Kinetic Study of the Activation of the Neutrophil NADPH Oxidase by Arachidonic Acid. Antagonistic Effects of Arachidonic Acid and Phenylarsine Oxide†

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Received June 29, 1999; Revised Manuscript Received September 16, 1999

ABSTRACT: The O_2^- generating NADPH oxidase complex of neutrophils comprises two sets of components, namely a membrane-bound heterodimeric flavocytochrome b which contains the redox centers of the oxidase and water-soluble proteins of cytosolic origin which act as activating factors of the flavocytochrome. The NADPH oxidase can be activated in a cell-free system consisting of plasma membranes and cytosol from resting neutrophils in the presence of GTPγS and arachidonic acid. NADPH oxidase activation is inhibited by phenylarsine oxide (PAO), a sulfhydryl reagent for vicinal or proximal thiol groups. The site of action of PAO was localized by photolabeling in the β-subunit of flavocytochrome b [Doussière, J., Poinas, A, Blais, C., and Vignais, P. V. (1998) *Eur. J. Biochem. 251*, 649−658]. Moreover, the spin state of heme b is controlled by interaction of arachidonic acid with the flavocytochrome b [Doussière, J., Gaillard, J., and Vignais, P. V. (1996) *Biochemistry 35*, 13400−13410]. Here we report that the promoting effect of arachidonic acid on the activation of NADPH oxidase is due to specific binding of arachidonic acid to flavocytochrome b. Elicitation of NADPH oxidase activity by arachidonic acid is in part associated with an increased affinity of flavocytochrome b for O_2, an effect that was counteracted by the methyl ester of arachidonic acid. On the other hand, the affinity for NADPH was not affected by arachidonic acid. We further demonstrate that PAO antagonizes the effect of arachidonic acid on oxidase activation by decreasing the affinity of the oxidase for O_2, but not for NADPH. PAO induced a change in the spin state of heme b, as arachidonic acid does, with, however, some differences in the constraints imposed to the heme. It is concluded that the opposite effects of arachidonic acid and PAO are exerted on the β-subunit of flavocytochrome b at two different interacting sites.

In its activated form, the O_2^- generating NADPH oxidase of phagocytic cells is a protein complex comprising a membrane-bound heterodimeric flavocytochrome b and a set of water-soluble activation factors of cytosolic origin that include a monomeric G protein (Rac1 or Rac2) and a protein triad p47phox, p67phox, and p40phox (for review, see refs *1*−*4*). The NADPH oxidase activity, which is latent in circulating neutrophils and resting macrophages, is fully expressed when these cells are challenged by specific stimuli, for instance at the onset of phagocytosis. Several signaling pathways appear to control oxidase activation. One of them involves specific protein kinases and the phosphorylation of serine and threonine residues in the cytosolic factors p47phox (*5*), p67phox (*6*), and p40phox (*7*). On the other hand, evidence from recent studies suggests that arachidonic acid, which is released by the action of the cytosolic phospholipase A2 ($cPLA_2$)[1] in stimulated neutrophils (*8*), may activate NADPH oxidase (*9*−*12*). $cPLA_2$ itself is activated by phosphorylation under the control of MAP kinases (*13*, *14*). These results corroborated early reports on the inhibitory effect of *p*-bromophenacyl bromide, an inhibitor of PLA_2, on the production of O_2^- by activated neutrophils (*15*, *16*). A convenient method to study the mechanism of NADPH oxidase activation by arachidonic acid is to use a cell-free system consisting of membranes and cytosol from resting neutrophils, GTPγS, ATP, and $MgSO_4$ (for review, see ref *1*). We report here a study of the kinetic parameters of the NADPH oxidase, activated in a cell-free system by different concentrations of arachidonic acid. We demonstrate that arachidonic acid acts primarily on the membrane-bound flavocytochrome b by increasing the affinity of the oxidase for O_2 and that this effect is counteracted by the methyl ester derivative. We also show that phenylarsine oxide (PAO), a reagent of vicinal and proximal thiol groups, counteracts the enhancing effect of arachidonic acid on oxidase activation and specifically antagonizes the increase in O_2 affinity induced by arachidonic acid.

MATERIALS AND METHODS

Chemicals. NADPH, ATP, and GTPγS were from Boehringer, horse heart cytochrome *c*, arachidonic acid, and

† This work was supported by funds from the Centre National de la Recherche Scientifique, the Commissariat à l'Energie Atomique, and the Université Joseph Fourier - Grenoble I and by specific grants from the Association pour la Recherche sur le Cancer (9996) and the Direction des Systèmes des Forces et Prospectives (DGA 96/2558A).

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[1] Abbreviations: PAO, phenylarsine oxide; $cPLA_2$, cytosolic phospholipase A_2.

10.1021/bi991502w CCC: $18.00
Published on Web 11/13/1999

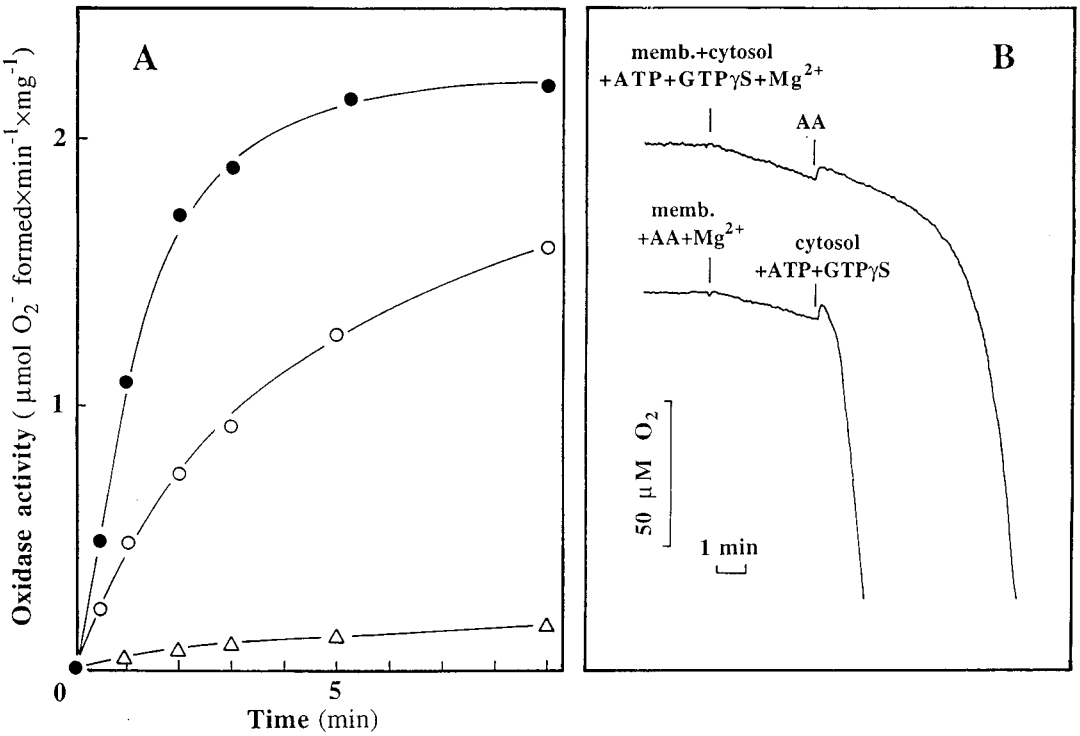

FIGURE 1: Effect of the sequence of preincubation of membranes and cytosol from resting neutrophils with arachidonic acid on oxidase activation. (A) Elicited oxidase activity measured by the rate of O_2^- production after different times of activation. (Top curve) Membranes from resting neutrophils (600 μg of protein) were preincubated for 5 min at 20 °C in 100 μL of PBS with arachidonic acid, using a ratio of arachidonic acid (μmol) to membrane protein (mg) of 1.2 found to be optimum in a parallel titration assay (●). Ten microliter aliquots were withdrawn and mixed with 30 μM GTPγS, 250 μM ATP, 2.5 mM $MgSO_4$, and cytosol from resting neutrophils, using a ratio of cytosolic protein to membrane protein of 10. The volume was adjusted to 0.1 mL with PBS. After different periods of time of activation in the complete system ranging from 30 s to 9 min, 50 μL aliquots of the mixtures were transferred to photometric cuvettes containing the assay medium consisting of 100 μM cytochrome *c*, 250 μM NADPH, 2.5 mM $MgSO_4$ in 2 mL of PBS. The rate of O_2^- production (micromoles of O_2^- formed per minute per milligram of membrane protein) was calculated from the rate of the superoxide-inhibitable reduction of cytochrome *c* at 550 nm. Inhibition of cytochrome *c* reduction by superoxide dismutase was more than 98%. Time zero on the abscissa corresponds to the addition of GTPγS, ATP, $MgSO_4$, and cytosol to the arachidonic acid pretreated membranes. (Middle curve) Same protocol as in the top curve, except that the preincubation of membranes with arachidonic acid was omitted. Membranes and cytosol, GTPγS, ATP and $MgSO_4$ were mixed together with arachidonic acid at time zero on the abscissa (○). (Bottom curve) The sequence of preincubations corresponding to oxidase activation in the top curve was reversed, i.e., arachidonic acid was incubated first with cytosol for 5 min. This was followed by addition of membranes, GTPγS, ATP, and $MgSO_4$, which corresponds to time zero on the abscissa (△). (B) Dependence of the elicited oxidase activity measured by the rate of O_2 uptake on the sequence of addition of arachidonic acid and the other components of the cell free system, namely neutrophil membranes (800 μg), neutrophil cytosol (ratio of cytosolic protein to membrane protein equal to 10), 30 μM GTPγS, 250 μM ATP, and 2.5 mM $MgSO_4$. Arachidonic acid was used at the optimal concentration of 1.2 μmol/mg membrane protein.

arachidonic methyl ester were from Sigma, and phenylarsine oxide was from Aldrich.

Biological Preparations. Bovine neutrophils were used as a source of a particulate fraction enriched in plasma membranes and a water soluble fraction referred as cytosol (*10*). The fractions were taken up in saline phosphate buffer (PBS) consisting of 2.7 mM KCl, 136.7 mM NaCl, 1.5 mM KH_2PO_4, and 8.1 mM Na_2HPO_4, pH 7.4. Protein concentration was assayed with the BCA reagent using bovine serum albumin as standard.

Oxidase Activity and Oxidase Activation. All experiments were conducted at a temperature of 20 °C. NADPH oxidase activity corresponding to the reaction, NADPH + 2 $O_2 \rightarrow$ $NADP^+ + 2\ O_2^- + H^+$, was assayed either by measurement of the production of the superoxide O_2^-, using a photometric assay based on the reduction of ferricytochrome *c* by O_2^- or by polarographic measurement of O_2 uptake, using a Clark electrode polarized at a voltage of 0.8 V. In both cases, the assay of oxidase activity was preceded by an activation step which consisted in mixing plasma membranes and cytosol prepared from resting neutrophils. Except when indicated, the cytosol to membrane protein ratio was adjusted to a value of 10 or close to 10. The mixture was supplemented with 2.5 mM $MgSO_4$, 30 μM GTPγS, 250 μM ATP, and an optimal amount of arachidonic acid predetermined in a parallel titration assay to elicit maximal oxidase activity. This optimal amount of arachidonic acid depended on membrane preparations and ranged between 1.0 and 1.4 μmol/mg of membrane protein. Following the activation step, oxidase activity was assayed. When the oxidase activity was assayed by the rate of production of O_2^-, a 20 μL aliquot of the suspension of activated particles containing 20−40 μg of membrane protein were transferred to a photometric cuvette containing 100 μM cytochrome *c* in 2 mL of air-saturated PBS in which the O_2 concentration (230 μM) was saturating for the NADPH oxidase complex. The oxidase reaction was initiated by addition of NADPH at final concentration of 250 μM. Cytochrome *c* reduction was recorded at 550 nm for 1−2 min, and 50 μg of superoxide dismutase was added to quench O_2^-. The residual reduction of cytochrome *c* was recorded for 2−3 min. With our preparations of neutrophil plasma membranes, cytochrome *c* reduction was virtually

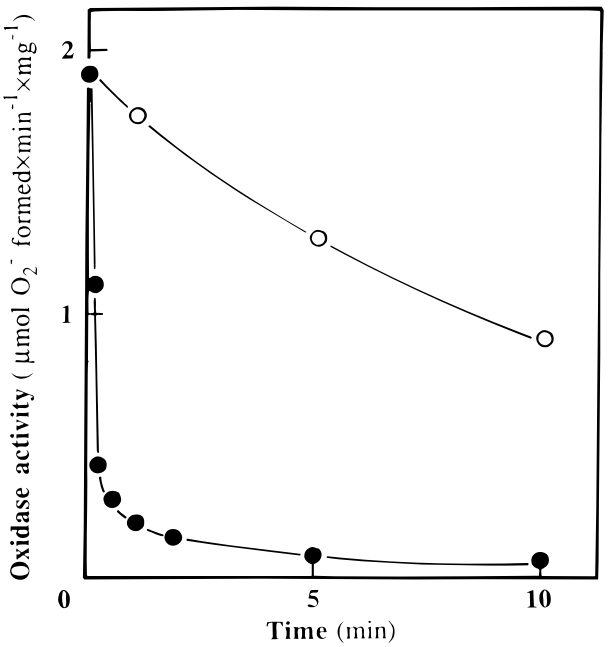

FIGURE 2: Time course of the inhibition of oxidase activation by preincubation of cytosol with arachidonic acid. Preincubation of arachidonic acid with neutrophil cytosol was carried out at 20 °C (●) as described in Figure 1, bottom curve, or at 0 °C (○) for different periods of time, up to 10 min as indicated on the abscissa. After the preincubation step, the medium was supplemented with neutrophil membranes, using a cytosol to membrane protein ratio of 10, 30 μM GTPγS, 250 μM ATP, and 2.5 mM $MgSO_4$. After 10 min of incubation at 20 °C, the oxidase activity was assayed by the production of O_2^- as in the experiment of Figure 1.

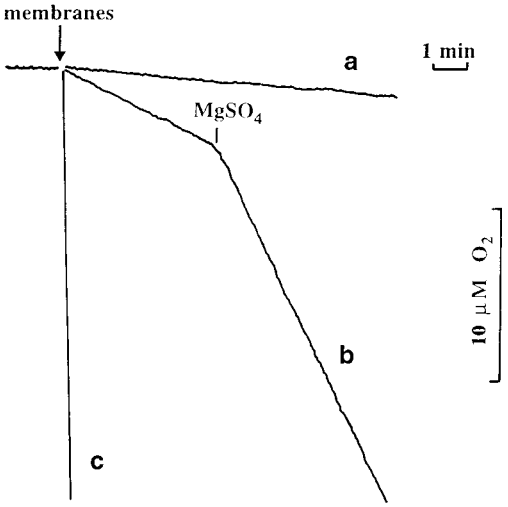

FIGURE 3: Direct activation of the membrane-bound flavocytochrome b by arachidonic acid. Oxygen uptake was monitored with an oxygraph and a Clark electrode (cf. Materials and Methods). Three membrane samples corresponding to 800 μg of protein were tested for oxidase activity in PBS supplemented with 250 μM NADPH. The first membrane sample was used as a control (trace a). The second membrane sample was treated with the optimal concentration of arachidonic acid (1 μmol/mg membrange protein) for 5 min at 20 °C before the assay of O_2 uptake (trace b). As indicated on trace b, 2.5 mM $MgSO_4$ was added during the course of O_2 uptake. The third membrane sample was supplemented with cytosol (cytosol to membrane protein ratio of 10), 30 μM GTPγS, 250 μM ATP, 2.5 mM $MgSO_4$ and arachidonic acid (1 μmol/mg membrane protein); incubation lasted for 10 min before the assay of O_2 uptake (trace c). For convenience, traces a, b and c were assembled on the same graph.

fully quenched upon addition of superoxide dismutase. The K_M NADPH was determined under the above conditions except that the concentration of NADPH was varied. When the oxidase activity was assayed by the rate of O_2 uptake, the suspension of activated particles was transferred to an oxygraphic cuvette containing 1.5 mL of PBS supplemented with NADPH at saturating concentration (250 μM). For convenience of measurement, the amount of membranes used in the oxygraphic assay was at least 20 times higher than that used in the photometric assay. For measurement of the K_M O_2, the O_2 concentration in the aerated PBS medium contained in the oxygraphic cuvette was decreased 60−70% by controlled N_2 bubbling prior to the addition of NADPH. Below 50−40 μM, the oxygraphic traces curved inward. The rates of O_2 uptake were deduced from the slopes of the tangents to the oxygraphic traces, and the contact points of the tangents with the curves were used to determine the mean average of the O_2 concentrations at which O_2 uptake proceeds (*10*). Both data were used to calculate the K_M values.

In a number of experiments, either the membrane fraction or the cytosolic fraction was incubated separately with arachidonic acid in the presence or absence of $MgSO_4$ for 5 min, and this was followed by addition of GTPγS, ATP, $MgSO_4$, and the complementary fraction, cytosol or membranes, respectively. In most of the cases, after a further 10 min incubation, oxidase activity was assayed. In some experiments, oxidase activity was assayed on the membrane fraction alone incubated for 5 min with arachidonic acid, with or without $MgSO_4$.

EPR Spectroscopy. EPR spectra were recorded with an X-band Bruker EMX spectrometer equipped with an Oxford Instrument ESR-900 continuous-flow helium cryostat. The reaction mixtures containing the neutrophil membranes were transferred to EPR quartz tubes.

RESULTS

Preincubation of Neutrophil Membranes with Arachidonic Acid Prior to the Addition of Cytosol, GTPγS and ATP Accelerates the Time Course of NADPH Oxidase Activation. In the cell-free system assay commonly used to promote NADPH oxidase activation, referred to here as routine cell-free system, membranes and cytosol from resting neutrophils are mixed with GTPγS, ATP, magnesium ions, and an anionic amphiphile. Although ATP is not an absolute requirement for the expression of the respiratory burst in the cell-free assay of oxidase activation, its addition resulted in our system in a 10−20% increase in the elicited superoxide production. Arachidonic acid has proved to be one of the most efficient amphiphilic activators (*17*−*23*). Maximal oxidase activation depends on an optimal concentration of arachidonic acid (*24*). After a few min of incubation at room temperature with arachidonic acid to allow oxidase activation to proceed to completion, the elicited NADPH oxidase activity was assessed by measuring the rate of O_2 uptake and the rate of O_2^- production (cf. Materials and Methods). With the particulate neutrophil fraction used, enriched in plasma membranes, the rate of O_2^- release was virtually the same as that of O_2 uptake.

To determine which of the two subcellular fractions, membranes or cytosol, was the preferential target responsible for the activating effect of arachidonic acid on NADPH

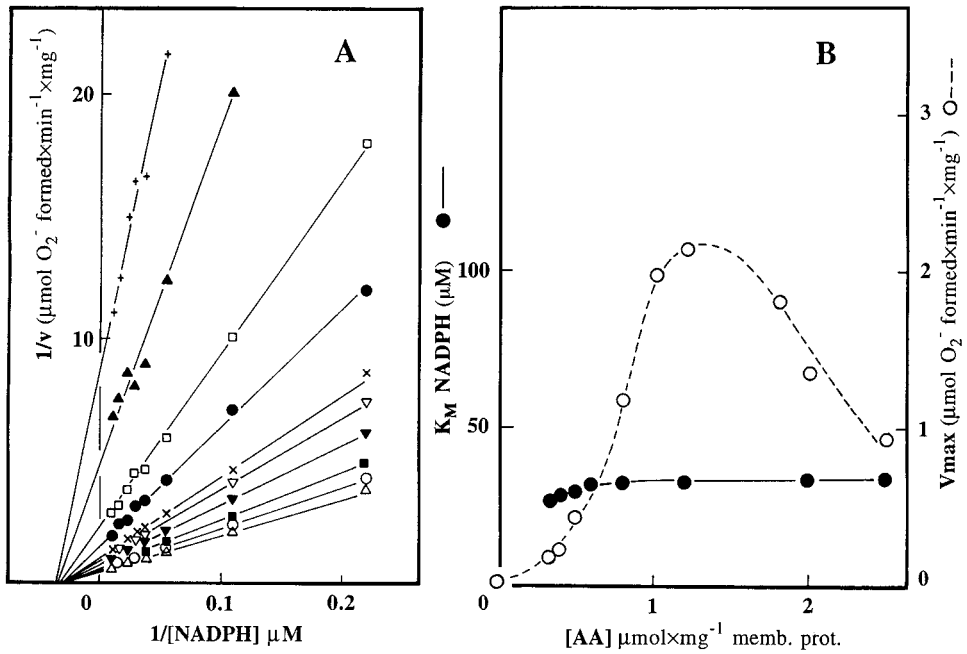

FIGURE 4: Effect of various fixed concentrations of arachidonic acid on the kinetic parameters of the elicited oxidase activity, using NADPH as varying substrate and an air-saturated medium. (A) The neutrophil membrane suspension was fractionated in a number of samples. Each of them was pretreated for 5 min with 2.5 mM $MgSO_4$ and arachidonic acid, using for each sample a fixed ratio of arachidonic acid (μmol) to membrane protein (mg), ranging 0.35−2.50 (0.35 (+); 0.40 (▲); 0.50 (□); 0.60 (●); 0.80 (▽); 1.0 (○); 1.20 (△); 1.80 (■); 2.00 (▼); 2.50 (×)].This preincubation step was followed by addition of 30 μM GTPγS, 250 μM ATP, and cytosol, using a cytosol to membrane protein ratio of 10. After 10 min, aliquots of the mixture were assayed for the production of O_2^-, with different concentrations of NADPH in an air-saturated PBS medium (O_2 = 230 μM), using the SOD-inhibitable reduction of cytochrome *c* (cf. Materials and Methods). (B) The calculated V_{max} and K_M values from Figure 4A were replotted as a function of the ratios of arachidonic acid (μmol) to membrane protein (mg).

oxidase, we set up a protocol in which arachidonic acid was first preincubated for 5 min at 20 °C with either membranes or cytosol from resting neutrophils (Figure 1A, top and bottom traces). The amount of added arachidonic acid in the preincubation step was the same as that which elicited maximal oxidase activity in the routine cell-free system. The mixture was then supplemented with the complementary subcellular fraction, GTPγS, ATP and $MgSO_4$ and after different periods of time, aliquot fractions were withdrawn and oxidase activity was assayed by the rate of production of O_2^- at 20 °C. When membranes were preincubated first with arachidonic acid, followed after 5 min by cytosol, GTPγS, ATP and $MgSO_4$, oxidase activation was markedly accelerated, and the level of oxidase activity was also increased (Figure 1A, top trace), compared to the routine assay, in which arachidonic acid was mixed together with neutrophil membranes, cytosol, GTPγS, ATP, and $MgSO_4$ (Figure 1, middle trace). A similar level of oxidase activity was obtained after preincubation of membranes with arachidonic acid alone as in Figure 1A, top trace, or with arachidonic acid together with GTPγS, ATP, and $MgSO_4$ (not shown). When cytosol was first preincubated with arachidonic acid, followed by addition of membranes, GTPγS, ATP, and $MgSO_4$, oxidase activation was markedly decreased (Figure 1A, bottom trace). In the oxygraphic experiment illustrated in Figure 1B, the time course of O_2 uptake was recorded in two conditions which differed by the order of addition of arachidonic acid with respect to the other components of the cell-free system. Preincubation of neutrophil membranes with arachidonic acid and $MgSO_4$ resulted in quasi immediate O_2 uptake upon the further addition of cytosol, GTPγS, and ATP. In contrast, upon preincubation of neutrophil membranes with cytosol, GTPγS, ATP, and $MgSO_4$, followed by that of arachidonic acid, the maximal rate of O_2 uptake was observed only after a delay of 4−5 min. In summary, the order of addition of arachidonic acid with respect to neutrophil membranes and cytosol is critical for elicitation of the NADPH oxidase activity. Taken together, the results of experiments of Figure 1 indicate that a specific target for arachidonic acid acting as activator of NADPH oxidase is located in the membrane fraction.

The deleterious effect of preincubation of arachidonic acid with neutrophil cytosol was analyzed in more detail by studying the time course of this process (Figure 2). At 20 °C, a 1 min contact of cytosol with arachidonic acid led to more than 75% loss of oxidase activation; at 0 °C, half decrease required about 10 min. Even at a concentration of arachidonic acid 10 times lower, loss of oxidase activation, although less rapid, was still quite noticeable, of the order of 10−20%/min at 20 °C.

Assuming that the membrane-bound component of NADPH oxidase is a target site which accounts for the promoting effect of arachidonic acid on oxidase activation, we reasoned that preincubation of arachidonic acid for a few minutes with neutrophil membranes alone, in the absence of cytosol, should result in the emergence of some oxidase activity due to direct activation of flavocytochrome b by arachidonic acid. The oxygraphic experiment illustrated in Figure 3 (trace b) shows that this is the case. Activation by arachidonic acid was significantly enhanced without delay by addition of magnesium ions (Figure 3, trace b), but not by GTPγS nor ATP (not shown). In the absence of arachidonic acid, whether $MgSO_4$ was present, O_2 uptake was hardly detected (Figure 3, trace a). The oxidase activity of neutrophil membranes

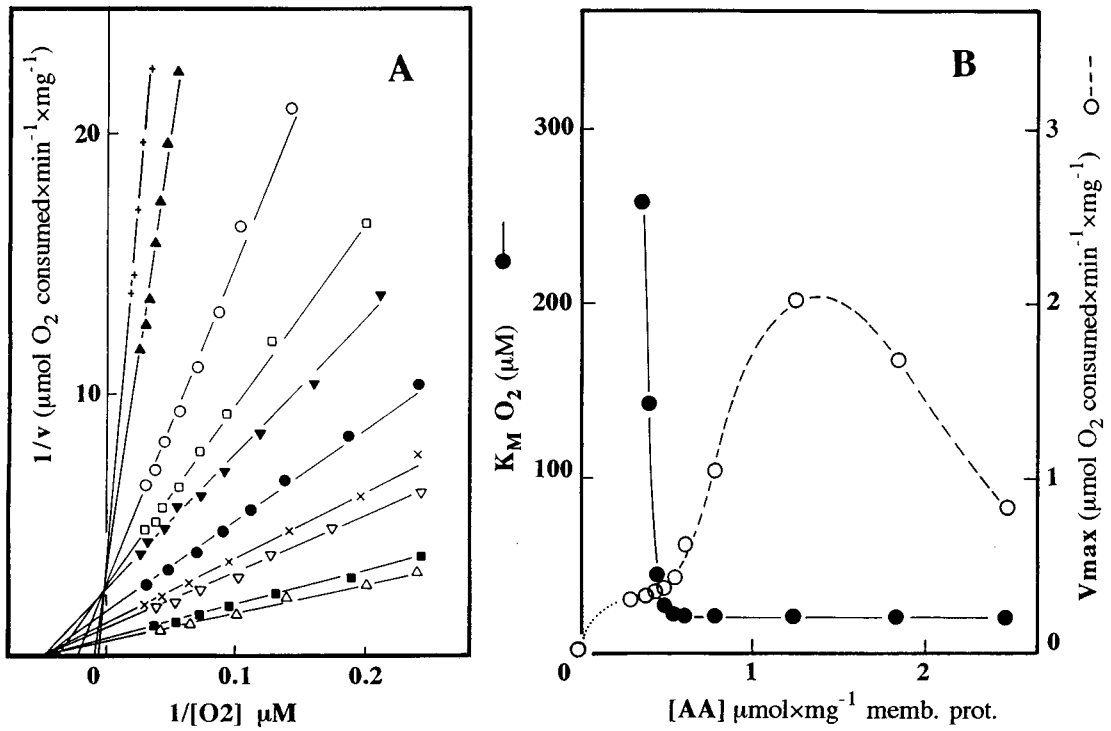

FIGURE 5: Effect of various fixed concentrations of arachidonic acid on the kinetic parameters of the elicited oxidase activity, using O_2 as varying substrate and NADPH at a saturating level. (A) Oxidase activation was carried out using a 5 min preincubation step with different concentrations of arachidonic acid, in the presence of 2.5 mM $MgSO_4$ as in Figure 4. The following ratios of arachidonic acid (μmol) to membrane protein (mg) were used: 0.35 (+); 0.40 (▲); 0.45 (○); 0.50 (□); 0.55 (▼); 0.60 (●); 0.80 (▽); 1.20 (△); 1.80 (■); 2.50 (×). This was followed by addition of 30 μM GTPγS, 250 μM ATP, and cytosol, using a cytosol to membrane protein ratio of 10. After 10 min, the elicited oxidase for each ratio of arachidonic acid to membrane protein was assayed by oxygraphy, using NADPH (250 μM) as electron donor in the absence of cytochrome *c* (cf. Materials and Methods). The initial O_2 concentration (230 μM) of the medium was decreased by two-thirds or more by controlled bubbling of nitrogen prior to the assay of O_2 uptake. The rate of O_2 uptake was calculated from the slopes of the tangents to the oxygraphic traces, and the K_M O_2 values were calculated as described in Materials and Methods. (B) The calculated V_{max} (○) and K_M (●) values from Figure 5A were replotted as a function of the ratios of arachidonic acid (μmol) to membrane protein (mg).

challenged with arachidonic acid in the absence of cytosol was, however, much lower than that elicited with the complete cell-free system including cytosol, GTPγS, and ATP (Figure 3, trace c). Since, in these experiments, large amounts of neutrophil membranes were used, we included a control with 1 mM sodium azide, an inhibitor of the mitochondrial cytochrome *c* oxidase (not shown). No modification in the rate of O_2 uptake was observed, excluding any contribution of the mitochondrial respiration

Effect of Varying the Concentration of Arachidonic Acid Preincubated with Neutrophil Membranes on the K_M O_2 and the K_M NADPH of the Reconstituted NADPH Oxidase. In the following experiments illustrated by Figures 4 and 5, samples of neutrophil membranes were preincubated for 5 min with $MgSO_4$ and different fixed concentrations of arachidonic acid. The mixtures were then supplemented with GTPγS, ATP, and cytosol, using a cytosol to membrane protein ratio of 10, to fully reconstitute NADPH oxidase activity. After 10 min, the elicited oxidase activity was assayed for the V_{max} values, the K_M NADPH, and the K_M O_2. Under these conditions, maximal oxidase activity was elicited for a ratio of arachidonic acid (μmol) to membrane protein (mg) of 1.2−1.3.

The oxidase activity followed by the production of O_2^- was measured photometrically by the reduction of cytochrome *c* in an air-saturated medium, in the presence of increasing concentrations of NADPH (Figure 4A). The reciprocal plots of the rate of O_2^- production by NADPH oxidase activated by different levels of arachidonic acid vs the NADPH concentration intersected the 1/[NADPH] axis in a close range of values from which a K_M NADPH of 26−33 μM could be calculated. In a parallel experiment (Figure 5A), the oxidase activity was measured polarographically by the rate of O_2 uptake at different concentrations of O_2 below 80−60 μM until anaerobiosis was attained, using a saturating concentration of NADPH (cf. Materials and Methods). When the amount of arachidonic acid preincubated with the neutrophil membranes increased from 0.35 to 0.60 μmol/mg of membrane protein, the reciprocal plots were found to pivot clockwise about the same point of intersection slightly on the left of the 1/*v* axis above the 1/[O_2] axis. The V_{max} was slightly increased, and the K_M O_2 was decreased by about 10-fold from more than 200 to 20 μM. When the level of arachidonic acid in the preincubation step was increased to values higher than 0.60 μmol/mg membrane protein to the optimal value of 1.2−1.3 μmol/mg membrane protein, the reciprocal plots converged to the same point on the 1/[O_2] axis, corresponding to a value of K_M O_2 of 20 μM, and at the same time, the V_{max} increased to a value of 2 μmol of O_2 uptake/min/mg membrane protein. For concentrations of arachidonic acid higher than the optimal concentration, the V_{max} for O_2 uptake decreased, but the K_M O_2 was not modified. This observation holds in the case of Figure 4A where the varying parameter was NADPH.

From the data of Figures 4A and 5A, the same bell-shaped curves for the NADPH oxidase activities were obtained as

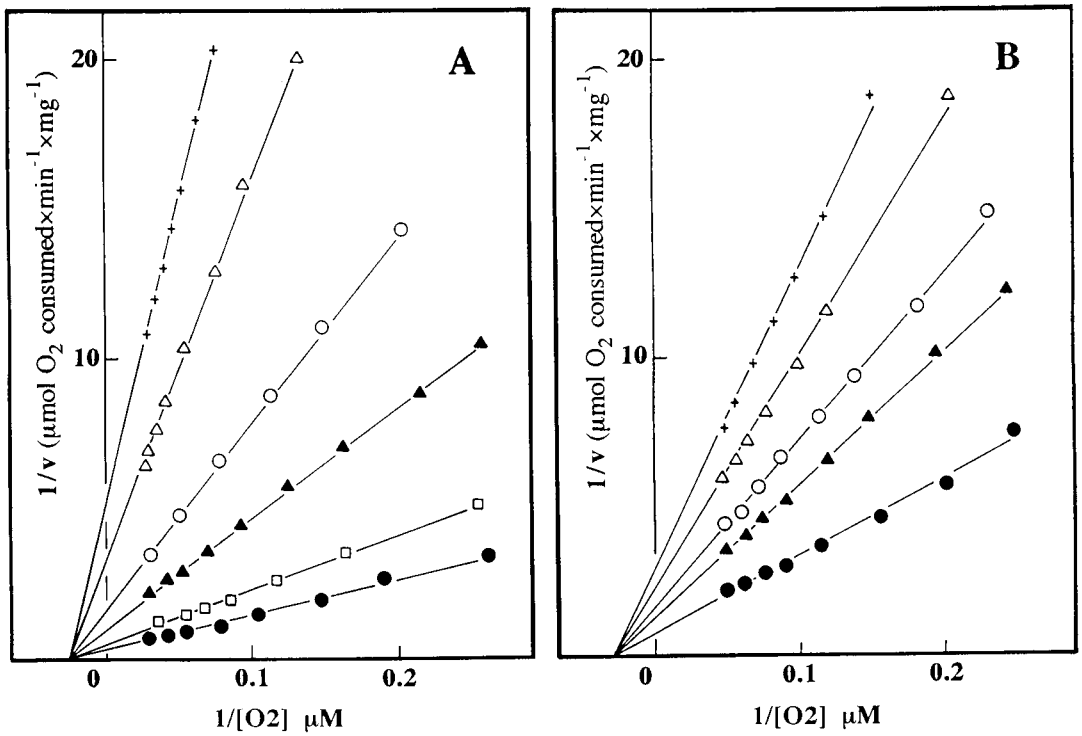

FIGURE 6: Dependence of the kinetic parameters of the NADPH oxidase activated by arachidonic acid on the cytosol to membrane protein ratio and the GTPγS concentration. (A) Neutrophil membranes were preincubated for 5 min with 2.5 mM $MgSO_4$ and an optimal amount of arachidonic acid (1.2 μmol/mg membrane protein). This was followed by addition of 30 μM GTPγS, 250 μM ATP, and increasing amounts of cytosol, so that the cytosol to membrane protein ratio was raised from 2.5 to 10.0 [2.5 (+); 3.5 (△); 4.2 (○); 5.0 (▲); 7.0 (□); 10 (●)]. After 10 min, the elicited oxidase was assayed by the rate of O_2 uptake as in Figure 5. (B) The experimental protocol was similar to that of Figure 6A, except that the varying parameter was the GTPγS concentration that was raised from 0 to 30 μM [0 (+); 0.2 (△); 0.7 (○); 3 (▲); 30 (●)], and that the cytosol to membrane protein ratio was 10.

a function of the amount of arachidonic acid present in the preincubation step, whatever the mode of assay used, i.e., O_2^- production or O_2 uptake with maximal oxidase activity elicited for the same ratio of arachidonic acid (μmol) to membrane protein (mg) (Figures 4B and 5B). However, the K_M O_2 and the K_M NADPH were differently affected by low levels of arachidonic acid corresponding to a range of ratios of arachidonic acid (μmol) to membrane protein (mg) of 0.35−0.55, with no effect on the K_M NADPH and a marked effect on the K_M O_2. The dual effect of arachidonic acid at low concentrations on the K_M O_2 and at high concentrations on the V_{max} of NADPH oxidase is noteworthy. The requirement of both cytosol and high concentrations of arachidonic acid for maximal activity of NADPH oxidase might be explained by a rate-limiting step in electron transfer between NADPH and heme b within flavocytochrome b. In the presence of high concentrations of arachidonic acid and cytosolic factors, the electron transfer between NADPH and heme b would not be rate limiting any longer.

In two additional experiments, neutrophil membranes were preincubated with the optimal concentration of arachidonic acid, the varying parameters being the cytosol to membrane protein ratio and the GTPγS concentration. In both cases, the reciprocal plots relative to O_2 uptake intersected the 1/[O_2] axis at the same point (Figure 6), from which a K_M value of 30−20 μM could be calculated. These kinetics differed from those obtained by varying the arachidonic acid levels, suggesting that the cytosolic factors and GTPγS on one hand and arachidonic acid on the other act at two different sites on flavocytochrome b.

Inhibitory Effect of Arachidonic Acid Methyl Ester on NADPH Oxidase Activation. The free carboxylic group of arachidonic acid is essential for the functioning of arachidonic acid as an activator of the NADPH oxidase (*10*, *21*, *23*). As shown in Figure 7, the methyl ester of arachidonic acid was not only unable to elicit oxidase activation, but it counteracted the oxidase activation elicited by arachidonic acid. Since arachidonic acid had a strong effect on the K_M O_2 of NADPH oxidase (cf. Figure 5), we tested whether arachidonic acid methyl ester antagonized the effect of arachidonic acid on the affinity of the reconstituted oxidase for O_2. Neutrophil membranes were incubated with various fixed concentrations of arachidonic acid methyl ester and the optimal concentration of arachidonic acid for 5 min. This was followed by addition of cytosol, GTPγS, ATP, and $MgSO_4$. After 10 min, the rate of O_2 uptake was measured. The corresponding reciprocal plots are illustrated in Figure 8A. Interestingly, at low concentrations of arachidonic acid methyl ester (<0.2 μmol/mg membrane protein), only the K_M O_2 was modified, increasing from 20 μM to nearly 100 μM. Above 0.3 μmol/mg membrane protein, arachidonic acid methyl ester modified only the V_{max}, decreasing its value. The dual effect of arachidonic acid methyl ester on the V_{max} of O_2 uptake and the K_M O_2 is illustrated in Figure 8B. It appears to be the mirror image of the activating effect of arachidonic acid on oxidase activation (Figure 5B), suggesting that inhibition of oxidase activation by arachidonic acid methyl ester results from direct substitution at the target site of arachidonic acid.

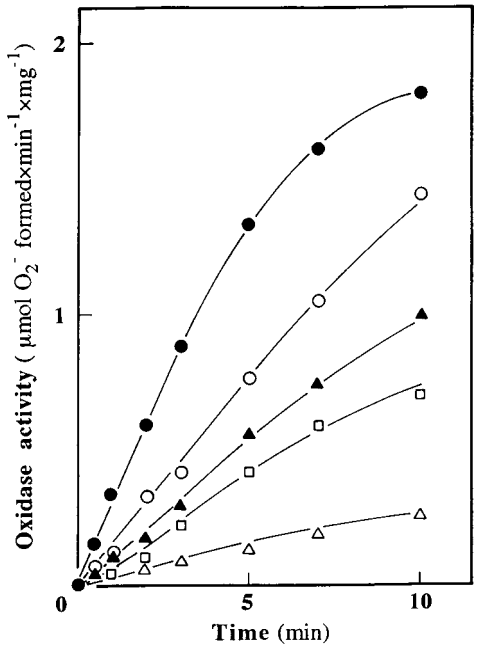

FIGURE 7: Inhibitory effect of arachidonic acid methyl ester on the arachidonic acid-dependent activation of NADPH oxidase. Neutrophil membranes were preincubated for 5 min at 20 °C in the presence of 2.5 mM $MgSO_4$ with the optimal amount of arachidonic acid for maximal oxidase activation (1.2 μmol/mg membrane protein) and various amounts of arachidonic acid methyl ester up to 0.8 μmol/mg membrane protein [zero (●); 0.1 (○); 0.2 (▲); 0.4 (□); 0.8 (△)]. This was followed by addition of cytosol, using a cytosol to membrane protein ratio of 10, 30 μM GTPγS, and 250 μM ATP, and incubation for different periods of time up to 10 min, as indicated in the abscissa of the Figure. Then, oxidase activity was determined by the rate of O_2^- production (cf. Materials and Methods).

Effect of Varying the Concentration of Arachidonic Acid on Oxidase Activation, Using the Neutrophil Membrane Fraction Alone, in the Absence of Neutrophil Cytosol. In the preceding experiments, O_2 uptake was assayed using a fully reconstituted oxidase complex, in which membranes preincubated with arachidonic acid and $MgSO_4$ were supplemented with cytosol, GTPγS, and ATP. The experiment of Figure 9 shows that, in a simple system consisting of neutrophil membranes and $MgSO_4$, in the absence of cytosol, arachidonic acid also acted as an activator of NADPH oxidase, although the rate of O_2 uptake was more than 100 times lower than in the presence of cytosol, GTPγS, and ATP. Following incubation of membranes with various fixed concentrations of arachidonic acid for 5 min, O_2 uptake was measured polarographically. The reciprocal plots of the rates of O_2 uptake as a function of O_2 concentration exhibited curvatures that could be interpreted either by the presence of two different enzymes participating in O_2 uptake or by negative cooperativity. Contamination by cytochrome oxidase was excluded since sodium azide had no effect on the shape of the curves. Since the shapes of the curves were similar at all concentrations of arachidonic acid, the negative cooperativity explanation appeared to be a plausible one, leading us to infer that addition of cytosol to membranes induces the transition to Michaelian kinetics. The linear portions of the curves corresponding to O_2 concentrations higher than 30 μM intersected at a point above the $1/[O_2]$ axis slightly on the left of the $1/v$ axis as in Figure 5A in the case of oxidase activation by low concentrations of arachidonic acid. As the concentration of arachidonic acid increased, the plots pivoted clockwise about the point of intersection. For the highest oxidase activation, the V_{max} value for O_2 uptake amounted to 20 nmol/mg protein/min, and a K_M O_2 of 30 μM could be calculated. Thus, whereas the V_{max} value was strongly decreased with the neutrophil membranes alone, compared to the fully reconstituted system with membranes plus cytosol, the K_M O_2 for maximal oxidase activation was virtually the same in the two systems, which again points to the role played by arachidonic acid in the control of the affinity of the membrane-bound flavocytochrome b for O_2. Decreasing the concentration of arachidonic acid preincubated with membranes resulted in a decreased affinity for O_2 and a decreased velocity of O_2 uptake. For concentrations of O_2 lower than 30 μM, the plots could be resolved into a family of parallel lines corresponding to V_{max} values for O_2 uptake lower than 10 nmol/mg protein/min and K_M O_2 values lower than 10 μM. In all the assays, the medium was supplemented with $MgSO_4$. Omission of $MgSO_4$ decreased the activating effect of arachidonic acid by 4−5-fold (not shown).

The results of the experiments on NADPH oxidase activation presented in the above sections led us to hypothesize that arachidonic acid and the cytosolic factors trigger oxidase activation by interacting with different regions of flavocytochrome b. The effect of arachidonic acid is to increase the affinity of the heme component of flavocytochrome b for O_2, whereas the effect of the cytosolic factors is rather directed to the control of the turnover of flavocytochrome b. Nevertheless, the enhancing effect of the cytosolic factors on oxidase activation requires a conformation of flavocytochrome b capable of reacting with O_2, and therefore a prior contact with arachidonic acid.

Antagonistic Effects of Phenylarsine Oxide and Arachidonic Acid on the Kinetic Parameters of the Activated NADPH Oxidase Relative to O_2 Uptake. Inhibition of NADPH oxidase activation by PAO, a reagent of vicinal or proximal thiol groups in proteins, is due to the binding of PAO to the β-subunit of flavocytochrome b (*25*). Once activated, the NADPH oxidase is no longer inhibited by PAO (*25*, *26*). In the following experiment, neutrophil membranes were incubated with various fixed concentrations of PAO for 5 min. Then the oxidase was activated using the routine cell-free system, in which membranes are mixed with arachidonic acid, cytosol, GTPγS, ATP, and $MgSO_4$. The amount of arachidonic acid used in all the conditions was chosen as that eliciting maximal oxidase activation in the absence of PAO. Oxidase activity was then assayed either by the rate of production of O_2^- in an air-saturated medium at increasing concentrations of NADPH (Figure 10A) or by the rate of O_2 uptake in the presence of a saturating level of NADPH and at different concentrations of O_2 (Figure 10B). Striking differences were apparent in the reciprocal plots of oxidase activity depending on the oxidase assay used. In the first case (Figure 10A), the reciprocal plots intersected the 1/[NADPH] axis at a common intercept and the $1/v$ axis at different intercepts, a feature typical of a noncompetitive inhibition. In the second case (Figure 10B), the reciprocal plots intersected the $1/[O_2]$ axis at different points and the $1/v$ axis at a common intercept, a feature typical of a competitive inhibition. These results suggest that the O_2-binding site of flavocytochrome b loses progressively its

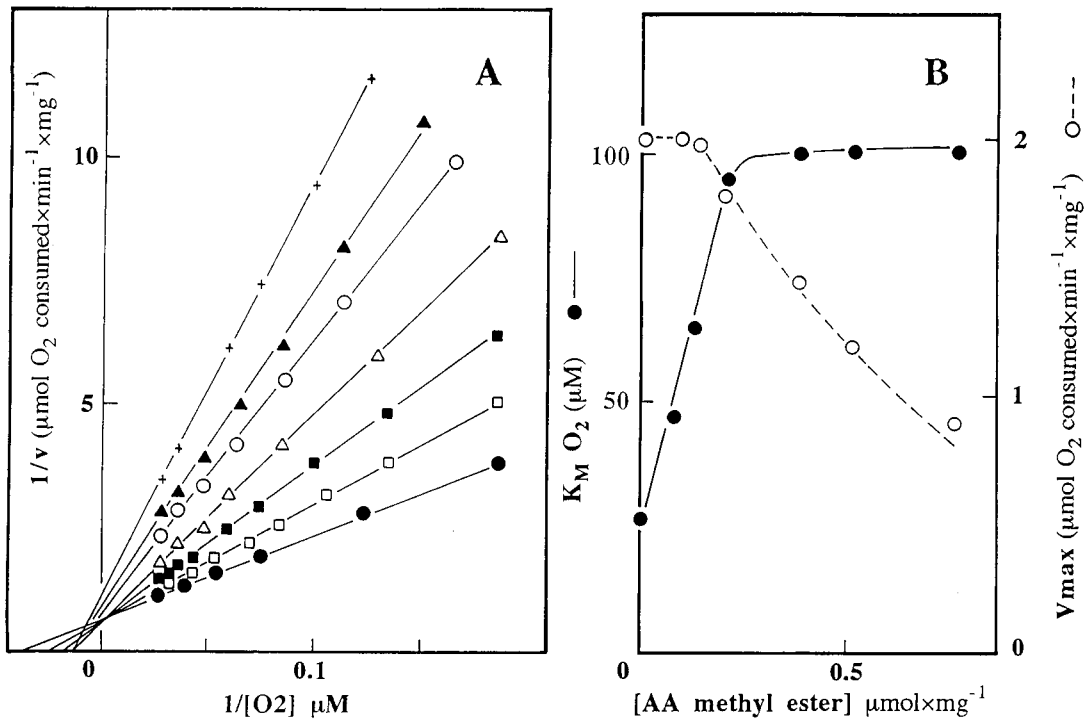

FIGURE 8: Kinetic parameters of the inhibition of oxidase activation by arachidonic acid methyl ester. (A) The neutrophil membrane suspension was fractionated in a number of samples. Each of them was pretreated for 5 min in the presence of 2.5 mM $MgSO_4$ with a different fixed concentration of arachidonic acid methyl ester and a same fixed concentration of arachidonic acid. The ratios of arachidonic acid methyl ester (μmol) to membrane protein (mg) ranged between 0 and 0.80 [zero (●); 0.1 (□); 0.15 (■); 0.20 (△); 0.40 (○); 0.50 (▲); 0.80 (+)]. Arachidonic acid in all membrane samples was used at the optimal concentration determined for control membranes in the absence of arachidonic acid methyl ester [ratio of arachidonic acid (μmol) to membrane protein (mg) of 1.0]. This was followed by addition of 30 μM GTPγS, 250 μM ATP, and cytosol, using a cytosol to membrane protein ratio of 10. After 10 min, the elicited oxidase activity was assayed by oxygraphy, using NADPH (250 μM) as electron donor in the absence of cytochrome *c*. The rate of O_2 uptake was determined for different concentrations of O_2, as described in Figure 5A. (B) The calculated values of V_{max} (○) and K_M O_2 (●) from Figure 8A were replotted as a function of the ratios of arachidonic acid methyl ester (μmol) to membrane protein (mg) used in the preincubation step.

affinity for O_2 upon the binding of increasing amounts of PAO. Most likely, PAO modified the O_2 affinity by an indirect effect on the heme, since it was unable to prevent the binding of butylisocyanide to heme b assessed by the red-shift of the Soret band at 425 nm in optical spectrum (not shown). This is in contrast with the inhibitory effect of arachidonic acid methyl ester on the binding of butylisocyanide to the heme component of flavocytochrome b (*10*).

In the experiment illustrated in Figure 11, neutrophil membranes were pretreated by various fixed concentrations of arachidonic acid for 5 min and for another 3 min by increasing concentrations of PAO. The mixture was then supplemented with cytosol, GTPγS, ATP, and additional arachidonic acid to attain the optimal concentration. Oxidase activation was allowed to proceed for 10 min, and the oxidase activity was measured by O_2^- production, using an air-saturated medium and a saturating concentration of NADPH. In accordance with the results of Figure 1, preincubation of the membranes with increasing concentrations of arachidonic acid (up to 1.4 μmol/mg of membrane protein), in the absence of PAO, enhanced the elicited oxidase activity, doubling the rate of O_2^- production. As shown by the different curves corresponding to the various fixed concentrations of arachidonic acid, PAO antagonized oxidase activation brought about by arachidonic acid in an apparent competitive manner. For concentrations of arachidonic acid in the preincubation medium ranging 0.2−1.4 μmol/mg membrane protein, the Dixon plots of the elicited oxidase activity vs the PAO concentration were characterized by straight lines intersecting at a point corresponding to a K_i value of 2 nmol of PAO/mg of membrane protein (Insert of Figure 11). When arachidonic acid was omitted in the preincubation medium and added later with cytosol, GTPγS, and ATP, the Dixon plots departed from linearity. Thus, in the absence of arachidonic acid, the membrane-bound flavocytochrome b displayed a heterogeneous sensitivity to PAO, probably due to the presence of two populations of flavocytochrome b differing by their affinity for PAO. Arachidonic acid at concentrations as low as 0.2 μmol/mg membrane protein appeared to induce a shift toward a single population of flavocytochrome b with an homogeneous sensitivity to PAO.

EPR Spectra of Neutrophil Membranes Treated by Arachidonic Acid and PAO. It was previously reported (*10*) that neutrophil membranes prepared from resting neutrophils exhibit, in their oxidized state, a low-spin signal at $g = 3.26$ typical of the hexacoordinated state of the heme iron in the nonactivated flavocytochrome b and that upon addition of arachidonic acid, the $g = 3.26$ signal disappears and two new signals emerged, a high-spin signal at $g = 6.00$ and a low-spin signal at $g = 2.20$. Figure 12 shows the low magnetic field region corresponding to control neutrophil membranes (trace a) and to membranes treated with an optimal amount of arachidonic acid (1.2 μmol/mg of protein) (trace b). Trace b shows the $g = 6.00$ signal typical of the heme of activated flavocytochrome b. The signals at $g = 6.85$ and 5.02 are characteristic of contaminant myeloperoxidase. Comparatively to arachidonic acid, treatment of

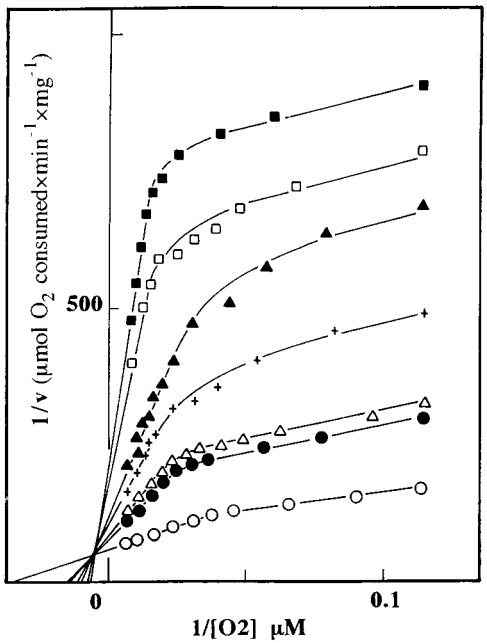

FIGURE 9: Effect of varying the concentration of arachidonic acid on oxidase activation, using neutrophil membranes in the absence of cytosol. Samples of neutrophil membranes (4 mg of protein) were preincubated for 5 min with 2.5 mM $MgSO_4$ and different fixed concentrations of arachidonic acid, corresponding to arachidonic acid (μmol) to membrane protein (mg) ratios ranging between 0.1 and 1.0 [0.14 (■); 0.21 (□); 0.30 (▲); 0.40 (+); 0.50 (△); 0.60 (●); 0.70 (○)]. The elicited oxidase activity was determined by the rate of O_2 uptake using NADPH (250 μM) as electron donor in the absence of cytochrome *c*, as described in Figure 5A.

neutrophil membranes by an amount of PAO capable of fully inhibiting oxidase activation (40 nmol/mg of protein) was found to generate a high-spin signal with two components at $g = 6.19$ and 5.80 (trace c). This indicates that PAO induces a change in the spin state of heme b, as arachidonic acid does, with, however, some differences in the constraint imposed to the heme. Upon addition of arachidonic acid to neutrophil membranes pretreated by PAO, the splitting between the two *g* values at 6.19 and 5.80 increased, and a more rhombic signal with *g* values of 6.30 and 5.50 emerged together with a nearly axial signal at $g = 6.01$, typical of the binding of arachidonic acid (trace d). This observation suggests that both PAO and arachidonic acid bind to flavocytochrome b to form a ternary complex, imposing additional constraints to the heme component of flavocytochrome b. A binary complex consisting of arachidonic acid bound to flavocytochrome b appears to coexist with the ternary complex, possibly due to displacement of PAO by arachidonic acid.

DISCUSSION

The redox component of the phagocyte NADPH oxidase is a membrane-bound flavocytochrome b, localized in the plasma membrane of the cell. Its kinetic competence to transfer electrons from NADPH to O_2 has been demonstrated (*27*, *28*). Of the two subunits that compose flavocytochrome b, the larger β-subunit contains the redox centers involved in NADPH oxidation, namely FAD and heme (*29*). In early studies on oxidase activation in cell-free systems, arachidonic acid and a few other long-chain unsaturated fatty acids were used as activators of flavocytochrome b, in addition to a cytosolic fraction which provided water-soluble proteins referred as oxidase activating factors (for review, see ref *1*). It was soon recognized that the activating unsaturated fatty

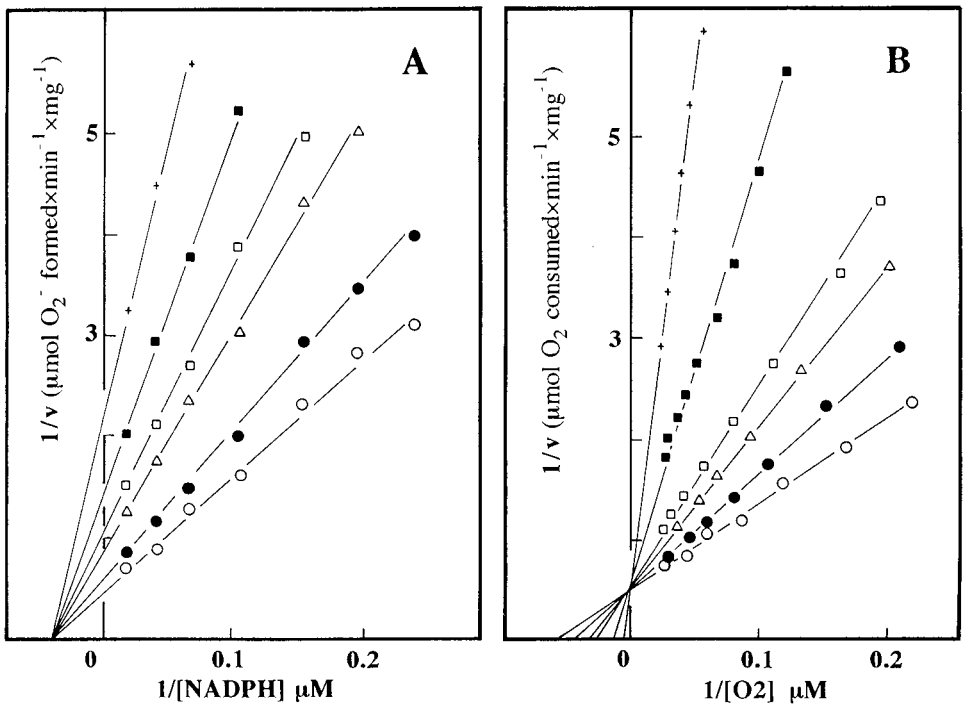

FIGURE 10: Effect of PAO on the K_M NADPH and the K_M O_2 of the elicited NADPH oxidase activity. (A) Neutrophil membranes were preincubated for 5 min with different fixed concentrations of PAO. The PAO (nmol) to membrane protein (mg) ratios ranged between 0 and 10 [zero (○); 0.5 (●); 1.0 (△); 4.0 (□); 6.0 (■); 9.0 (+)]. Then, oxidase activation was carried out by mixing membranes with arachidonic acid (1.2 μmol/mg membrane protein), and cytosol, using a cytosol to membrane protein ratio of 10, and adding 30 μM GTPγS, 250 μM ATP, and 2.5 mM $MgSO_4$. After 10 min, the elicited oxidase activity was assayed by the rate of production of O_2^- in the presence of different concentrations of NADPH (cf. Materials and Methods). (B) Same protocol as in panel A except that the elicited oxidase activity was assayed by the rate of O_2 uptake at different concentrations of O_2 and in the presence of 250 μM NADPH as described in Figure 5A.

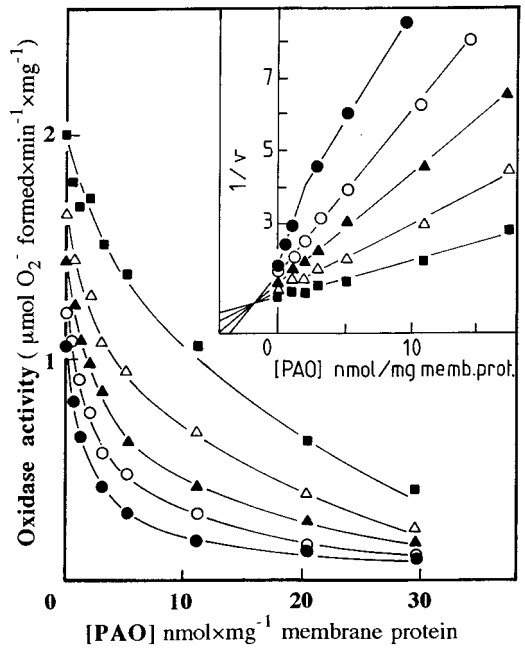

FIGURE 11: Antagonistic effect of arachidonic acid and PAO on oxidase activation. Aliquot samples of neutrophil membranes (370 μg of protein/sample) were preincubated for 5 min in PBS with different fixed concentrations of arachidonic acid, using a range of arachidonic acid (μmol) to membrane protein (mg) ratios of 0 to 1.2 [zero, (●); 0.2 (○); 0.3 (▲); 0.5 (△); 1.2 (■)], final volume 275 μL. Each sample of membranes treated with a fixed concentration of arachidonic acid was fractionated in nine fractions. Each of them was treated with a different concentration of PAO for 3 min. The ratios of PAO (nmol) to membrane protein (mg) were between 0 and 30. Each sample was then supplemented with cytosol, using a cytosol to membrane protein ratio of 10, 30 μM GTPγS, 250 μM ATP, 2.5 mM $MgSO_4$, and a complementary amount of arachidonic acid adjusted with respect to that present in the preincubation step to reach the optimal ratio of arachidonic acid (μmol) to membrane protein (mg) (1.4 in the present experiment). After an additional incubation period of 10 min, the elicited oxidase activity was assayed by the rate of O_2^- production in the presence of 250 μM NADPH. The plots of oxidase activity vs the concentration of PAO (nmol/mg protein) are given for the different fixed concentrations of arachidonic acid in the preincubation step. The Dixon plots of the data are given in the inset of the figure.

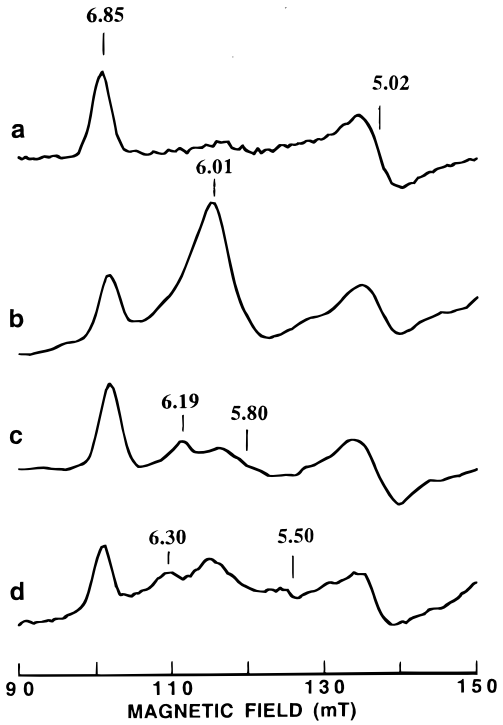

FIGURE 12: Effect of PAO and arachidonic acid on the EPR spectrum of neutrophil membranes. In each assay, samples of neutrophil membranes at 50 mg of protein/mL were used. Trace a corresponds to control membranes. Trace b corresponds to membranes incubated for 5 min at 20 °C with 1.2 μmol of arachidonic acid/mg of protein, an amount found to induce maximal oxidase activation. Trace c corresponds to membranes incubated for 5 min at 20 °C with 40 nmol of PAO/mg of protein, an amount sufficient to fully inhibit oxidase activation. In the assay corresponding to trace d, membranes were preincubated with PAO (40 nmol/mg protein) for 5 min at 20 °C, and then treated with 1.2 μmol of arachidonic acid/mg of protein for 5 min at 20 °C. In all cases, the samples were frozen, and the EPR spectra were recorded under the following conditions: microwave power, 10 mW; frequency modulation, 100 kHz; amplitude modulation, 1.25 mT; microwave frequency, 9.655 GHz. Temperature, 4 K. Only the low field region of the EPR spectra is given in the figure.

acids could be replaced by artificial anionic amphiphiles, such as sodium dodecyl sulfate (*30*), and it was inferred that the activating effect of the fatty acids was probably the result of nonspecific conformational changes brought about by hydrophobic interactions. However, this view must be qualified by accumulating evidence pointing to physiological and specific functions of arachidonic acid in stimulated neutrophils and macrophages. First, attention was early drawn to the inhibition of the respiratory burst by inhibitors of PLA_2 (*15*, *16*). Second, in activated neutrophils, arachidonic acid is released from phospholipids by $cPLA_2$ (*31*), whose activity depends on phosphorylation by MAP kinase (*32*). Third, the implication of $cPLA_2$ in the activation of phagocyte NADPH oxidase was recently highlighted by the results of experiments carried out with a human myeloid cell line deficient in $cPLA_2$ and unable to produce arachidonic acid (*12*). These cells failed to activate NADPH oxidase in response to a variety of soluble and particulate stimuli, but addition of arachidonic acid restored the production of O_2^-. Fourth, a role of arachidonic acid in NADPH oxidase activation was also suggested by the results of transfection experiments using CHO cells transfected with a fragment of the β-subunit of flavocytochrome b that contains the transmembrane domain of the β-subunit. The transfected cells were able to exhibit an arachidonate-activable H+ flux (*11*, *33*). Finally, through the use of EPR spectroscopy, it has been shown that arachidonic acid facilitates the transition of the hexacoordinated form of the heme iron of flavocytochrome b to a pentacoordinated form capable of reacting with O_2 (*10*). The aim of the present study was to explore the kinetic competence of arachidonic acid as an activator of the neutrophil NADPH oxidase and to clarify on a kinetic basis the antagonistic effects of arachidonic acid as an activator and PAO as an inhibitor of NADPH oxidase.

Kinetic Competence of Arachidonic Acid as an Activator of NADPH Oxidase. The experiments described here show that a target of arachidonic acid which is responsible for the functioning of arachidonic acid as activator of NADPH oxidase is located in the membrane fraction of resting neutrophils. In fact, preincubation of neutrophil membranes with arachidonic acid, prior to the addition of cytosol, GTPγS, ATP, and $MgSO_4$ proved to be much more efficient for oxidase activation than the addition of arachidonic acid to the mixture of membranes and cytosol, enhancing both

the rate and the maximal level of O_2^- production by the activated oxidase. Reversing the sequence of preincubation, i.e., mixing first arachidonic acid with cytosol prior to the addition of membranes impeded oxidase activation. This result, however, does not preclude the possibility that binding of arachidonic acid to cytosolic factors can exert positive effects on NADPH oxidase activation under appropriate conditions, e.g., the simultaneous presence of neutrophil membranes and cytosolic factors, by inducing a transient conformation of cytosolic factors which enables them to bind to flavocytochrome b. In the absence of flavocytochrome b, a prolonged contact between arachidonic acid and the cytosolic factors would be deleterious as shown in the present study. In this context, the requirement for the simultaneous presence of p47phox and flavocytochrome b for the activation of NADPH oxidase by anionic amphiphiles was recently stressed (*34*). It was explained that the favorable effect of arachidonic acid on p47phox could be due to the unmasking of the N-terminal SH_3 domain which is masked by a proline-rich stretch in the native p47phox and becomes exposed upon addition of arachidonic acid (*35*). In addition, the arachidonic acid-induced conformational change in p47phox detected by monitoring intrinsic fluorescence was found to be similar to that revealed by phosphorylation of p47phox by protein kinase C (*36, 37*). It has been reported that arachidonic acid increases the affinity of the assembled NADPH oxidase for NADPH in neutrophil membranes (*9*). In these experiments, arachidonic acid was added to membranes prepared from PMA-stimulated neutrophils. Under these conditions, the membrane-bound flavocytochrome b was partially activated by loose association with the cytosolic factors, and the effect of arachidonic acid was likely to strengthen this association and to stabilize the active form of the oxidase complex.

Our kinetic experiments were designed to detect modifications in the elicited oxidase activity brought about by incubation of arachidonic acid with neutrophil membranes, followed or not by addition of neutrophil cytosol. Two types of experimental protocol were utilized. In the first, membranes preincubated with arachidonic acid and $MgSO_4$ were supplemented with cytosol, GTPγS, and ATP, and after the membrane-bound oxidase was fully activated, its kinetic parameters were determined (V_{max}, K_M NADPH, K_M O_2) (Figures 4 and 5). In the second, the kinetic parameters of the activated oxidase were determined on membranes preincubated with arachidonic acid and $MgSO_4$ in the absence of cytosol, GTPγS and ATP (Figure 9). Using the first protocol, we observed that, in contrast to the K_M NADPH, which remains constant whatever the conditions, the K_M O_2 dropped from a value higher than 200 μM to a value of 20 μM when the concentrations of arachidonic acid used (μmol/mg membrane protein) during the preincubation step increased up to a critical value of 0.55 μmol/mg membrane protein. Above 0.60 μmol of arachidonic acid/mg membrane protein, only the V_{max} value increased until the optimal concentration of arachidonic acid was attained. Thus, whereas at low concentrations, arachidonic acid increases the affinity of flavocytochrome b for O_2, at higher concentrations, it promotes the efficient interaction between the cytosolic factors and flavocytochrome b, so that the maximal catalytic competence is attained. This sequence of activation steps by arachidonic acid is confirmed by the inhibitory effect of arachidonic acid methyl ester, which is the mirror image of the activating effect of arachidonic acid (Figures 7 and 8). Another interesting observation was that, above the optimal concentration of arachidonic acid, the rates of production of O_2^- and O_2 uptake decreased, but the values of K_M NADPH and the K_M O_2 remained stable. This could be readily explained by the fact that, when arachidonic acid is used above the concentration required to saturate the neutrophil membrane fraction, the excess arachidonic acid exerts a deleterious effect on the activation factors present in the cytosolic fraction. However, the affinities for NADPH and O_2 are not modified by the excess of arachidonic acid, indicating the absence of deleterious effects on the membrane-bound flavocytochrome b component of the NADPH oxidase complex. In the second protocol, although the measured oxidase activity of membranes incubated with $MgSO_4$ and arachidonic acid was markedly lower than that obtained with the complete cell-free system, the kinetic behavior with respect to O_2 was more or less the same.

Taken together, the above results suggest that the activity of NADPH oxidase is under the control of both arachidonic acid and the cytosolic factors, and that these two types of activators interact with flavocytochrome b at two different binding sites. Arachidonic acid appears to act as an activator that primarily controls the interaction of the heme component of the flavocytochrome b with O_2, increasing the affinity of the heme for O_2 and, consequently, the turnover of the oxidase. The arachidonic acid-dependent increase in O_2 affinity was consistent with the results of EPR spectroscopy experiments that showed that arachidonic acid promotes the transition of the heme iron of flavocytochrome b from a hexacoordinated form unable to bind O_2 to a pentacoordinated form competent for reacting with O_2 (*10*). In brief, a critical step in oxidase activation by stimulated neutrophils would be the increased production of arachidonic acid, and the interaction of arachidonic acid with the α-helix domain of the β-subunit of the flavocytochrome b, which contains the heme-binding site. As for the cytosolic factors of oxidase activation, they most likely interact with the domain of flavocytochrome b exposed to the cytosol, which contains the NADPH- and FAD-binding sites, and induce in the presence of arachidonic acid some conformational change in the β-subunit, allowing a more efficient interaction between the NADPH- and FAD-binding sites or facilitating electron transfer from FAD to hemes b.

The role played by the carboxylic group of arachidonic acid in oxidase activation appears to be critical. In fact the methyl ester of arachidonic was not only unable to promote activation, but it also counteracted the activating effect of arachidonic acid, probably by direct competition at the target site. When neutrophil membranes alone, in the absence of cytosol, were mixed with arachidonic acid, elicitation of oxidase activity was markedly enhanced by the addition of $MgSO_4$. This suggests that the ionization state of arachidonic acid modulates its efficiency to activate NADPH oxidase. In this respect, it is worth recalling that the insertion of an ionizable molecule, for example a fatty acid, in a neutral bilayer induces appreciable shifts in its apparent p*K*, which may amount to 2−3 units (*35*). The kinetic modifications of flavocytochrome b tentatively ascribed to the binding of arachidonic acid and the cytosolic factors are depicted in the scheme of Figure 13.

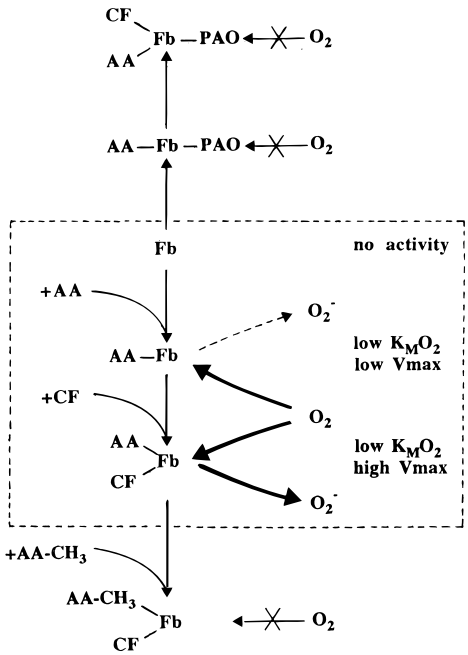

FIGURE 13: Scheme depicting the effect of arachidonic acid, arachidonic acid methyl ester, cytosolic factors, and phenylarsine oxide on the flavocytochrome b component of the neutrophil NADPH oxidase. Full activation of the flavocytochrome b (Fb) component of the NADPH oxidase corresponds to the dual binding of arachidonic acid (AA) and cytosolic factors (CF) to flavocytochrome b. The primary step of activation corresponds to the binding of arachidonic acid to flavocytochrome b, with the kinetic modification being restricted to the increased affinity of flavocytochrome b for O_2. The further binding of cytosolic factors results in the marked increase on the flavocytochrome b turnover. This sequence of reactions is surrounded by a dotted line. Inhibition of activation by arachidonic acid methyl ester (AA CH_3) is caused by the displacement of arachidonic acid, and the loss of affinity for O_2. The simultaneous binding of PAO and arachidonic acid is deduced from EPR spectroscopy experiments.

PAO as Antagonist of Arachidonic Acid Acting as Activator of NADPH Oxidase. PAO, a reagent of vicinal or proximal thiol groups in proteins, inhibits NADPH oxidase activation by interacting with the β-subunit of the flavocytochrome b component of the oxidase complex (*25*). PAO might target cysteinyl residues present in the β-subunit, namely Cys 84, Cys 85, Cys 368, and Cys 370. However, PAO might also bind to other Cys residues which are distant in the β-subunit sequence and are brought close to each other in the tertiary structure, as the result of the juxtaposition of two α-helices of the transmembrane domain of the β-subunit. In this paper, we show that the affinity of NADPH oxidase for O_2 is increased by arachidonic acid and on the contrary decreased by PAO. EPR spectroscopy data indicate that both arachidonic acid and PAO bind to flavocytochrome b. The two molecules being structurally different should therefore bind to two different interacting sites in the β-subunit of flavocytochrome b as illustrated in the scheme of Figure 13. Thus, a structural modification of the β-subunit imposed by the binding of arachidonic acid to its specific site is likely to be propagated to the PAO binding site decreasing its affinity for PAO. The presence of two populations of flavocytochrome b with different affinities for PAO (see Results) is consistent with the idea of a reversible transition between the two populations, under the control of arachidonic acid.

ACKNOWLEDGMENT

We thank Dr. J. Willison for careful reading of the manuscript and Mrs. J. Bournet-Cauci for excellent secretariat assistance.

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BI991502W