Schulman, L. H., & Pelka, H. (1988) *Science 242*, 765–768.
Schulman, L. H., & Pelka, H. (1989) *Science 246*, 1595–1597.
Schulman, L. H., & Pelka, H. (1990) *Nucleic Acids Res.* (in press).
Schulman, L. H., Valenzuela, D., & Pelka, H. (1981) *Biochemistry 20*, 6018–6023.
Schulman, L. H., Pelka, H., & Susani, M. (1983) *Nucleic Acids Res. 11*, 1439–1455.
Schulman, L. H., Pelka, H., & Leon, O. (1987) *Nucleic Acids Res. 15*, 10523–10530.
Somerville, C. R., & Ahmed, A. (1977) *J. Mol. Biol. 111*, 77–81.
Stern, L., & Schulman, L. H. (1977) *J. Biol. Chem. 252*, 6403–6408.
Studier, F. W., & Moffatt, B. A. (1986) *J. Mol. Biol. 189*, 113–130.
Valenzuela, D., & Schulman, L. H. (1986) *Biochemistry 25*, 4555–4561.
Wilkinson, A. J., Fersht, A. R., Blow, D. M., & Winter, G. (1983) *Biochemistry 22*, 3581–3586.
Woo, N. H., Roe, B. A., & Rich, A. (1980) *Nature 286*, 346–351.
Zelwer, C., Risler, J. L., & Brunie, S. (1982) *J. Mol. Biol. 155*, 63–81.

## *Articles*

# Cytosolic Factors in Bovine Neutrophil Oxidase Activation. Partial Purification and Demonstration of Translocation to a Membrane Fraction†

Jacques Doussière,* Marie-Claire Pilloud, and Pierre V. Vignais

*LBio/Biochimie, Département de Recherche Fondamentale, Centre d'Etudes Nucléaires, 85X, 38041 Grenoble Cedex, France*

*Received August 1, 1989; Revised Manuscript Received October 23, 1989*

ABSTRACT: The $O_2^{\cdot-}$-generating oxidase of bovine neutrophils is activated in a cell-free system consisting of a particulate fraction enriched in plasma membrane and containing the dormant oxidase, a high-speed supernatant from neutrophil homogenate (cytosol), Mg ions, GTPγS, and arachidonic acid [Ligeti, E., Doussiere, J., & Vignais, P. V. (1988) *Biochemistry 27*, 193–200]. The cytosolic components participating in the activation of the membrane-bound oxidase have been investigated. These components were resolved into several active peaks by Q Sepharose chromatography. The oxidase-activating potency of these peaks was synergistically enhanced by combining samples from separate peaks, or by supplying them with a threshold amount of crude cytosol. Partial purification of two active fractions containing a limited number of proteins of 65, 56, 53, and 45 kDa was achieved by gel filtration of cytosol on Ultrogel AcA44, followed by chromatography on hydroxylapatite and Mono Q. The specific oxidase-activating potency of these partially purified fractions (activating potency per milligram of soluble protein) was 6–8-fold higher than that of crude cytosol; it was enhanced up to 75-fold by complementation with a minute amount of crude cytosol, which per se had a limited efficiency. These data indicate that oxidase activation requires more than one cytosolic component to be activated. To check whether translocation of cytosolic proteins to the membrane occurred concomitantly with oxidase activation, use was made of radiolabeled cytosolic proteins. Cytosol was treated with phenyl[$^{14}$C]isothiocyanate ([$^{14}$C]PITC], such that 60% of its activation potency was still present. Translocation was studied under conditions in which production of $O_2^{\cdot-}$ was largely modulated by varying the amount of arachidonic acid added to the cell-free system. Maximal oxidase activation with optimal concentration of arachidonic acid resulted in the selective translocation of labeled cytosolic proteins of 65, 53, 45, and 17 kDa to the membrane.

The plasma membrane bound oxidase of neutrophils, which is responsible for the production of large quantities of superoxide ($O_2^-$) during phagocytosis, is dormant in circulating neutrophils. It becomes activated upon binding of particulate or soluble ligands to specific receptors of the plasma membrane [see Rossi (1986) and Bellavite (1988)]. Elaboration of a cell-free system for oxidase activation in guinea pig macrophages (Bromberg & Pick, 1984, 1985) and in neutrophils from different species (Heyneman & Vercauteren, 1984; Curnutte, 1985; Mc Phail et al., 1985; Clark et al., 1987; Gabig et al., 1987; Seifert & Schultz, 1987; Ligeti et al., 1988; Tanaka et al., 1988) has led to the conclusion that a cytosolic factor of protein nature is required for activation of the membrane-bound oxidase. The search for the isolation and identification of this factor has been intensive. So far, attempts to purify this cytosolic factor by size-exclusion chromatography (Gabig et al., 1987; Curnutte et al., 1987; Fujita et al., 1987; Ishida et al., 1989) and affinity chromatography on 2′,5′-ADP–agarose (Sha'ag & Pick, 1988) or GTP–agarose (Volpp et al., 1988) have resulted in the recovery of fractions only partially enriched in cytosolic factor activity. It was recently recognized that neutrophils of patients suffering from an autosomal recessive form of chronic granulomatous disease (CGD)[1] are deficient in cytosolic proteins (Curnutte et al.,

† This work was supported by grants from the "Centre National de la Recherche Scientifique" (U.A 1130) and from the "Université Joseph Fourier, Faculté de Médecine de Grenoble".

0006-2960/90/0429-2225$02.50/0 

1988). Variants of the autosomal form of CGD have been correlated with defects in distinct cytosolic proteins, indicating that the cytosolic factor consisted of different components (Volpp et al., 1988; Nunoi et al., 1988; Caldwell et al., 1988; Curnutte et al., 1989; Bolscher et al., 1989).

Parameters of oxidase activation with a bovine neutrophil cell-free system were previously analyzed (Ligeti et al., 1988, 1989; Doussière et al., 1988; Pilloud et al., 1989). The large amount of neutrophils which can be obtained from bovine blood makes it possible to undertake the purification of the components of the oxidase activation system on a large scale. In this paper, experiments are described which point to the multiplicity and synergism of cytosolic factors responsible for oxidase activation in bovine neutrophils. We also report the preparation of a partially purified fraction containing a limited number of proteins from bovine neutrophil cytosol, and we demonstrate that cytosolic proteins of the same size as those present in this purified fraction are translocated to membrane during oxidase activation.

## Materials and Methods

*Materials.* Arachidonic acid, nitroblue tetrazolium, ferricytochrome *c* (horse heart, grade VI), and PMSF were purchased from Sigma; NADPH superoxide dismutase and guanine nucleotides from Boehringer; Percoll, Sephadex G-50 fine, Q Sepharose fast flow, and Mono Q column (HR5/5) from Pharmacia; Ultrogel AcA44 from IBF; and hydroxylapatite from Bio-Rad. Arachidonic acid was dissolved in absolute ethanol and stored at −80 °C under $N_2$ until used. [$^{14}$C]PITC was purchased from Amersham and dissolved in DMSO.

*Preparation of Cytosol and Membrane Fractions.* Neutrophils were prepared from bovine blood (Doussière & Vignais, 1985). The cells (about 1 g of protein) obtained from 6 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, and supplemented with 1 mM PMSF and 1 mM EDTA as antiproteases. All further treatments were carried out at 2–4 °C. Disruption of the cells was achieved by ultrasonic treatment for 4 × 15 s with a Branson sonifier at 40-W output. Nondisrupted cells as well as nuclei and granular material were eliminated by centrifugation at 6000*g* for 10 min in a Sorvall rotor. The resulting supernatant was spun for 60 min at 130000*g* in a Beckman 40 rotor. The 130000*g* pellet (about 100 mg of protein) was resuspended in 10 mL of PBS plus 1 mM EGTA by sonication for 4 × 15 s at 40-W output. A previous analysis (Morel et al., 1985) showed that this particulate fraction is significantly enriched in plasma membrane. When prepared from unstimulated cells, it contains the $O_2^{\cdot-}$-generating oxidase in its resting form. In order to eliminate any membranous particles, the 130000*g* supernatant was centrifuged at 300000*g* for 60 min in a Beckman 65Ti rotor. The resulting supernatant, termed cytosol, was adjusted to 18–20 mg/mL and stored at −80 °C.

*Assays of the Oxidase-Activating Potency of Cytosol and Purified Cytosolic Fractions.* The oxidase-activating potency of fractions purified from bovine neutrophil cytosol was evaluated at 25 °C by a rapid screening assay, followed by a more quantitative assay on the active fractions. Screening was conducted in two steps consisting of activation of the membrane-bound oxidase and of evaluation of the elicited oxidase activity, respectively. First, 100-μL aliquots of cytosolic fractions were mixed with 100 μL of PBS containing 100 μg of membrane protein in the presence of 5 mM $MgCl_2$, 10 μM GTPγS, and 110 nmol of arachidonic acid. As several cytosolic components are probably required for oxidase activation, and a given eluate might be deprived of some of these components, another incubation was conducted in parallel with the same medium supplemented with a threshold amount of crude cytosol (30 μg of protein) which served to supply the complementary components. In both cases, incubation was allowed to proceed for 10 min at room temperature. In the second step, the elicited oxidase activity was revealed by addition of 1 mL of PBS containing 5 mM $MgCl_2$, 200 μM NADPH, and 200 μM NBT. The reaction was allowed to proceed for 5 min, and was stopped by addition of 100 μL of 10% SDS. $O_2^{\cdot-}$ production was estimated from the absorbancy at 570 nm.

Active fractions identified by the above screening assay and corresponding to a same peak of elution were pooled for a quantitative assay of oxidase activation. The assay was performed in two steps. First, an aliquot of each pool was preincubated for 10 min at 25 °C with 250 μg of membrane protein in 100 μL of PBS in the presence of 5 mM $MgCl_2$, 10 μM GTPγS, and an optimal concentration of arachidonic acid. The elicited oxidase activity was determined after transfer of the preincubation mixture to 2 mL of PBS supplemented with 5 mM $MgCl_2$, 100 μM ferricytochrome *c*, and 200 μM NADPH. Reduction of ferricytochrome *c* was followed spectrophotometrically at 550 nm. After a linear phase of 3–4 min, 50 μg of superoxide dismutase was added and recording continued for another 3 min. The difference between the two slopes corresponded to the SOD-inhibitable reduction of ferricytochrome *c*, and was taken as oxidase activity. In a parallel assay, it was checked that ferricytochrome *c* reduction continued in a linear fashion in the absence of SOD.

*$NaDodSO_4$–Polyacrylamide Gel Electrophoresis.* Aliquots of crude cytosol or purified cytosolic fractions were freeze-dried. The residue was treated by a mixture of 90% acetone and 10% water. The resulting suspension was centrifuged at 10000*g* for 10 min; the supernatant enriched in salt was discarded, and the pellet was dissolved in 20 μL of 2% SDS, 20% sucrose, 10% mercaptoethanol, 0.1% bromophenol blue, 0.05 M Tris, and 0.38 M glycine, final pH 8.0. The solubilized proteins were subjected to gel electrophoresis as described by Laemmli and Favre (1973), using a 4% stacking gel and a 12% resolving gel. The molecular weight standards used were phosphorylase *b* ($M_r$ 94000), bovine serum albumin ($M_r$ 67000), ovalbumin ($M_r$ 43000), carbonic anhydrase ($M_r$ 30000), soybean trypsin inhibitor ($M_r$ 20100), and α-lactalbumin ($M_r$ 14400). Gels were stained with Coomassie blue R250.

*Labeling of Cytosolic Proteins by [$^{14}$C]PITC.* The pH of the cytosol was adjusted to 8.0 by addition of 1 N NaOH. To 1 mg of cytosolic protein in 100 μL were added increasing amounts of [$^{14}$C]PITC (11 mCi/mmol) up to 450 nmol, the final volume being adjusted to 130 μL. After 30 min at 2–4 °C, the reaction was terminated by addition of 10 μL of 1 M Tris, pH 8. After 15 min of contact, the pH was brought back to 7.5 with 1 N HCl. Protein-bound [$^{14}$C]PITC was separated from free [$^{14}$C]PITC by centrifugation filtration on small columns of G50 Sephadex (Penefsky, 1977), and the volume of the filtrate was adjusted to 150 μL.

[1] Abbreviations: GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); PITC, phenyl isothiocyanate; NBT, nitroblue tetrazolium; SOD, superoxide dismutase; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; CGD, chronic granulomatous disease; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; DMSO, dimethyl sulfoxide.

Transfer of [$^{14}$C]PITC-labeled cytosolic proteins to the membrane fraction was assayed as follows. The filtrates containing the labeled cytosolic proteins (900 $\mu$g) were incubated with 1 mg of membrane protein in 250 $\mu$L of PBS in the presence of 10 $\mu$M GTP$\gamma$S, 5 mM $MgCl_2$, and increasing concentrations of arachidonic acid for 10 min at 25 °C. The mixture was placed in centrifuge tubes on a layer of 10% sucrose (w/w) in PBS. Centrifugation was run for 1 h at 40 000 rpm in a Beckman rotor SW41. After centrifugation, the supernatant was removed, the tubes were carefully rinsed and dried with blotting paper, and the pellets were resuspended in 300 $\mu$L of PBS. One part of the suspension was used to determine the oxidase activity by following the kinetics of the SOD-inhibitable reduction of cytochrome *c*. Another part was solubilized in SDS, and the lysate was subjected to slab gel electrophoresis. The gel was stained with Coomassie blue R250. For autoradiography, the gel was impregned with EN$^3$hance (Laskey & Mills, 1975), dried, and exposed to a Fuji RX film at −70 °C with a Cronex intensifying screen.

*Determination of Protein Concentration.* Protein concentration of cytosol and cytosolic fractions was measured by the method of Bradford (1976). Protein of the membrane fraction and neutrophils was determined by the biuret procedure, as previously described (Morel et al., 1985).

## Results

*Optimal Conditions for in Vitro Assay of Cytosolic Factors Involved in Oxidase Activation.* The cell-free system from bovine neutrophils routinely used for elicitation of $O_2^{\bullet-}$ production consists of a membrane fraction enriched in plasma membrane which contains the dormant oxidase, cytosol, $MgCl_2$, GTP$\gamma$S, and arachidonic acid (Ligeti et al., 1988). The ability of cytosol to confer activation to the membrane-bound oxidase depends on a number of parameters that include the relative amounts of arachidonic acid, membrane, and cytosol, the concentrations of GTP$\gamma$S and $Mg^{2+}$, and finally the ionic strength and the volume of the incubation medium. The optimal concentration of GTP$\gamma$S is around 10 $\mu$M (Ligeti et al., 1988). Maximum oxidase activation occurs for $MgCl_2$ concentrations ranging between 5 and 15 mM (data not shown).

The amount of arachidonic acid needed for full oxidase activation is linearly related to the salt concentration of the medium (Pilloud et al., 1989). This parameter is important to control during the course of purification of cytosolic factors, as the amount of salt present in the assayed fractions depends on the step of purification. The amount of arachidonic acid must also be properly adjusted with respect to the amount of membrane and cytosol protein (Ligeti et al., 1988; Pilloud et al., 1989). However, when low amounts of cytosol ($\leq$100 $\mu$g of protein) are used, contrasting with an excess of membrane protein (200–250 $\mu$g), the optimal concentration of arachidonic acid depends essentially on the amount of membrane protein and no longer on that of cytosol.

As previously reported (Pilloud et al., 1989), Michaelian kinetics are displayed when the extent of oxidase activation under optimal conditions of assay is plotted against increasing amounts of cytosol. At low concentrations of cytosol or purified cytosolic fractions, the relationship between the amount of cytosolic protein and the measured oxidase activating potency is approximately rectilinear. This makes it possible to readily calculate the specific cytosolic activation potency, that is defined as the membrane-bound oxidase activity (nmol of $O_2^{\bullet-}$/min) elicited per milligram of cytosolic protein.

*Multiplicity and Synergism of Cytosolic Components Involved in Oxidase Activation.* Subjecting bovine cytosol to

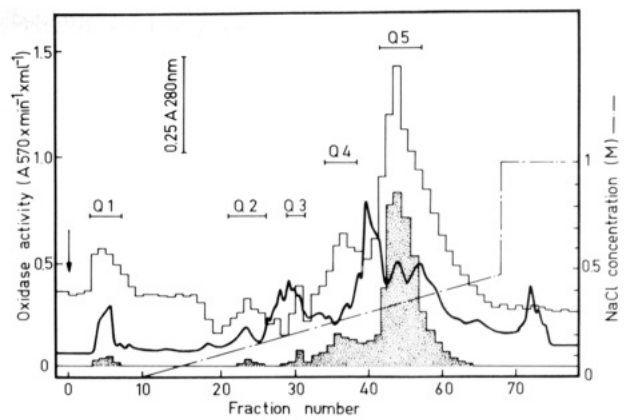


FIGURE 1: Fractionation of bovine neutrophil cytosol on Q Sepharose. Synergistic effect of cytosol on the activating potency of cytosolic fractions resolved by Q Sepharose chromatography. Experimental details are given under Results. Fractions of 3 mL were collected, and 100-$\mu$L aliquots were assayed for their activating potency on the membrane-bound NADPH oxidase without added cytosol (dotted bars) or with added cytosol (open bars), using the screening assay described under Materials and Methods. The column was finally washed with 1 M NaCl. The oxidase-activating potency was eluted in five peaks termed Q1, Q2, Q3, Q4, and Q5. The thick line corresponds to absorbance.

Q Sepharose chromatography yielded a complicated pattern of protein fractionation, consistent with the conclusion that several cytosolic proteins participate in the activation of membrane-bound oxidase. The high-speed supernatant (cytosol) from bovine neutrophils (300 mg of protein) was dialyzed against 10 mM Tris, pH 7.5, 0.1 mM EGTA, and 0.1 mM PMSF for 5 h and then chromatographed, using a Q Sepharose column (40 cm × 1 cm) equilibrated with the same buffer supplemented with 0.5 mM EGTA and 0.5 mM PMSF. After the column was washed with the equilibrium buffer, a linear 0–0.5 M NaCl gradient was applied. The eluted fractions were first analyzed for their potency to activate the membrane-bound oxidase by a rapid screening assay based on the use of NBT (cf. Materials and Methods). Five active peaks termed Q1–Q5 were identified (dotted bars in Figure 1). When the screening assay was conducted in the presence of a threshold amount of cytosol (30 $\mu$g of protein), just sufficient to provide a limited activation of the membrane-bound oxidase, the $O_2^{\bullet-}$ production by the fractions of peaks Q1, Q4, and Q5 was markedly enhanced (open bars in Figure 1). The enhanced oxidase activity was much higher than the sum of the oxidase activities generated by the separate eluted fractions on one hand and the crude cytosol on the other. A likely explanation for this synergistic effect is that the fractions of peaks Q1, Q4, and Q5 are deficient in one or several components necessary for oxidase activation and that these components were supplied by added cytosol.

In contrast to peaks Q1, Q4, and Q5, oxidase activation by fractions of peaks Q2 and Q3 was not synergistically enhanced by cytosol. Moreover, by mixing cytosol with fractions eluted just before peak Q2 (fraction 18–23) or between peaks Q2 and Q3 (fractions 28–32), the elicited oxidase activity was lower than that elicited by cytosol alone, as if an inhibitory component was present in these fractions. This unexpected inhibition suggested that the protein material eluted in this region of the NaCl gradient contained a component capable of scavenging the generated $O_2^{\bullet-}$. A test was therefore performed to verify the presence of $O_2^{\bullet-}$ scavenger by assaying the eluates in a medium where $KO_2$ was used as a source of $O_2^{\bullet-}$. In brief, a 30-$\mu$L sample of 7 mM $KO_2$ in dimethyl sulfoxide was introduced in 1 mL of PBS, pH 7.8, in the photometric cuvette in the presence or absence of eluates. After 1 min of incu-

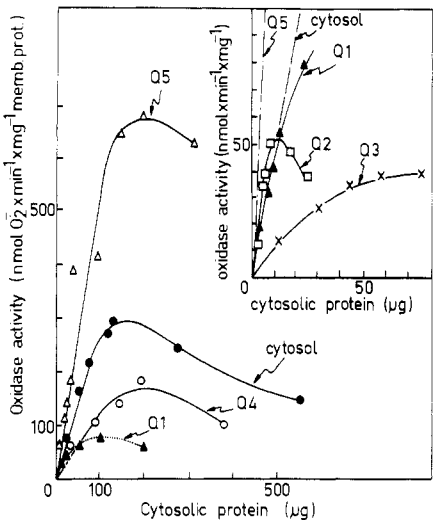


FIGURE 2: Kinetics of activation of membrane-bound oxidase by cytosol or purified cytosolic fractions from Q Sepharose. The experiment was performed in two steps. In the preincubation step, the membrane-bound oxidase was activated by cytosol or the cytosolic Q fractions in the presence of GTPγS, $MgCl_2$, and arachidonic acid. This was followed by the assay of the activated oxidase. Experimental conditions are detailed under Materials and Methods. $O_2^{\cdot-}$ production is expressed in nmol min$^{-1}$ (mg of membrane protein)$^{-1}$. The specific activating potency of cytosolic Q fractions (see Results) corresponds to the initial slope of the curves.

bation at 25 °C, the remaining $O_2^{\cdot-}$ was determined by spectrophotometry at 570 nm after addition of 1 mL of 200 μM NBT in PBS, pH 7.8. Fractions 18–32, including peaks Q2 and Q3, were indeed able to scavenge very efficiently $O_2^{\cdot-}$. A plausible scavenger candidate is superoxide dismutase. In summary, although peaks Q2 and Q3 might contain components able to activate the membrane-bound oxidase, the full expression of these components is probably obscured by the presence of contaminating $O_2^{\cdot-}$ scavenger.

SDS–PAGE analysis revealed a discrete number of protein bands in the case of peaks Q1 and Q2, with predominance of 55- and 45-kDa bands in Q1 and a 65-kDa band in Q2. A much more complex pattern was observed with the material of peaks 3–5 (data not shown).

*Kinetic and Complementation Studies with Q Sepharose Active Fractions.* For a more precise evaluation of the oxidase-activating potency of components present in peaks Q1–Q5 in the preceding experiment, the eluates corresponding to each peak were pooled and assayed for the rate of $O_2^{\cdot-}$ production by the SOD-inhibitable reduction of ferricytochrome *c*. Appropriate conditions were devised to accurately determine the oxidase-activating potency related to the cytosolic protein in a simple way. For this purpose, the amounts of the membrane fraction and arachidonic acid were maintained at the fixed values of 250 μg of protein and 100 nmol, respectively. In this experiment, the oxidase capacity of the membrane fraction was in excess, relative to the activating factors of the added cytosolic fractions. The data assembled in Figure 2 refer to the oxidase activities elicited under these conditions by increasing amounts of either cytosol or samples of the Q cytosolic pools. Virtually linear kinetics of $O_2^{\cdot-}$ production were elicited by amounts of cytosol and the Q pools 1, 4, and 5 in a range of 50–100 μg of protein. Above 100 μg of protein, the curves deviated from linearity, and for concentrations higher than 200 μg of protein oxidase, activity was decreased, which was consistent with the fact that the optimal ratio of arachidonic acid by cytosol was not any longer fulfilled [see Pilloud et al. (1989)]. In the case of pools Q2 and Q3, the elicited oxidase activities deviated from linearity for much lower amounts of protein, namely, 10 and 30 μg, respectively. As discussed above, this might be related to contaminating $O_2^{\cdot-}$ scavenger.

Table I: Reconstitution of Oxidase Activity in the Cell-Free System by a Combination of Separate Cytosolic Active Fractions Resolved by Q Sepharose Chromatography[a]

| Q pools | $O_2^{\cdot-}$ formed in cell-free system [nmol min$^{-1}$ (100 μL of fraction)$^{-1}$] | factor of enhancement resulting from combination of separate fractions |
|---|---|---|
| 1 | 1.6 | |
| 2 | 2.7 | |
| 3 | 1.0 | |
| 4 | 8.3 | |
| 5 | 18.9 | |
| 4 + 5 | 37.9 | 1.4 |
| 1 + 4 | 27.7 | 2.8 |
| 1 + 5 | 45.7 | 2.2 |
| 1 + (4 + 5) | 67.5 | 2.3 |
| 2 + (4 + 5) | 26.3 | 0.9 |
| 3 + (4 + 5) | 31.3 | 1.1 |
| 2 + 3 + (4 + 5) | 28.4 | 0.9 |
| 2 + (1 + 4 + 5) | 61.2 | 1.9 |
| 3 + (1 + 4 + 5) | 46.3 | 1.5 |
| 2 + 3 + (1 + 4 + 5) | 66.6 | 2.0 |

[a] The contents of cytosolic peaks Q1–Q5 recovered by Q Sepharose chromatography (cf. Figure 1) were assayed for their activating potency on membrane-bound oxidase using the cell-free system described under Materials and Methods. They were used either separately or in combination, after 5-fold concentration. Twenty microliters of each peak (10–50 μg of protein) was allowed to stand at 25 °C for 10 min in PBS, 5 mM $MgCl_2$, and 10 μM GTPγS, final volume 200 μL. Then, 100 μL of membrane fraction (250 μg of protein) was added, followed by 120 nmol of arachidonic acid, and incubation was allowed to proceed for another 10 min at 25 °C before assay of the elicited oxidase activity by the SOD-inhibitable reduction of cytochrome *c* (cf. Materials and Methods). The results are given as oxidase activity (nanomoles per minute) elicited by 100-μL samples from the different Q peaks (prior to the 5-fold concentration). The enhancement factor resulting from combination of separate Q fractions corresponds to the ratio of elicited oxidase activity after mixing of different Q fractions to the arithmetic sum of the elicited oxidase activities obtained with the separate Q fractions.

Comparison of the slopes of the initial portions of the oxidase activation curves for cytosol and the Q pools 1, 4, and 5 (Figure 2) indicated that only pool Q5 had a better specific activating potency than cytosol. Possibly, several components necessary for oxidase activation are present in crude cytosol in a given stoichiometry. As pool Q5 has a higher specific activation potency than crude cytosol, it is clear that it contains all the active components present in cytosol; the enhancement of its activation potency by cytosol might be due to a disproportion in the stoichiometry of its active components (cf. Figure 1).

A series of complementation assays was performed using different modes of combination of the Q pools (Table I). The amount of the cytosolic fractions used corresponded to the linear portion of the dose effect curves in Figure 2. A typical synergism for activating potency was observed upon mixing Q pools 4 and 5, 1 and 4, 1 and 5, or 1, 4, and 5. Only additive effects or even less than additive effects resulted from mixing the Q pools 2 or (and) 3 to pools 4 and 5. Replacing Q pools 2 or 3 by Q pool 1 resulted in the reappearance of some synergism. These results are consistent with the finding that Q pools 2 and 3 are contaminated by an $O_2^{\cdot-}$-scavenging component, which prevents a correct evaluation of the expressed oxidase activation. Most probably, the different active peaks obtained by Q Sepharose chromatography of neutrophil cytosol contain protein aggregates. In other words, a given protein participating in oxidase activation might be present in several Q peaks, in different states of aggregation.

*Partial Purification of Cytosolic Components Involved in Oxidase Activation.* For routine purification experiments, 5–6 L of bovine blood was used. An illustration of partial purification of cytosolic components required for activation of the

Table II: Partial Purification of Cytosolic Components Required for Oxidase Activation in a Cell-Free System[a]

| step of purification | protein (mg) | sp act. potency: $O_2^{\cdot-}$ formed [nmol $min^{-1}$ (mg of cytosolic fraction protein)$^{-1}$] | total act. potency: $O_2^{\cdot-}$ formed (nmol $min^{-1}$) |
|---|---|---|---|
| cytosol | 360 | 90 | 32400 |
| ammonium sulfate ppt | 270 | 113 | 30510 |
| A[b] | 31 | 144 | 4464 |
| HA[c] | 8.8 | 254 | 2235 |
| MQA[d] | 0.5 | 534 (6732)[e] | 267 (3366)[e] |
| B[b] | 36 | 80 | 2880 |
| HB[c] | 2.1 | 309 | 648 |
| MQB[d] | 0.2 | 708 (5659)[e] | 142 (1130)[e] |

[a] The oxidase-activating potency of cytosol and purified cytosolic components was assayed as described under Materials and Methods. The cytosolic fractions used contained 2–30 μg of protein. The cell-free system in PBS contained an excess of membrane protein (200 μg) relative to the cytosolic fraction, 5 mM $MgCl_2$, and 10 μM GTPγS, and 120 nmol of arachidonic acid. [b] A and B correspond to the two active peaks eluted from Ultrogel AcA44 (Figure 3). [c] Fractions HA and HB correspond to active fractions eluted from HTP and originating from A and B fractions, respectively (cf. Figure 3). [d] Fractions MQA and MQB correspond to active fractions eluted from Mono Q and originating from HA and HB fractions, respectively (cf. Figure 3). [e] Values in parentheses correspond to the specific or total activating potency of fractions MQA and MQB supplemented with crude cytosolic protein. The oxidase-activating potency proper to this amount of crude cytosol was deduced for calculation of the oxidase activity elicited by combination of fractions MQA or MQB with cytosol.

membrane-bound oxidase is given in Table II. Neutrophils were disrupted by ultrasonic irradiation in a medium consisting of PBS, 1 mM PMSF, and 1 mM EGTA. The high-speed supernatant (cytosol) of neutrophil homogenate (360 mg of protein) was diluted with the sonication medium to a concentration of 18–20 mg/mL. Powdered ammonium sulfate was added to 60% saturation, and the mixture was gently stirred at 2–4 °C for 2 h. The precipitate was pelleted by centrifugation at 20000*g* for 15 min and then taken up in a volume of PBS, 1 mM PMSF, and 1 mM EDTA corresponding to one-fifth of the initial volume of cytosol. Virtually all the oxidase-activating potency of the crude cytosol was recovered in the ammonium sulfate precipitate. The solubilized proteins (270 mg) in 3–4 mL were further fractionated by filtration on a column of Ultrogel AcA44 (2 cm × 90 cm) equilibrated with PBS, 0.5 mM PMSF, and 0.5 mM EGTA (Figure 3A). The active fractions were eluted in two distinct peaks, A and B, each peak corresponding to 31 and 36 mg of protein, respectively. The content of each peak was processed separately by chromatography on hydroxylapatite (Figure 3B,B′) and then on Mono Q (Figure 3C,C′). First, the material of peak A was placed on a hydroxylapatite column (0.9 cm × 20 cm) equilibrated with a mixture of PBS and 0.5 mM PMSF. Elution with a linear gradient of 0–250 mM sodium phosphate, pH 7.5, in PBS yielded two active peaks, a minor one corresponding to the flow-through fraction and consisting of protein aggregates and a major one, termed HA, eluted with 90–120 mM phosphate (Figure 3B). The content of peak HA was dialyzed against 10 mM Tris, pH 7.5, 0.1 mM EGTA, and 0.1 mM PMSF for 5 h at 2 °C and chromatographed on a column of Mono Q equilibrated with the same buffer. The column was eluted with a gradient of 0–0.5 M NaCl in the equilibrating buffer programmed in an FPLC system. Active fractions were eluted with 280–300 mM NaCl and pooled (pool MQA) (Figure 3C).

The material of peak B recovered from the first chromatography on Ultrogel AcA44 (Figure 3A) was processed like that of pool A. Here again, elution of hydroxylapatite with the phosphate gradient yielded two active peaks. The first one corresponded to the flow-through fraction, and the second, termed HB, was eluted with 90–120 mM phosphate (Figure 3B′). After dialysis, the content of the HB peak was applied to a Mono Q column. Elution with the NaCl gradient yielded an active peak MQB at 280–300 M NaCl (Figure 3C′).

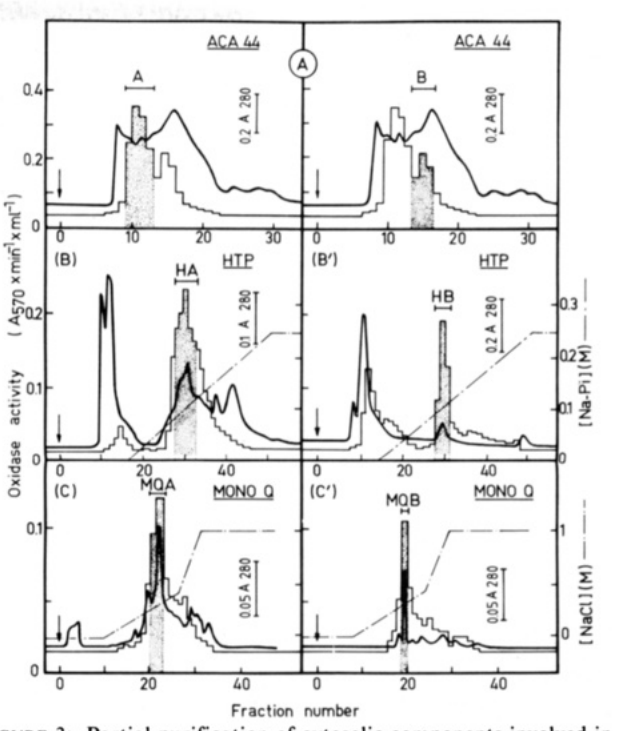


FIGURE 3: Partial purification of cytosolic components involved in the activation of membrane-bound oxidase. The cytosolic proteins responsible for the activation of the membrane-bound oxidase were precipitated from 20 mL of cytosol (360 mg of protein) by ammonium sulfate at 60% saturation as detailed under Results. The further steps of purification, namely, AcA44 gel filtration, hydroxylapatite chromatography, and Mono Q chromatography, are illustrated in pannels A, B and B′, and C and C′, respectively. The volume of the recovered fractions was 4 mL in panel A, 2 mL in panels B and B′, and 0.5 mL in panels C and C′ (for details, see Results). The oxidase activity elicited by the cytosolic fractions was determined by the rapid screening assay (cf. Materials and Methods) and expressed as the increase in absorbance (reduction of NBT) at 570 nm per minute and for 1 mL of each fraction assayed.

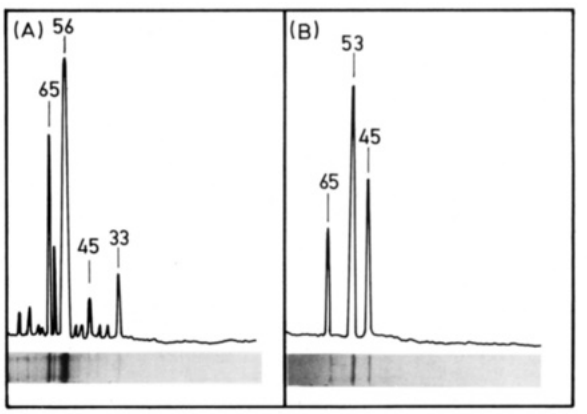


FIGURE 4: SDS–PAGE analysis of the MQA and MQB fractions recovered from Mono Q chromatography (cf. Figure 3). The amounts of protein used were 24 and 10 μg, respectively. The gels were stained with Coomassie blue.

SDS–PAGE analysis of peaks MQA and MQB showed somewhat similar protein profiles characterized by two common bands with molecular masses of 65 and 45 kDa (Figure 4), which suggests that the two corresponding proteins were already present in pools A and B recovered from the first chromatographic step on Ultrogel AcA44, probably in different states of aggregation. Peak MQA contained three additional proteins with molecular masses of 60, 56, and 33 kDa, and

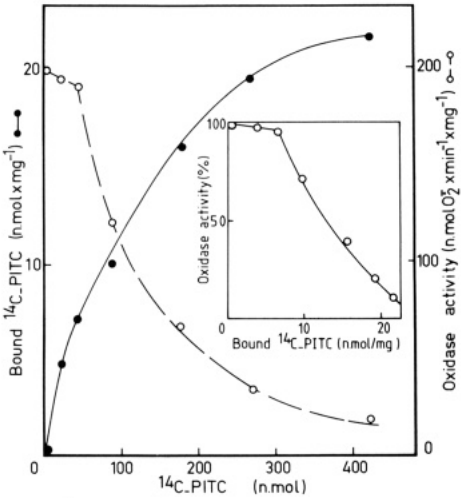


FIGURE 5: Modification of the oxidase-activating potency of cytosol proteins upon binding of [$^{14}$C]PITC. Following radiolabeling, free [$^{14}$C]PITC and protein-bound [$^{14}$C]PITC were separated by Sephadex G50 centrifugation filtration (Penefsky, 1977) as described under Materials and Methods. Part of the filtrate was used for determination of protein-bound [$^{14}$C]PITC by liquid scintillation counting, the other part being used for evaluation of the oxidase activation potency, with the cell-free system described under Materials and Methods. $O_2^{\bullet-}$ production was expressed as nanomoles per minute per milligram of membrane protein. Insert: The oxidase-activating potency (percent of the maximum) was plotted as a function of the bound [$^{14}$C]PITC.

peak MQB an additional one of 53 kDa. The specific oxidase-activity potency of the factors present in peaks MQA and MQB (activity of membrane-bound oxidase elicited per milligram of added soluble fraction) was 6–8-fold higher than that of crude cytosol. It was markedly enhanced by addition of a threshold amount of crude cytosol (Table II). In agreement with the results of the fractionation of cytosolic components by Q Sepharose chromatography (Table I), the present data on partial purification indicate that several cytosolic factors concur to activate the membrane-bound oxidase. In brief, fractions MQA and MQB contain only part of the necessary components required for oxidase activation; the missing components are supplied by addition of a suboptimal amount of cytosol.

*Evidence That Chemically Labeled Cytosolic Proteins Are Selectively Translocated to the Membrane during the Course of Oxidase Activation.* Translocation of cytosolic factors to the membrane is a required step for activation of the membrane-bound oxidase (Doussière et al., 1988; Ligeti et al., 1989). In the experiments to be described now, proteins of bovine neutrophil cytosol were labeled with [$^{14}$C]PITC, and an attempt was made to correlate the extent of oxidase activation with the translocation of specific labeled cytosolic proteins to the membrane. The first step in this experimentation was an analysis of the modification of the oxidase-activating potency of the labeled cytosol. Below 7 nmol of bound [$^{14}$C]PITC/mg of cytosolic protein, no deleterious effect was observed. Above this amount of bound PITC, the activating potency of cytosol diminished abruptly, with a 50% decrease for 11 nmol of bound [$^{14}$C]PITC/mg of cytosolic protein (Figure 5). For the study of translocation of cytosolic proteins to the membrane, we decided to use a sample of cytosol labeled with [$^{14}$C]PITC, such that 60% of its activating potency was still present.

As the extent of oxidase activation depends on the amount of arachidonic acid present in the cell-free system (Ligeti et al., 1988; Pilloud et al., 1989), an experiment was set up in which the amount of arachidonic acid was varied, and the translocation of labeled cytosolic proteins to the membrane

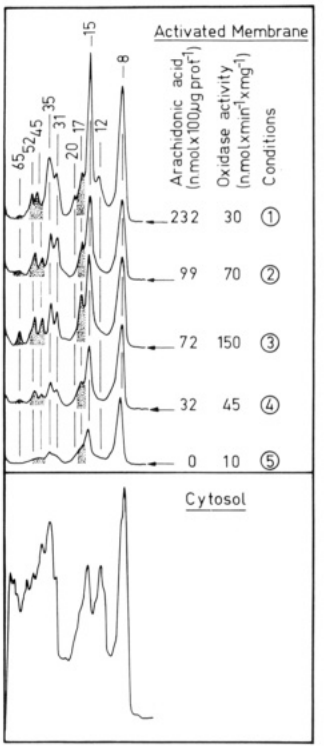


FIGURE 6: Transfer of [$^{14}$C]PITC-labeled cytosolic proteins to the membrane fraction of bovine neutrophils during in vitro oxidase activation. A sample of cytosol was labeled by [$^{14}$C]PITC to such an extent that 60% of the oxidase-activation potency was left (cf. Materials and Methods and Figure 5). Five separate incubations in PBS were then carried out with cytosol (900 µg), the membrane fraction (1 mg), 5 mM $MgCl_2$, 10 µM GTPγS, and different amounts of arachidonic acid that were chosen to elicit different levels of oxidase activation. Condition 3 corresponds to the optimal amount of arachidonic acid (72 nmol/100 µg of protein) resulting in maximal oxidase activity (150 nmol min$^{-1}$ mg$^{-1}$). Arachidonic acid was also used at higher concentrations (conditions 1 and 2) or a lower concentration (condition 4) relative to the optimal concentration of arachidonic acid. In condition 5, no arachidonic acid was added. After a 10-min-preincubation at 25 °C, the suspension was centrifuged through a layer of sucrose in PBS as described under Materials and Methods. The sedimented pellet was suspended in PBS. Part of the suspension was used to assay the activated oxidase, and the other (200 µg of protein) after concentration was lysed by SDS and subjected to SDS–PAGE. The gels were stained by Coomassie blue, treated with EN$^3$hance, and then autoradiographed. The densitometric scans of the autoradiographies are shown in the upper part of the figure. Incorporation of radioactivity in protein bands apparently related to an increase of of oxidase activity is illustrated by dots. The lower part of the figure shows the densitometric scan of the autoradiography of a gel after electrophoresis of 10 µg of [$^{14}$C]PITC-labeled cytosol.

fraction was analyzed in parallel with the extent of oxidase activation. For this purpose, after the activation step, the membrane fraction was separated from cytosol by centrifugation through a sucrose layer (cf. Materials and Methods), and the radiolabeled proteins present in this membrane fraction were separated by SDS–PAGE and revealed by autoradiography.

In Figure 6, the values of oxidase activities elicited with the different concentrations of arachidonic acid are shown together with the scans of the autoradiographies of the respective gels. The areas of the radioactive peaks visualized on the scans were measured and compared to the elicited oxidase activity. Clearly, oxidase activation was related to the number and the nature of the labeled cytosolic proteins translocated to the membrane. When arachidonic acid was omitted, a condition resulting in negligible elicited oxidase activity, only the 8- and 15-kDa proteins were translocated in significant amounts. With an excess of arachidonic acid, which leads to a somewhat

depressed oxidase activation, additional proteins with molecular masses of 52, 45, 35, 31, 20, 17, and 12 kDa were translocated. With the optimal concentration of arachidonic acid yielding maximal oxidase activation, the translocation of the 8-, 15-, 31-, and 35-kDa proteins was lowered, and that of the 12- and 20-kDa proteins was abolished. In contrast, translocation of the 52-, 45-, and 17-kDa proteins was significantly enhanced, and, further, a 65-kDa protein was selectively transferred to the membrane, suggesting a central function of this latter protein in the activation process.

## Discussion

*Participation of Several Cytosolic Factors in the Activation of the Membrane-Bound Oxidase.* Up to the present, the participation of several cytosolic factors in oxidase activation has been demonstrated by complementation studies with human neutrophils isolated from patients suffering from the autosomal recessive, cytochrome *b* positive form of CGD, using a cell-free system of activation. A special interest was directed to a 47-kDa phosphoprotein located both in a membrane fraction and in the cytosol of neutrophils (Hayakawa et al., 1986; Kramer et al., 1988). The phosphorylation of this protein was defective in autosomal CGD (Segal et al., 1985), suggesting that oxidase activation depends on the phosphorylation of this protein (Heyworth et al., 1989). The defective phosphorylation of CGD neutrophils was restored by addition of the cytosol of neutrophils from healthy donors (Caldwell et al., 1988). A purified fraction isolated from the cytosol of normal neutrophils by (carboxymethyl)-Sepharose chromatography and containing a similar size protein was shown to restore oxidase activation in GGD neutrophils (Bolscher et al., 1989). Two variants of autosomal CGD lacking either the 47-kDa protein or another protein of 67 kDa were also reported (Volpp et al., 1988; Nunoi et al., 1988). More recently, the 47-kDa cytosolic protein was cloned from an expression library (Lomax et al., 1989; Volpp et al., 1989). From other complementation studies dealing with active cytosolic fractions from human neutrophils isolated by isoelectric focusing, two biochemically distinct forms of autosomal CGD were identified (Curnutte et al., 1989).

The work described in the present paper was carried out with bovine neutrophils. In fact, bovine neutrophils offer the advantage of providing large amounts of protein material for purification studies, and the cell-free system of activation described for bovine neutrophil oxidase (Ligeti et al., 1988) exhibits virtually the same properties as those reported in the case of human neutrophils. Direct evidence is provided in the present study that several cytosolic components are necessary for oxidase activation to occur. First, Q Sepharose chromatography of cytosol yielded a number of active fractions distributed in several peaks. The oxidase-activating potency of these peaks was synergistically enhanced by the addition of a limited amount of crude cytosol which per se had a low efficiency. Combining the different peaks also resulted in a synergistic enhancement of oxidase activation. A second line of evidence stems from the partial purification of cytosolic factors involved in oxidase activation. A partially purified fraction containing proteins of 65, 56, 53, and 45 kDa was shown to exhibit a significant oxidase-activating potency, that was synergistically enhanced by crude cytosol, indicating that other cytosolic proteins were needed for full oxidase activation. Translocation of specific proteins from the cytosol to the membrane during the course of oxidase activation was assessed with cytosolic proteins labeled with [$^{14}$C]PITC to a low extent, to avoid loss of activation capacity. Proteins of the same sizes as those identified in the partially purified fraction and in addition a 17-kDa protein were preferentially translocated to the membrane when oxidase was maximally activated by an optical concentration of arachidonic acid, suggesting the formation of a complex between cytosolic factors and specific membrane components.

*Methodological Aspects.* The low yield of bovine cytosolic factors in purification (Table II) might be explained by a loss of protein due to the strong propensity of the cytosolic proteins to aggregation. The quantitative assay of $O_2^{\bullet-}$ production by the oxidase system deserves some comments. Oxidase activation is strongly dependent on the ratios between the concentrations of arachidonic acid, membrane, and cytosol (Pilloud et al., 1989). When optimal concentrations of all components are provided, the elicited oxidase activity varies hyperbolically as a function of the amount of cytosol used (Pilloud et al., 1989). A sigmoidal response was observed under nonoptimal conditions of oxidase activation (Ligeti et al., 1988; Babior et al., 1988). The sigmoidal response was interpreted in terms of cooperative interaction between several cytosolic factors (Babior et al., 1988), which might fit with the synergistic effect of these cytosolic factors on oxidase activation. The transition between the sigmoidal and hyperbolic kinetics, depending on conditions of oxidase activation, probably reflects the complexity of the interactions between the cytosolic factor(s) involved and their target site(s) on the membrane.

## Acknowledgments

We thank J. Bournet for secretarial assistance.

**Registry No.** NADPH oxidase, 9032-22-8.

## References

Babior, B. M., Kuver, R., & Curnutte, J. T. (1988) *J. Biol. Chem. 263*, 1713–1718.

Bellavite, P. (1988) *Free Radical Biol. Med. 4*, 225–261.

Bolscher, B. G. J. M., Van Zwieten, R., Kramer, J., Weening, R. S., Verhoeven, A. J., & Roos, D. (1989) *J. Clin. Invest. 83*, 757–763.

Bromberg, Y., & Pick, E. (1984) *Cell Immunol. 88*, 213–221.

Bromberg, Y., & Pick, E. (1985) *J. Biol. Chem. 260*, 13539–13545.

Caldwell, S. E., Mc Call, C. E., Hendricks, C. L., Leone, P. A., Bass, D. A., & Mc Phail, L. C. (1988) *J. Clin. Invest. 81*, 1483–1496.

Clark, R. A., Leidal, K. C., Pearson, D. W., & Nauseef, W. M. (1987) *J. Biol. Chem. 262*, 4065–4074.

Curnutte, J. T. (1985) *J. Clin. Invest. 75*, 1740–1743.

Curnutte, J. T., Kuver, R., & Scott, P. J. (1987) *J. Biol. Chem. 262*, 5563–5569.

Curnutte, J. T., Berkow, R. L., Roberts, R. L., Shurin, S. B., & Scott, P. J. (1988) *J. Clin. Invest. 81*, 606–616.

Curnutte, J. T., Scott, P. J., & Mayo, L. A. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 825–829.

Doussière, J., & Vignais, P. V. (1985) *Biochemistry 24*, 7231–7239.

Doussière, J., Pilloud, M.-C., & Vignais, P. V. (1988) *Biochem. Biophys. Res. Commun. 152*, 993–1001.

Fujita, I., Takehige, K., & Minakami, S. (1987) *Biochim. Biophys. Acta 931*, 41–48.

Gabig, T. G., English, D., Akard, L. P., & Schell, M. J. (1987) *J. Biol. Chem. 262*, 1685–1690.

Hayakawa, T., Suzuki, K., Suzuki, S., Andrews, P. C., & Babior, B. M. (1986) *J. Biol. Chem. 261*, 9109–9115.

Heyneman, R. A., & Vercauteren, R. E. (1986) *J. Leukocyte Biol. 36*, 751–759.

Heyworth, P. G., Shrimpten, C. F., & Segal, A. W. (1989) *Biochem. J. 260*, 243-248.
Ishida, K., Takeshige, K., Takasugi, S., & Minakami, S. (1989) *FEBS Lett. 243*, 169-172.
Kramer, I. M., Verhoeven, A. J., Van der Bend, R. L., Weening, R. S., & Roos, D. (1988) *J. Biol. Chem. 263*, 2352-2357.
Laemmli, U. K., & Favre, M. (1973) *Nature 227*, 680-685.
Laskey, R. A., & Mills, A. (1975) *Eur. J. Biochem. 56*, 335-341.
Ligeti, E., Doussière, J., & Vignais, P. V. (1988) *Biochemistry 27*, 193-200.
Ligeti, E., Tardif, M., & Vignais, P. V. (1989) *Biochemistry 28*, 7116-7123.
Lomax, K. J., Leto, T. L., Nunoi, H., Gallin, J. I., & Malech, H. L. (1989) *Science 245*, 409-412.
Mc Phail, L. C., Shirley, P. S., Clayton, C. C., & Snyderman, R. (1985) *J. Clin. Invest. 75*, 1735-1739.
Morel, F., Doussière, J., Stasia, M.-J., & Vignais, P. V. (1985) *Eur. J. Biochem. 152*, 669-679.
Nunoi, H., Rotrosen, D., Gallin, J. J., & Malech, H. I. (1988) *Science 242*, 1298-1300.
Penefsky, H. S. (1977) *J. Biol. Chem. 252*, 2891-2899.
Pilloud, M.-C., Doussière, J., & Vignais, P. V. (1989) *Biochem. Biophys. Res. Commun. 159*, 783-790.
Rossi, F. (1986) *Biochim. Biophys. Acta 853*, 63-89.
Seifert, R., & Schultz, G. (1987) *Eur. J. Biochem. 162*, 563-569.
Sha'ag, D., & Pick, E. (1988) *Biochim. Biophys. Acta 952*, 213-219.
Tanaka, T., Makino, R., Iizuka, T., Ishimura, Y., & Kanegasaki, S. (1988) *J. Biol. Chem. 263*, 13670-13676.
Volpp, B. D., Nauseef, W. M., & Clark, R. A. (1988) *Science 242*, 1295-1297.
Volpp, B. D., Nauseef, W. M., Dondson, J. E., Moser, D. R., & Clark, R. A. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 7195-7199.

# Kinetics of the Interaction of a 41-Kilodalton Macrophage Capping Protein with Actin: Promotion of Nucleation during Prolongation of the Lag Period†

Clarence L. Young, Frederick S. Southwick, and Annemarie Weber*
*Infectious Diseases Section, Department of Medicine, and Department of Biochemistry and Biophysics, University of Pennsylvania, Philadelphia, Pennsylvania 19104*
*Received June 21, 1989; Revised Manuscript Received October 25, 1989*

ABSTRACT: A 41-kilodalton macrophage capping protein (MCP) has been isolated which is capable of forming complexes with actin monomers in addition to capping the barbed ends of actin filaments (Southwick & DiNubile, 1986). The protein is calcium activated in a fully reversible manner. Using kinetic assays, we determined a capping constant, defined here as a modified $K_d$, of 1 nM and a $K_d$ of 3-4 $\mu$M for MCP-actin monomer complex formation. MCP weakly nucleates actin polymerization: more than 0.5 $\mu$M MCP is necessary to shorten the lag period, and 1 $\mu$M MCP at an actin/MCP ratio of 10 reduces the average length of actin filaments to about 200 molecules per filament. We determined that the actin nucleus that survives MCP inactivation contains a minimum number of five actin molecules. These experiments also make a point with respect to the interpretation of the prolongation of the lag period. We directly demonstrate that in the presence of an actin binding protein a prolongation of the lag period can be associated with increased nucleation, contrary to the usual interpretation in the literature that it indicates no or decreased nucleation by the actin binding protein.

Control of actin filament assembly plays an important role in such diverse physiological processes in nonmuscle cells as platelet activation, chemotaxis, phagocytosis, granule secretion, and cytokinesis [cf. Fox and Phillips (1983) and Korn (1978)]. A number of actin binding proteins are thought to participate in the regulation of filament assembly, and substantial progress has been made in recent years in elucidating the details of the interaction of some of these proteins with actin in vitro [cf. Pollard and Cooper (1986) and Stossel et al. (1985)]. Two classes of actin binding proteins may be of particular importance in regulating actin filament assembly: the monomer binding proteins, such as profilin, and the barbed end capping proteins. Among the latter, villin, which is found in the epithelial microvilli of intestines and kidney proximal tubules, and gelsolin, which is present in most cells and in mammalian blood, have been best characterized (Mooseker, 1985; Janmey et al., 1985; Kwiatkowski et al., 1988).

Recently, Southwick and DiNubile (1986) isolated a new capping protein from macrophage extracts, called here macrophage capping protein (MCP).[1] MCP is activated by calcium; in contrast to gelsolin and similar to villin, the calcium activation is rapidly and completely reversible. In contrast to both gelsolin and villin, this protein does not sever actin filaments. In order to better evaluate the biological potential

†Supported by Robert Wood Johnson Foundation Grant 12957 (C.L.Y.), National Institute of Allergy and Infectious Diseases Grant RO1 Al-23262-04 (F.S.S.), National Heart, Lung, and Blood Institute Grant HLB 15836 (A.W.) (Pennsylvania Muscle Institute), and National Institute of Digestive and Kidney Diseases Grant D.K. 25387 (Mark S. Mooseker).
*Author to whom correspondence should be addressed at the Department of Biochemistry and Biophysics, University of Pennsylvania.

[1] Abbreviations: ATP, adenosine 5'-triphosphate; EGTA, ethylene glycol bis($\beta$-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; MCP, macrophage capping protein.

0006-2960/90/0429-2232$02.50/0 