nucleotides? The possibility that the disappearance of the cytosolic activating capacity depends on G nucleotides was tested in the experiment of Table II. Incubation was carried out either in the presence of 20 μM GTP-γ-S, or 1 mM GDP-β-S, or 20 μM GTP-γ-S plus 10 mM EDTA. As previously shown (Ligeti et al., 1988), GTP or GTP analogues, like GTP-γ-S, markedly potentiate the oxidase activity in a reconstituted system. This was confirmed in the present experiment together with the fact that GDP-β-S was inefficient and that EDTA, a chelator which removes Mg^{2+}, impeded activation. In all cases, namely, in the presence of GTP-γ-S, GDP-β-S, and GTP-γ-S plus EDTA, the activating capacity disappeared from the cytosol. The possibility that GDP-β-S or EDTA present in the cytosolic concentrates could interfere with the assay itself was excluded on the following basis. First, it was checked that the activating capacity of the cytosol was not recovered by removing GDP-β-S from

Table II: Effect of GTP-γ-S, GDP-β-S, and EDTA on the Distribution of the Cytosolic Factor between Membrane and Soluble Fractions[a]

addition to preincubation mixture	activity of reconstituted system (A),[b] $O_2^{\cdot-}$ formed[c]	activating potency of concd supernatant (B),[b] $O_2^{\cdot-}$ formed[c]	activity of separated membranes (C),[b] $O_2^{\cdot-}$ formed[c]
GTP-γ-S	637	60	189
GDP-β-S	177	65	23
GTP-γ-S + EDTA	258	68	34

[a] Membranes (1 mg of protein) and cytosol (18 mg of protein) were preincubated with 2.4 μmol of arachidonic acid in a final volume of 3 mL of PBS containing 2.5 mM Mg^{2+} and either 20 μM GTP-γ-S, 1 mM GDP-β-S, or 20 μM GTP-γ-S plus 10 mM EDTA (cf. Materials and Methods). After separation by high-speed centrifugation, the supernatants were concentrated to 850 μL and passed through Sephadex G-50 columns. Membranes were resuspended in 300 μL of PBS. [b] A, B, and C refer to the scheme of Figure 1. [c] $O_2^{\cdot-}$ production is expressed as nmol min^{-1} (mg of membrane protein)$^{-1}$.

the concentrated supernatants by filtration through a Sephadex G-50 column or by counteracting the effect of EDTA by an increased concentration of Mg^{2+}. Second, it was verified that the concentrated supernatants passed through the gel filtration column did not interfere with the oxidase activation achieved with fresh membranes and fully active cytosol. It can therefore be concluded that the disappearance of the oxidase activating potency from cytosol does not obligatorily depend on the presence of guanine nucleotides. Yet GTP-γ-S is required for full elicitation of oxidase activity. These results prompted us to investigate the location of G proteins in bovine neutrophils and their function in the process of oxidase activation. Through the use of the nonhydrolyzable analogue GTP-γ-S in its radiolabeled form, it was indeed possible to quantitate both ligand binding and its effect on oxidase activation in the same sample, under conditions where the GTP-binding protein is postulated to be fixed in its active form.

Binding of [^{35}S]GTP-γ-S to Neutrophil Cytosolic Proteins. Response of the Membrane-Bound Oxidase Activity to the Cytosolic Fraction Preloaded with GTP-γ-S. The cytosolic fraction was freed from endogenous nucleotides by filtration through Sephadex G-50 and subsequently incubated with radiolabeled GTP-γ-S in the presence of various concentrations of the unlabeled nucleotide. Free and bound ligands were separated by gel filtration. The rate of association of GTP-γ-S to cytosolic proteins depended on the concentration of the ligand, the plateau being attained in approximately 45 min when 0.05 μM GTP-γ-S was used, but in only 4 min in the presence of 50 μM GTP-γ-S. In contrast, dissociation of radiolabeled bound GTP-γ-S was a slow process; in the presence of a 100-fold excess of unlabeled GTP-γ-S, 3% and 40% of bound ligand were released in 5 and 90 min, respectively. As the $O_2^{\cdot-}$ production stimulating activity of the cytosol was preserved even after a prolonged incubation at 30 °C, the binding studies were carried out for 45 min.

Binding of radiolabeled GTP-γ-S to cytosolic proteins exhibited a hyperbolic dependence on GTP-γ-S concentration (Figure 5A), saturation being approached around 30 μM. In four different preparations, the maximal amount of bound GTP-γ-S ranged between 45 and 80 pmol/mg of protein. GDP-β-S efficiently competed against GTP-γ-S for binding (Figure 5A). The cytosolic fractions containing increasing amounts of bound GTP-γ-S were tested for in vitro activation of the membrane-bound oxidase in the reconstituted system without further addition of any G nucleotides (Figure 5B). The dose effect curve showed a lack of response of oxidase activation up to 0.3 μM GTP-γ-S, despite a noticeable binding of GTP-γ-S to cytosolic proteins. A clear correlation between GTP-γ-S binding and oxidase activation was observed between 0.3 and 2.5 μM GTP-γ-S, the half-maximal GTP-γ-S-dependent stimulation of the oxidase being attained with about 1 μM GTP-γ-S. For GTP-γ-S concentrations higher than 10 μM, a small, but significant, increase in oxidase activation was observed; for example, raising the concentration of GTP-γ-S

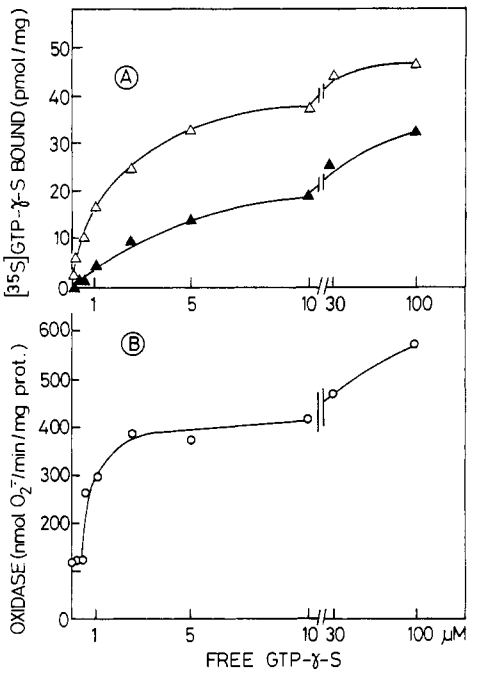

FIGURE 5: Binding of [^{35}S]GTP-γ-S to cytosolic proteins and effect on oxidase activation. (A) Binding of [^{35}S]GTP-γ-S to cytosolic proteins in the absence (△) or presence of 100 μM GDP-γ-S (▲) was carried out as detailed under Materials and Methods. (B) Oxidase activating effect of cytosol loaded with [^{35}S]GTP-γ-S was tested after 5 min of incubation with membranes and arachidonic acid, but without further addition of GTP-γ-S (○). When 10 μM GTP-γ-S was added, the maximal rate of $O_2^{\cdot-}$ production was 990 nmol min^{-1} (mg of membrane protein)$^{-1}$.

from 30 to 100 μM resulted in an increase of the elicited oxidase activity from 470 to 570 nmol $O_2^{\cdot-}$ min^{-1} (mg of membrane protein)$^{-1}$. Along this line, it is noteworthy that maximal oxidase activation was not fully achieved by using GTP-γ-S-saturated cytosol. In fact, when the incubation mixture was further supplemented with 10 μM GTP-γ-S, a higher level of oxidase activity was elicited, up to 900–1000 nmol of $O_2^{\cdot-}$ min^{-1} (mg of membrane protein)$^{-1}$.

Binding [^{35}S]GTP-γ-S to Neutrophil Membrane Proteins. Dependence of Oxidase Activity on GTP-γ-S Bound to Membrane and GTP-γ-S Bound to Cytosol. Radiolabeled GTP-γ-S was found to bind to the membrane fraction more slowly than to cytosol. In the presence of 50 μM GTP-γ-S, the maximal value of binding was only attained in about 8 min, whereas at 0.05 μM GTP-γ-S the plateau was not yet fully achieved after 45 min. When GTP-γ-S binding was explored in the concentration range of 0.1–100 μM, incubation was carried out for 45 min. From the GTP-γ-S binding curve (data not shown), 50% saturation at about 1 μM GTP-γ-S could be calculated, and the saturation plateau was attained at 10 μM GTP-γ-S. In four different preparations the maximal amount of GTP-γ-S bound to membrane ranged between 150 and 250 pmol/mg of protein, an amount 2–5 times higher

Table III: Dependence of Oxidase Activation on GTP-γ-S Bound to Membrane and Cytosolic Proteins and on Addition of GTP-γ-S to the Reconstituted System[a]

membrane and cytosolic fractions depleted in nucleotides or saturated in [^{35}S]GTP-γ-S	added GTP-γ-S (μM)	$O_2^{\bullet-}$ production [nmol min^{-1} (mg of membrane protein)$^{-1}$]
depleted membranes + depleted cytosol	none	193
depleted membranes + depleted cytosol	10	614
depleted membranes + GTP-γ-S-saturated cytosol	none	416
depleted membranes + GTP-γ-S-saturated cytosol	10	705
GTP-γ-S-saturated membranes + depleted cytosol	none	291
GTP-γ-S-saturated membranes + depleted cytosol	10	605
GTP-γ-S-saturated membranes + GTP-γ-S-saturated cytosol	none	385
GTP-γ-S-saturated membranes + GTP-γ-S-saturated cytosol	10	591

[a] The membrane and the cytosolic fractions previously depleted of their endogenous nucleotides by gel filtration were separately loaded with [^{35}S]GTP-γ-S by incubation with 100 μM [^{35}S]GTP-γ-S for 5 min at 30 °C. Separation of the free and bound [^{35}S]GTP-γ-S was by gel filtration. [^{35}S]GTP-γ-S and 182 pmol of [^{35}S]GTP-γ-S/mg of protein, respectively. The reconstituted system consisted of different combinations of cytosol and membranes depleted in nucleotide or saturated in [^{35}S]GTP-γ-S. The medium was supplemented with arachidonic acid, $MgCl_2$ (cf. Materials and Methods), and, when indicated, 10 μM GTP-γ-S. After preincubation, $O_2^{\bullet-}$ generation was assayed as described under Materials and Methods.

than that determined for cytosol.

The investigation of the effect of GTP-γ-S bound to the membrane on the process of activation of the oxidase in the in vitro system was seriously restricted by the poor stability of membrane preparations at room temperature. Although no loss in the ability to bind GTP-γ-S could be detected after either incubation for several hours at 30 °C or repeated freezing and thawing of membranes, both conditions abolished $O_2^{\bullet-}$ production. For this reason, the dependence of oxidase activation on the presence of GTP-γ-S bound to the membrane fraction was studied with membrane loaded for a brief period of time (about 5 min) with high concentrations of GTP-γ-S. A parallel study was carried out with GTP-γ-S-loaded cytosol, and the effect of added GTP-γ-S was also analyzed (Table III). Due to some variations in the recovery of proteins by gel filtration of either membranes or cytosol, the most comparable data are the paired values obtained in presence or absence of GTP-γ-S added to the incubation mixture. In the experiment summarized in Table III, the membrane suspension was divided into two fractions. One of the fractions was incubated with 100 μM radiolabeled GTP-γ-S and filtered on Sephadex G-50. This treatment resulted in the binding of 182 pmol of GTP-γ-S/mg of membrane protein. The other fraction was kept in ice without GTP-γ-S. When the oxidase in both membrane fractions was activated in the in vitro system in the presence of 10 μM GTP-γ-S and the depleted cytosol, almost identical values of $O_2^{\bullet-}$ production [nmol min^{-1} (mg of membrane protein)$^{-1}$] were obtained, namely, 614 and 605 (lines 2 and 6 of Table III). Omitting GTP-γ-S from the activation mixture seriously lowered $O_2^{\bullet-}$ production, down to 193 nmol of $O_2^{\bullet-}$ min^{-1} mg^{-1} in the case of control membranes (line 1 of Table III) and to 291 nmol of $O_2^{\bullet-}$ formed min^{-1} mg^{-1} for GTP-γ-S-saturated membranes (line 5 of Table III). Yet the oxidase activity of GTP-γ-S-saturated membranes significantly exceeded that of control membranes (291 vs 193 nmol of $O_2^{\bullet-}$ formed min^{-1} mg^{-1}) (lines 5 and 1 of Table III).

In the second part of the experiment, both membrane fractions were activated by GTP-γ-S-saturated cytosol. When the effect of GTP-γ-S-saturated membranes was compared to that of GTP-γ-S-saturated cytosol on oxidase activity, the former was less effective (291 vs 416 mol of $O_2^{\bullet-}$ formed min^{-1} mg^{-1}) (lines 5 and 3 of Table III). However, the combination of GTP-γ-S-saturated membranes with GTP-γ-S-saturated cytosol did not augment the extent of activation (385 vs 416 nmol of $O_2^{\bullet-}$ min^{-1} mg^{-1}). Yet, in this case, oxidase activation was significantly enhanced (up to 591 nmol of $O_2^{\bullet-}$ min^{-1} mg^{-1}) by supplementing the incubation mixture with 10 μM free GTP-γ-S (line 8 Table III). Taken together, the above results indicate that although the binding of GTP-γ-S to the membrane fraction promotes oxidase activation to some extent, predominant effects are due to GTP-γ-S bound to the cytosolic fraction. They also stress the point that the presence of free GTP-γ-S in the incubation mixture (in addition to the GTP-γ-S bound either to the membrane fraction or to the cytosolic fraction or to both) is required for maximal activation of the oxidase.

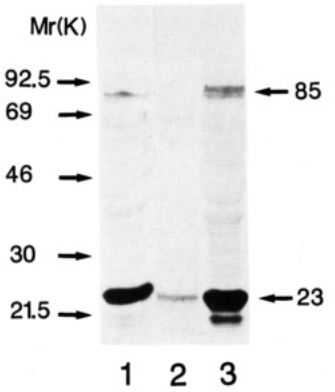

FIGURE 6: Detection of membrane and cytosolic GTP-binding proteins on nitrocellulose blots. Membrane and cytosolic proteins were separated by SDS–PAGE with 10% polyacrylamide and transferred onto nitrocellulose sheets. After incubation with [^{35}S]GTP-γ-S and washing as described under Materials and Methods, the sheets were autoradiographed. Lanes 1, 2, and 3 correspond to 400 μg of cytosolic protein, 350 μg of membrane protein, and 730 μg of cytosolic protein, respectively. The positions of the molecular weight standards (Amersham) are indicated: trypsin inhibitor (M_r = 21 500); carbonic anhydrase (M_r = 30 000); ovalbumin (M_r = 46 000); bovine serumalbumin (M_r = 69 000); and phosphorylase *b* (M_r = 92 500).

A scrutiny of major GTP-binding proteins in the membrane and cytosol fractions was attempted. After separation of proteins by SDS–PAGE and transfer to nitrocellulose sheets in the absence of SDS, the blots were treated with [^{35}S]-GTP-γ-S. A band of 23-kDa molecular mass was found to be highly labeled (Figure 6). Comparatively, in the membrane fraction, labeling of the 23-kDa protein was moderate. As the total binding of GTP-γ-S per milligram of protein was 2–5 times higher in the membrane than in the cytosolic fraction, it has to be supposed that the bulk of GTP-γ-S was fixed to other membrane proteins than the 23-kDa protein. Potential candidates are proteins in the 40–41-kDa molecular mass range, recently characterized as substrates for pertussis toxin (Murphy et al., 1987; Rotrosen et al., 1988; Uhing et al., 1987). These proteins might not have been detected by the present technique because of irreversible denaturation and loss of the GTP-binding ability during SDS–PAGE, as suggested

by Bokoch and Parkos (1988) and Bhullar and Haslam (1987).

Discussion

The mechanism of activation of the $O_2^{\bullet-}$-generating oxidase in neutrophils is a multistep process. Deciphering the identity of the components and the nature of the reactions involved in oxidase activation is now made possible through the use of a cell-free system consisting of a particulate fraction enriched in plasma membrane, a soluble cytosol, unsaturated long-chain fatty acids, and GTP (or GTP-γ-S). Clearly, a G-protein-dependent reaction is required for full expression of oxidase activation. The experiments described here provide further information on the dependence of oxidase activation on cytosolic factor(s) and on the role played by GTP in the activation process.

Disappearance of Activating Factor(s) from the Cytosol Occurs in Parallel with the Oxidase Activation Process. Through the use of a cell-free system, it has been possible to demonstrate clear parallelism between the activation of the $O_2^{\bullet-}$-producing oxidase of the plasma membrane and the disappearance of the cytosolic activating factor(s) from the soluble phase (Figures 2 and 3 and Table I). A critical event seems to be the interaction of fatty acids with some components of the membrane or the cytosol, as omission of fatty acids or the scavenging effect of albumin prevents the loss of the activating capacity. In contrast to recent findings (Tanaka et al., 1988), and in accordance with our previous results (Ligeti et al., 1988), unsaturated fatty acids, but not saturated ones, were effective in stimulating $O_2^{\bullet-}$ production. In the presence of albumin, a partial reversibility of these effects could be demonstrated (Figure 4).

As to the disappearance of the activating factor(s) from the cytosol, several mechanisms can be envisaged. The simplest mechanism is translocation of the cytosolic factor(s) to the membrane fraction and formation of a complex with the membrane-bound oxidase. This would be one of the steps of the activation process. In this context, the results of the experiments where the ratio of cytosolic protein to membrane protein (Figure 3) or the final volume of the incubation mixture was varied can be interpreted as indication for the presence in the membrane fraction of saturable binding sites with rather high affinity for the cytosolic factor(s). Although our results are compatible with this hypothesis, in the present state there is no unequivocal proof for it. An alternative and possibly complementary explanation is that one or more cytosolic factors are inactivated during the course of oxidase activation. Removal of an inhibitory membrane component by the cytosolic factor, with the consequent desinhibition of the oxidase activity, is another hypothesis that cannot be excluded. Which of these or other hypotheses provides the correct explanation for the observed phenomenon has to be decided in future experiments. Regardless of the exact mechanism disappearance of the activating capacity from the cytosol was always observed if an adequate fatty acid was present, and the type of the added G nucleotide or prevention of its binding to its target protein was, in this respect, without any influence (Table II). As the activation of the oxidase enzyme shows clear dependence on the species of G nucleotide present, we suggest that interaction of the cytosolic factor(s) and involvement of G nucleotides occur at distinct phases of the activation process.

Participation of G Nucleotides and G Proteins in the Activation Process. In the past few years much interest has been focused on the GTP-binding proteins of neutrophils. Pertussis toxin substrates were identified in human neutrophils as membrane proteins of 40 and 41 kDa (Uhing et al., 1987; Bokoch et al., 1988a,b; Goldsmith et al., 1987; Murphy et al., 1987; Rotrosen et al., 1988). In some membrane fractions, binding of GTP to 23–27-kDa proteins was also reported (Uhing et al., 1987; Bokoch & Parkos, 1988). Occurrence of 40- and 41-kDa GTP-binding proteins in the cytosolic fraction of neutrophil homogenates was attributed to the dissociation of the α subunits of membrane-bound heterotrimeric G proteins (Bokoch et al., 1988a,b; Rotrosen et al., 1988). Interaction with the chemotactic receptors was suggested as a function for these G proteins (Rotrosen et al., 1988; Gierschik & Jacobs, 1987). The role of smaller molecular weight G proteins remains unknown. In the experiments carried out on bovine neutrophils reported in this paper, GTP binding to both the membrane and cytosolic proteins was demonstrated. The extent of total GTP-γ-S binding to the membrane was in good agreement with data reported by Bokoch et al. (1988a,b) for human neutrophils, and the relative affinities for different G nucleotides and analogues were similar to those found in the case of various membrane-bound G proteins (Northup et al., 1982; Bokoch et al., 1984; Bhullar & Haslam, 1987; Bokoch & Parkos, 1988).

Saturation of specific binding sites in the membrane with GTP-γ-S had a moderate effect on oxidase activation in the reconstituted system. This is not the case for the G proteins of cytosol whose titration by GTP-γ-S resulted in a marked oxidase activation. The shape of the GTP binding curve relative to cytosol and that of the dose effect curve relative to oxidase activation (Figure 5A,B) slightly differ in that at low concentration of GTP-γ-S ($\simeq$0.3 μM) no activation of the oxidase was detected in spite of a clear increase in the amount of GTP-γ-S bound to cytosolic proteins. A likely explanation is that several GTP-binding proteins are present in the cytosol of neutrophils and are titrated in different ranges of GTP-γ-S concentrations. A GTP-binding protein specific for oxidase activation appears to be characterized by a K_d value of $\simeq$1 μM, and its response to GTP-γ-S would be specific for concentrations of GTP-γ-S ranging between 0.3 and 2.5 μM. Second, in the dose effect curve, the lack of response of activation to very low concentrations of GTP-γ-S (<0.1 μM) might be explained by the titration of one or several GTP-binding proteins showing very high affinity for GTP-γ-S but not being involved in the control of oxidase activation.

Binding of [^{35}S]GTP-γ-S to membrane and cytosolic proteins reached saturation in 4 and 8 min, respectively, provided that the ligand concentration was 50 μM, which is in large excess of the concentration needed to augment oxidase activity. At a lower concentration of [^{35}S]GTP-γ-S, still compatible with rapid expression of the oxidase activity, a significantly longer period of time was required for saturation to be attained. This apparent discrepancy between the time dependence of the oxidase activation and the binding of [^{35}S]GTP-γ-S to either membrane proteins or cytosolic proteins can be explained on the following basis. In the reconstituted system where membrane and cytosol are present together, the GTP-binding proteins involved in oxidase activation would assume a rapid change of conformation, resulting in acceleration of binding of [^{35}S]GTP-γ-S.

Maximal values of $O_2^{\bullet-}$ production could not be achieved when only protein-bound GTP-γ-S was present in the incubation mixture; in fact, supplementation with free GTP-γ-S resulted in a substantial increase of the activity of the enzyme (more than 25%). This increase could be due either to unmasking of additional GTP-γ-S binding sites or to a partial release of bound nucleotide from a GTP-binding protein during the activation process, possibly linked to a change of confor-

mation. Another possibility is that several cytosolic GTP-binding proteins are involved in oxidase activation and that, besides the relatively high affinity GTP-binding protein of $K_d \simeq 1$ μM, additional protein(s) of lower affinity may have a subsidiary function.

After separation of cytosolic proteins by SDS–PAGE and electrotransfer to nitrocellulose, a relatively abundant GTP-binding protein of 23 kDa was revealed (Figure 6). Further work is needed to determine whether this protein is involved in oxidase activation. In this context, it is noteworthy that small molecular weight G proteins have been recently reported in HL60 cells (Uhing et al., 1987) and human neutrophils (Bokoch & Parkos, 1988; Bokoch et al., 1988a,b); their function is still unknown.

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