Electron Transfer Across the O_2^- Generating Flavocytochrome *b* of Neutrophils. Evidence for a Transition from a Low-Spin State to a High-Spin State of the Heme Iron Component†

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ABSTRACT: The NADPH oxidase complex of activated neutrophils consists of a membrane-bound flavocytochrome *b* and cytosolic activation factors. Despite its ability to react with O_2, the heme *b* component of the flavocytochrome is insensitive to cyanide and CO, and slowly reactive to butyl isocyanide. We report here that arachidonic acid, an anionic amphophil which elicits oxidase activation in a cell-free system induces a transition of the heme iron of the neutrophil flavocytochrome *b* from a low-spin hexacoordinated state to a high-spin pentacoordinated state and promotes the binding of butyl isocyanide to the heme *b*. Low-temperature EPR spectra of air-oxidized flavocytochrome *b* either purified or in its membrane-bound form showed a low-spin signal at $g = 3.26$ and a high-spin signal at $g = 6.0$. Upon addition of arachidonic acid, the $g = 3.26$ signal vanished; a low-spin signal at $g = 2.23$ appeared, and the signal at $g = 6.0$ progressively increased. The subsequent addition of butyl isocyanide resulted in the decrease of the $g = 6.0$ and $g = 2.23$ signals and in the appearance of a new low-spin signal at $g = 2.33$. Consistent with the EPR results, upon addition of arachidonic acid to oxidized flavocytochrome *b*, a 2.5 nm blue shift of the Soret peak was detected in low-temperature optical spectra. The subsequent addition of butyl isocyanide resulted in the emergence of a peak at 432 nm reflecting the formation of a butyl isocyanide-oxidized heme *b* complex. In the case of sodium dithionite-reduced flavocytochrome *b*, arachidonic acid promoted the binding of butyl isocyanide to the reduced heme *b*, as shown by the emergence of a peak at 434 nm and the decrease of the α band at 558 nm. The same promoting effect was encountered with sodium dodecyl sulfate, an anionic amphophil capable of eliciting oxidase activation like arachidonic acid. In contrast to arachidonic acid, arachidonic acid methyl ester was ineffective and counteracted the effect of arachidonic acid. Butyl isocyanide added to intact neutrophils was found to bind to heme *b*, only after the cells have been activated. These data demonstrate the transient accumulation of a pentacoordinated form of the heme iron of flavocytochrome *b* under *in vitro* and *in vivo* conditions; the pentacoordinated form of the reduced heme *b* is postulated to react with O_2 to generate the superoxide anion.

The redox core of the NADPH oxidase complex of phagocytic cells, particularly well studied in neutrophils, consists of a flavocytochrome *b*, referred to earlier as cytochrome b_{558}, located in the plasma membrane and the membrane of the specific granules (Knoller et al., 1991; Koshkin & Pick, 1993). The neutrophil flavocytochrome *b* is an heterodimer consisting of a large glycosylated subunit of 91 kDa (gp91phox) and a small subunit of 22 kDa (p22phox) [for review Morel et al. (1991) and Trasher et al. (1994)]. The gp91phox subunit contains an NADPH-binding site (Segal et al., 1992; Rotrosen et al., 1992; Sumimoto et al., 1992; Doussière et al., 1993, Ravel & Lederer, 1993) and a FAD-binding site (Doussière et al., 1995). In unstimulated neutrophils, the flavocytochrome *b* is in a resting state. At the onset of phagocytosis, the activation factors, p47phox and p67phox of cytosolic origin, a monomeric G protein Rac2 and possibly another protein p40phox, bind to the flavocytochrome *b* to form an active oxidase complex (Trasher et al., 1994; Fuchs et al., 1995). In this configuration, the flavocytochrome *b* acquires the capacity to transfer electrons from NADPH to O_2 to generate the superoxide anion O_2^-, *via* FAD and the heme *b*. Although a large amount of information on the parameters of activation of the neutrophil NADPH oxidase complex has accumulated during the past few years, the mechanism by which flavocytochrome *b* is activated remains elusive. A particularly puzzling problem is the topological and functional status of the cytochrome *b* component. At least two hemes *b* are believed to be present in the flavocytochrome (Quinn et al., 1992; Cross et al., 1995). However, the location of the two hemes is still debated. Also the type of reaction of heme *b* with O_2 is not yet solved. The paradox that developed around the electron transfer from neutrophil heme *b* to O_2 stems from early observations that the reduced heme *b* is

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[1] Abbreviations: PMA, phorbol myristate acetate; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; SOD, superoxide dismutase.

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rapidly reoxidized by O_2 (Segal & Jones, 1979) but is virtually unreactive to CO (Morel and Vignais, 1984; Iizuka et al., 1985a); this is in contrast to classical hemoproteins such as cytochrome oxidase and cytochrome P450 which combine with CO and with O_2. On the other hand, optical spectroscopic studies have shown that heme ligands such as butyl isocyanide (Cross et al., 1984), imidazole, and pyridine (Iizuka et al., 1985b) bind to the flavocytochrome *b* solubilized in detergent. Binding resulted in inhibition of the production of O_2^- (Cross et al., 1984). The effect of pyridine, recently explored by the Kakinuma group using EPR spectroscopy, was found to result in a structural modification of the heme environment of the flavocytochrome *b* without direct binding to the heme (Fujii et al., 1995a). These results were discussed in the context of a previously reported work (Miki et al., 1992) showing that the heme component of flavocytochrome *b* was characterized by a low spin signal at $g = 3.26$; it was concluded that the six coordination sites of the heme iron are occupied by intrinsic ligands (Fuji et al., 1995a−c). A low-spin six-coordinated form of the heme iron of neutrophil flavocytochrome *b* was also suggested on the basis of resonance Raman spectra (Hurst et al., 1991). However the absence of EPR signals in the same flavocytochrome *b* preparation precluded any definite conclusion.

In the cell-free system of oxidase activation (Bromberg & Pick 1984; Heyneman & Vercauteren 1984), arachidonic acid as well as a few other anionic amphophils such as sodium dodecyl sulfate are required to promote oxidase activation. In the course of the exploration of the mechanism of action of butyl isocyanide, we found that the interaction of butyl isocyanide with the reduced flavocytochrome *b* in neutrophil membranes was markedly enhanced by arachidonic acid and sodium dodecyl sulfate. The aim of the present study was to determine, through the use of optical spectroscopy and EPR spectroscopy, the mechanism by which these amphophils increase the reactivity of butyl isocyanide toward neutrophil flavocytochrome *b*. The results indicate that these compounds are able to facilitate the transition of the hexacoordinated form of the heme *b* to a pentacoordinated form capable of reacting with an extrinsic ligand.

MATERIALS AND METHODS

Materials. NADPH, ATP, and GTP-γS were from Boehringer; horse heart cytochrome type III, arachidonic acid, arachidonic acid methyl ester, asolectin, leupeptin, pepstatin, TPCK, diisopropylfluorophosphate, Triton X-100, and PMA were from Sigma; *tert*-butyl isocyanide was from Fluka.

Biological Preparations. Bovine neutrophils were prepared routinely from 10 L of blood (Morel et al., 1985). For preparation of subcellular fractions, the neutrophils were homogenized by sonic disruption in 100 mL of a mixture of phosphate-buffered saline (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, and 10% sucrose, supplemented with the following cocktail of antiproteases, 1 mM diisopropylfluorophosphate, 1 mM EDTA, 1 μM leupeptin, 2 μM pepstatin, and 200 μM TPCK. The homogenate was subjected to two successive centrifugations of 10 min at 1000 and 10 000*g*. The 10 000*g* supernatant was subfractionated by a 75 min centrifugation at 100 000*g* on a sucrose gradient consisting of two sucrose layers of 18% and 38% (w/v) supplemented with 1 mM EDTA and adjusted to pH 7.4. Centrifugation was carried out in a SW25.2 swinging bucket rotor (Beckman). The cytosolic fraction corresponded to the supernatant fluid. The membrane fraction enriched in plasma membrane, but slightly contaminated with specific granule membranes, was collected at the 38%−18% sucrose interface. It was diluted twice with phosphate saline buffer supplemented with antiproteases and sedimented by centrifugation for 40 min at 150 000*g*. Depending on preparations, the heme b_{558} content of this particulate fraction ranged from 0.60 to 0.85 nmol/mg of protein. Flavocytochrome *b*, referred to earlier as cytochrome b_{558}, was extracted from the plasma membrane fraction with sucrose monolaurate and purified as described by Isogai et al. (1991) except that 0.1% Triton X-100 was used during the chromatographic steps. The purified flavocytochrome *b* was solubilized in PBS supplemented with 0.1% Triton X-100. Relipidation of flavocytochrome *b* in detergent was achieved by sonication in a mixture of phospholipids (asolectin) using a ratio of 8−10 mg of phospholipid per μmol of heme b_{558}. Arachidonic acid and butyl isocyanide were dissolved in ethanol to form stock solutions at 1 and 1.5 M, respectively. Appropriate dilutions were made in ethanol. The final concentration of ethanol in the assay medium was not more than 2%, except in Figure 6, and was routinely 1%.

EPR Spectra. EPR spectra were recorded with an X-band Varian E-109 spectrometer equipped with an Oxford Instruments ESR-900 continuous-flow helium cryostat. The reaction mixtures containing the neutrophil membranes or the purified flavocytochrome *b* were transferred to EPR quartz tubes. The tubes were frozen, and the EPR spectra were recorded. The experimental conditions are described in the figure legends.

Optical Spectra. Absorption spectra were recorded at room temperature or at 77 K with a Uvikon 930 spectrophotometer or with a double-beam Perkin-Elmer 557 spectrophotometer. Reduction was achieved with a few grains of sodium dithionite. The amount of reduced flavocytochrome *b* was determined from the absorbance at 558 nm using an ϵ_{558} value of 21.3 mM^{-1} cm^{-1}, and that of air-oxidized flavocytochrome *b* was determined from the absorbance at 415 nm using an ϵ_{415} value of 58.3 mM^{-1} cm^{-1}. Because of light diffusion by particulate suspensions, the spectral modifications of flavocytochrome *b* in neutrophil membranes, liposomes, or intact cells were followed by difference spectroscopy. Absolute spectra were recorded in the case of purified flavocytochrome *b* solubilized in detergent.

Assay of Oxidase Activity in the Cell-Free System. The assay was carried out in two steps (Ligeti et al., 1988). In the first step (activation step), plasma membranes (35−40 μg of protein) and cytosol (350−400 μg of protein) prepared from resting neutrophils were mixed and supplemented with 2 mM $MgSO_4$, 20 μM GTP-γS, 200 μM ATP, and an optimal amount of arachidonic acid in a final volume of 50 μL of PBS. Full oxidase activation was attained in 5−8 min at room temperature. In the second step (assay of oxidase activity), the 50 μL suspension was transferred to a photometric cuvette containing 200 μM NADPH and 100 μM cytochrome *c* in 2 mL of PBS. This resulted in a 40-fold

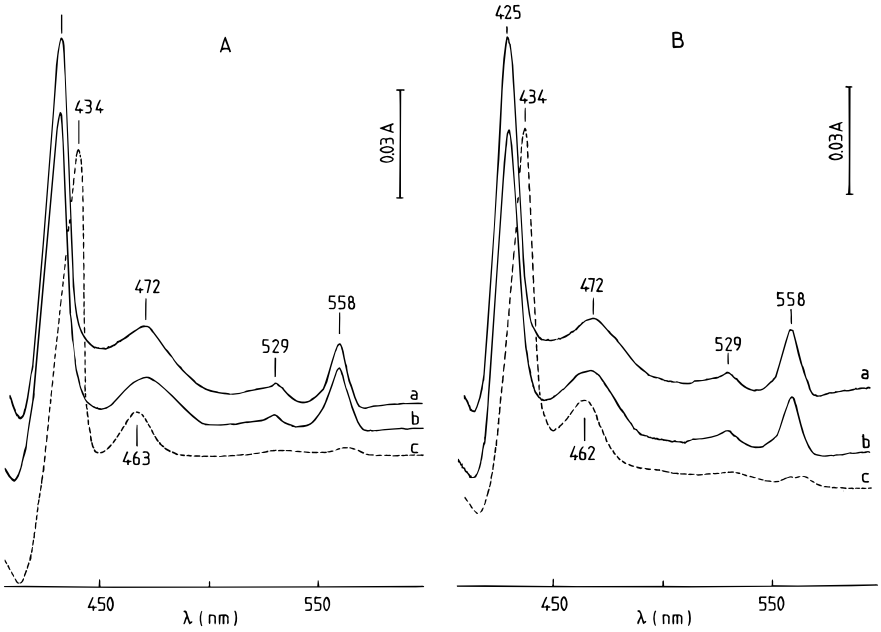

FIGURE 1: (A) Effect of arachidonic acid and butyl isocyanide on the optical difference spectra (dithionite-reduced minus oxidized) of neutrophil membranes. Membranes (2.2 mg of protein) were suspended in 2 mL of PBS and incubated at 22 °C for 10 min. The dithionite reduced membranes were treated with arachidonic acid or(and) butyl isocyanide as indicated. Samples corresponded to (a) control membranes or membranes treated with arachidonic acid (2 μmol/mg of membrane protein) (the two spectra were virtually identical); (b) membranes treated with 30 mM butyl isocyanide; (c) membranes treated with 30 mM butyl isocyanide and 2 μmol of arachidonic acid/mg of membrane protein. (B) Effect of sodium dodecyl sulfate and butyl isocyanide on the optical difference spectra (dithionite-reduced minus oxidized) of neutrophil membranes. Same conditions as in Figure 1A. The dithionite-reduced membranes were treated as follows: (a) control membranes or membranes treated with 0.13 mM sodium dodecyl sulfate; (b) membranes treated with 30 mM butyl isocyanide; (c) membranes treated with 30 mM butyl isocyanide and 0.13 mM sodium dodecyl sulfate.

dilution of the reagents present in the activation step, including arachidonic acid. The reduction of cytochrome *c* was recorded at 550 nm. After 2−3 min, 50 μg of superoxide dismutase was added to quench O_2^-, and the residual reduction of cytochrome *c* was recorded for another 2−3 min. The rate of O_2^- production was calculated from the differences between the two slopes. Activation of purified flavocytochrome *b* and assay of the O_2^- production by the activated flavocytochrome *b* wee carried out as described by Escriou et al. (1996).

Activation of Bovine Neutrophils. The neutrophils suspended in PBS supplemented with 10 mM glucose were activated by addition of 0.1 μM PMA in dimethyl sulfoxide.

Protein Assay. Protein concentration was assayed with the BCA reagent or by the biuret method, using bovine serum albumin as a standard.

RESULTS

Analysis by Difference Absorption Spectroscopy of the Effect of Arachidonic Acid and Sodium Dodecyl Sulfate on the Binding of Butyl Isocyanide to Dithionite-Reduced Flavocytochrome b. In contrast to CO and cyanide, which hardly react with neutrophil flavocytochrome *b* [see Morel et al. (1991) for review], butyl isocyanide has been reported to bind to the dithionite-reduced flavocytochrome *b* in neutrophil membranes solubilized in detergent (Cross et al., 1984). However, even with a concentration of butyl isocyanide as high as 10 mM, the reaction was slow, requiring about 10 min for completion, and the oxidase activity of intact phorbol-stimulated neutrophils in an aerated medium was far from being inhibited, suggesting that O_2 competed favorably with butyl isocyanide. In our hands, the Soret peak at 425 nm as well as the 558 and 529 nm peaks ascribed to heme *b* were decreased by less than 8% when resting neutrophil membranes reduced by sodium dithionite were incubated with 30 mM butyl isocyanide for 10 min at 22 °C (Figure 1A). The plasma membrane preparation used was slightly contaminated by myeloperoxidase originating from azurophil granules. In contrast to the modest spectral change observed for the flavocytochrome *b*, the peak of myeloperoxidase at 472 nm was enlarged, pointing to the propensity of butyl isocyanide to react spontaneously with reduced myeloperoxidase. Addition of 2 μmol of arachidonic acid per mg of protein to the dithionite-reduced neutrophil membranes treated by butyl isocyanide resulted in a shift of the Soret peak of flavocytochrome *b* from 425 to 434 nm, and the virtual disappearance of the peaks at 558 and 529 nm. The amount of arachidonic acid used was equivalent to that found to induce maximal oxidase activation in a cell-free system (Pilloud et al., 1989). Arachidonic acid, *per se*, did not modify significantly the reduced minus oxidized spectrum of the neutrophil membranes. These results indicate that arachidonic acid drastically modifies the reactivity of the reduced heme component of flavocytochrome *b* toward butyl isocyanide, possibly by displacing a loosely-bound group from the sixth heme iron coordination site.

The effect of sodium doecyl sulfate, another anionic amphophil which activates NADPH oxidase in the cell-free system (Pilloud et al., 1989), was tested on the dithionite-reduced spectrum of neutrophil membranes supplemented with butyl isocyanide, and compared to that of arachidonic acid (Figure 1B). At a concentration of 0.13 mM, optimal for oxidase activation, sodium dodecyl sulfate was able to promote interaction of butyl isocyanide with the reduced

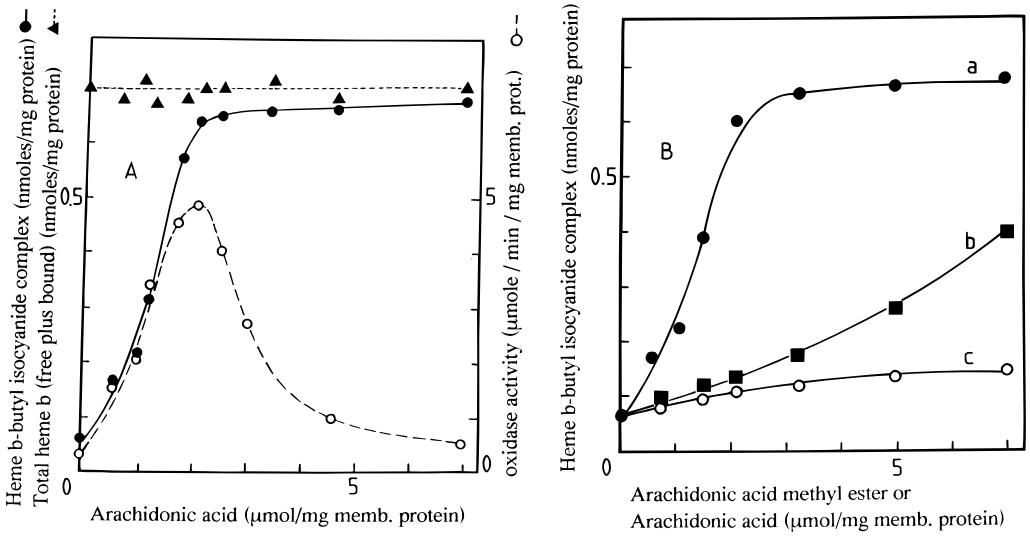

FIGURE 2: (A) Effect of increasing concentrations of arachidonic acid on the formation of the reduced heme *b*−butyl isocyanide complex in neutrophil membranes and on the O_2^- generating NADPH oxidase activity elicited in a cell-free system. Neutrophil membranes (2.1 mg of protein/mL of PBS) were incubated for 10 min at 22 °C with 30 mM butyl isocyanide and increasing concentrations of arachidonic acid up to 7 μmol/mg of membrane protein. The difference spectra (dithionite reduced plus butyl isocyanide and arachidonic acid minus oxidized) were then recorded. The amount of reduced heme *b*−butyl isocyanide complex (nmol/mg of membrane protein) was calculated from the value of the absorbance at 434 nm (cf. Figure 1), using an ϵ value of 115 mM^{-1} cm^{-1} (●). Conversely, the noncomplexed heme *b* was calculated from the height of the peak at 558 nm using an ϵ value of 21.3 mM^{-1} cm^{-1}. The curve ▲ represents the sum of the complexed and noncomplexed forms of heme *b* (nmol/mg of membrane protein). In a parallel experiment, the effect of increasing concentrations of arachidonic acid on NADPH oxidase activation in neutrophil membranes was determined. The membranes treated by arachidonic acid were incubated for 10 min at 22 °C with cytosol, $MgCl_2$, and GTPγS for oxidase activation (cf. Materials and Methods) and the elicited oxidase activity was assayed (○). (B) Compared effects of increasing concentrations of arachidonic acid (curve a) and arachidonic acid methyl ester (curve c) on the formation of the reduced heme *b*−butyl isocyanide complex in neutrophil membranes. Competition between arachidonic acid and arachidonic acid methyl ester (curve b). Conditions were the same as those of Figure 2A. In the case of curve b, arachidonic acid methyl ester was used at the fixed concentration of 5 μmol/mg of membrane protein and the concentration of arachidonic acid was varied.

heme of flavocytochrome *b*, at a rate similar to that induced by arachidonic acid. However at mM concentrations, above the critical micellar concentration, the flavocytochrome *b* spectrum was drastically altered, reflecting the denaturation of the protein.

When butyl isocyanide was replaced by potassium cyanide in a medium supplemented with arachidonic acid, there was no spectral modification of the reduced flavocytochrome. It therefore appears that the modification of the heme pocket induced by arachidonic acid is limited in its extent since the effect is restricted to an increase in the access of only hydrophobic reagents, such as butyl isocyanide, to the heme iron. In contrast to reduced flavocytochrome *b*, reduced myeloperoxydase reacted more readily with cyanide than with butyl isocyanide, even in the absence of arachidonic acid (data not shown).

The time course of the binding of butyl isocyanide to the dithionite-reduced flavocytochrome *b* in neutrophil membranes in the presence of arachidonic acid was monitored by following the rise of the emerging peak at 434 nm. The amount of the butyl isocyanide-reduced heme *b* complex was determined using an ϵ value of 115 mM^{-1} cm^{-1}, calculated from the peak at 434 nm of purified flavocytochrome *b* totally liganded by butyl isocyanide. Conversely, the disappearance of the free heme *b* was quantified by the decrease of the height of the reduced peak at 558 nm, using an ϵ value of 21.3 mM^{-1} cm^{-1}. The sum of the amounts of free and butyl isocyanide-bound flavocytochrome *b* remained constant for at least 20 min, indicating that the overall structure of the flavocytochrome was not altered during this period. The time course of butyl isocyanide binding to reduced heme *b* depended on the concentration of this ligand and on the temperature. Full binding was achieved in 20 min at room temperature, using 30 mM butyl isocyanide (data not shown).

Since arachidonic acid promotes oxidase activation in a cell-free system and the extent of oxidase activation depends on the amount of arachidonic acid present in the medium (Pilloud et al., 1989), we set up a dose−effect experiment in which we measured the effects of increasing concentrations of arachidonic acid on two parameters: the oxidase activity of the flavocytochrome *b* in a reconstituted cell-free system, and the accumulation of the butyl isocyanide-reduced heme *b* complex in neutrophil membranes. As shown in Figure 2A, the amounts of arachidonic acid necessary to elicit maximal oxidase activity and to promote maximal binding of butyl isocyanide to reduced heme *b* were the same, namely, about 2 μmol/mg of membrane protein. This correlation was shown to hold for a large range of concentrations of neutrophil membranes. The pH change resulting from addition of arachidonic acid was moderate. Upon addition of 2 and 7 μmol of arachidonic acid per mg of membrane protein, we observed pH shifts from 7.4 to about 7.0 and 6.0, respectively. As shown below, these variations of medium pH had negligible effects on the binding of butyl isocyanide to the reduced heme *b*.

In contrast to arachidonic acid, the effect of arachidonic acid methyl ester on the binding of butyl isocyanide to reduced heme *b* in neutrophil membranes was negligible, even at high concentrations (Figure 2B, trace c compared to trace a). Moreover, arachidonic acid methyl ester counteracted the free fatty acid-induced formation of the butyl isocyanide-reduced heme *b* complex (Figure 2B, trace b), suggesting that the carboxyl group of arachidonic acid interacted with strategic residues located in heme *b* or in

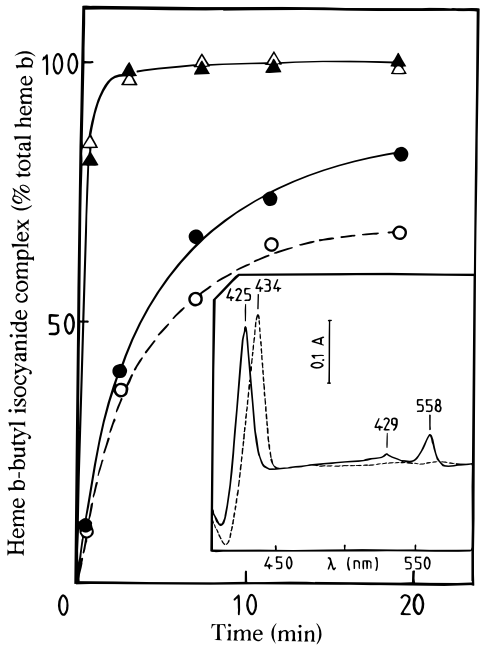

FIGURE 3: Time course of the binding of butyl isocyanide to the reduced heme *b* component of purified flavocytochrome *b* in Triton X-100 before or after relipidation. The effect of butyl isocyanide on the difference spectra of purified flavocytochrome *b* in 0.1% Triton X-100 is shown in the insert. The dithionite-reduced minus oxidized spectrum of purified flavocytochrome (2 μM) was recorded before and after a 1 min incubation at 22 °C with 30 mM butyl isocyanide. (The same spectra were obtained with 12 and 3 mM butyl isocyanide.) The extent of the binding of butyl isocyanide to reduced flavocytochrome *b* was determined by the peak at 434 nm. Purified flavocytochrome *b* (2 μM) relipidated with asolectin (see Materials and Methods) or not, or treated with arachidonic acid was mixed with 30 mM butyl isocyanide, and then the difference spectra (dithionite reduced minus oxidized) were recorded between 400 and 600 nm at 2-min intervals. (△) Control flavocytochrome *b*; (▲) flavocytochrome *b* plus arachidonic acid (2 μmol/nmol of heme *b*); (○) flavocytochrome relipidated with 8 mg of asolectin/μmol of heme *b*; (●) flavocytochrome *b* relipidated with asolectin and supplemented with arachidonic acid (2 μmol/nmol of heme *b*).

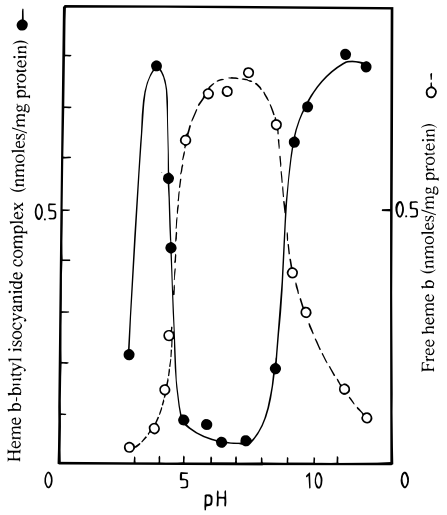

FIGURE 4: Effect of pH on the binding of butyl isocyanide to the reduced heme *b* component of flavocytochrome *b* in neutrophil membranes. Membranes (1.1 mg of protein/mL of PBS) were supplemented with 30 mM butyl isocyanide and then fractionated into several samples. The pH of each sample was adjusted to the desired value by addition of 1 N HCl or 1 N NaOH with stirring. After a 10 min incubation at 20 °C, the dithionite reduced minus oxidized spectra of the different samples were recorded as in Figure 1. Measurement of the size of the peaks at 434 and at 558 nm allowed the determination of the amount of reduced heme *b*−butyl isocyanide complex (●) and noncomplexed heme *b* (○), respectively.

the close neighborhood to facilitate the access of butyl isocyanide to the heme iron. Consistent with this finding was the fact that arachidonic acid methyl ester is unable to elicit oxidase activation in a cell-free system (Ligeti et al., 1988).

Like arachidonic acid, sodium dodecyl sulfate promoted the binding of butyl isocyanide to reduced heme *b* in neutrophil membranes at the same concentrations as those used for the activation of NADPH oxidase in a cell-free system. Triton X-100 was also able to promote the generation of the butyl isocyanide-reduced heme *b* complex in neutrophil membranes, but it does not elicit oxidase activation. Thus, the link between the ability of a reagent to enhance oxidase activation and that of promoting the binding of butyl isocyanide to the reduced heme *b* was apparently restricted to amphophilic anions such as arachidonic acid and sodium dodecyl sulfate.

In the experiment illustrated in Figure 3, we compared by difference optical spectroscopy the reactivity of the dithionite-reduced heme toward butyl isocyanide in purified flavocytochrome *b* in 0.1% Triton X-100 before (control), or after, relipidation with asolectin. In the control, the formation of the butyl isocyanide-reduced heme *b* complex attained completion in less than 2 min in the absence or presence of arachidonic acid and was most likely facilitated by the presence of the detergent. Binding of butyl isocyanide was at least 20 times more rapid in the case of purified reduced flavocytochrome *b* than in that of membrane-bound flavocytochrome *b*. Relipidation markedly slowed the reactivity of reduced heme *b* toward butyl isocyanide, and in this case, arachidonic acid added to the relipidated flavocytochrome *b* significantly accelerated the binding of butyl isocyanide to the reduced heme. This effect mimicked the arachidonic acid-enhanced reactivity of the membrane-bound flavocytochrome *b* to butyl isocyanide illustrated in Figure 1, suggesting that liposomes like the lipid core of the neutrophil membranes generate certain physical constraints restricting the access of butyl isocyanide to the heme iron of the flavocytochrome.

Effect of pH on the Binding of Butyl Isocyanide to Dithionite-Reduced Membrane-Bound Flavocytochrome b. Fujii et al. (1995c) reported that acidification or alkalinization of solubilized neutrophil flavocytochrome *b* converts the low-spin state of the heme iron to a high-spin state, suggesting that the sixth coordination site of the iron becomes open for ligand binding. If this were the case, one might expect that shifting the pH of the medium to acidic or alkaline values would facilitate the binding of butyl isocyanide to the reduced flavocytochrome in the absence of arachidonic acid. The results of this experiment carried out with a plasma membrane preparation of neutrophils are presented in Figure 4. As predicted, the amount of the butyl isocyanide-reduced heme *b* complex calculated from the rise of the peak at 434 nm markedly increased between pH 5 and 4 and above pH 9. A satisfactory correlation between the amount of reduced heme *b* bound to butyl isocyanide and the free form of reduced heme *b* was found to exist between pH 4 and 12. At pH lower than 4, the peak at 434 nm specific of the butyl isocyanide-reduced heme *b* complex decreased, but without

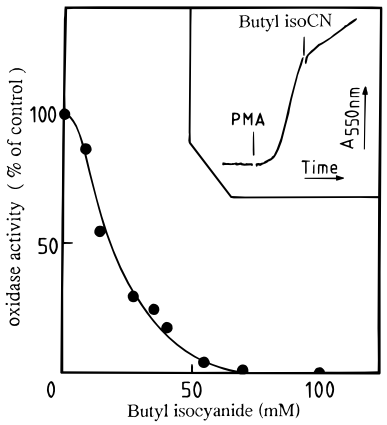

FIGURE 5: Inhibition of NADPH-oxidase activity of PMA-activated neutrophils by butyl isocyanide. Neutrophils (0.3 mg of protein) were introduced into a photometric cuvette containing 2 mL of PBS supplemented with 10 mM glucose and 100 μM cytochrome *c*, and thermostated at 37 °C. The absorbance at 550 nm was recorded. After 1 min, activation of respiration was induced by addition of PMA in 2 μL of dimethyl sulfoxide (final concentration 0.1 μM) as shown in the insert. After a 2 min linear phase of reduction of cytochrome *c*, butyl isocyanide was added, and the recording continued for an additional 2 min as indicated in the insert. The difference between the rate of reduction of cytochrome *c* before and after addition of butyl isocyanide was used to calculate the percentage of inhibition of the respiratory activity as a function of the concentration of butyl isocyanide. In parallel assays (not shown), the effect of superoxide dismutase on the rate of reduction of cytochrome *c* was determined to assess the rate of production of the superoxide O_2^-. Inhibition of cytochrome *c* reduction by 50 μg of superoxide dismutase was more than 95%, indicating that the rate of reduction of cytochrome *c* reflected the NADPH oxidase activity of the neutrophils.

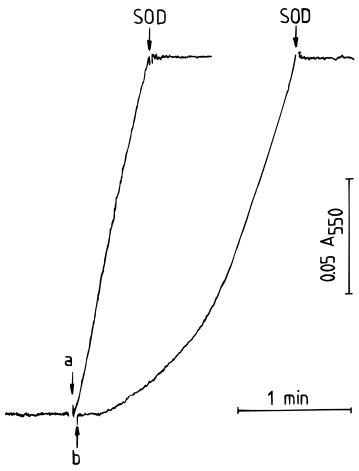

FIGURE 6: Competition between O_2 and butyl isocyanide for NADPH oxidase of PMA activated neutrophils. Two 100 μL samples of neutrophils corresponding to 3.3 mg protein in PBS were preincubated for 2 min at 30 °C with 0.1 μM PMA. Incubation of the first sample (control, a) was continued for 10 min. The second sample was supplemented with butyl isocyanide (final concentration 100 mM), and incubation was carried out for 10 min as for the first sample. Aliquot fractions of 10 μL (0.33 mg of protein) were withdrawn from each sample, a and b, and placed in a photometric cuvette thermostated at 37 °C and containing 2 mL of PBS supplemented with 10 mM glucose and 100 μM cytochrome *c*. In the case of sample *b*, the 200-times dilution resulted in the decrease of the concentration of free butyl isocyanide to 0.5 mM. The NADPH oxidase activity was assessed as the rate of reduction of cytochrome *c* inhibitable by SOD (50 μg). In a parallel experiment, it was checked that the concentration of ethanol (about 6%) brought by addition of the ethanolic solution of butyl isocyanide during the preincubation step had no deleterious effect on neutrophils.

concomitant increase in the peaks at 425 and 558 nm, this lack of correlation being probably due to the denaturation of the protein and the release of the heme iron.

The enhanced capacity of butyl isocyanide to bind to reduced heme *b* in neutrophil membranes either at pH values between 4 and 5 and above 9 (Figure 4), or in the presence of amphophilic anions such as arachidonic acid (Figure 1A) and sodium dodecyl sulfate (Figure 1B) or in detergent like Triton X-100, means that the sixth coordination site of the heme iron which is masked in the membrane-bound flavocytochrome *b* can be opended to extrinsic ligands, using a variety of non-physiological conditions. The experiments to be described in the next section show that the opening of the sixth coordination site also occurs under physiological conditions.

Activation of NADPH Oxidase in Whole Neutrophils Promotes the Binding of Butyl Isocyanide to the Heme Component of Flavocytochrome b. Addition of butyl isocyanide to a suspension of neutrophils activated by PMA in a medium supplemented with glucose resulted in inhibition of the production of the superoxide anion O_2^- (insert, Figure 5). Under our experimental conditions, half-inhibition required 17 mM butyl isocyanide (Figure 5). In a complementary experiment (Figure 6), PMA-activated neutrophils were preincubated at 30 °C with a fully inhibitory concentration of butyl isocyanide (100 mM) for 10 min. For the assay of oxidase activity, a small aliquot (10 μL) of the neutrophil suspension was transferred to a photometric cuvette containing 2 mL of reaction medium consisting of PBS supplemented with glucose and cytochrome *c*. This transfer resulted in an immediate decrease of the concentration of free butyl isocyanide to a final value of 0.5 mM which is not inhibitory. The consequence was a slow recovery of the oxidase activity from a totally inhibited state which lasted for about 20 s. Half of the activity in the control assay was recovered in about 80 s at 37 °C (Figure 6). This result indicates that O_2 dissolved in aerated PBS in the photometric cuvette competed efficiently with butyl isocyanide for binding to the heme component of flavocytochrome *b*. In other words, the inhibitory effect of butyl isocyanide was fully reversible. It is noteworthy that the high concentration of butyl isocyanide initially present in the preincubation medium was not harmful to neutrophils since, following dilution of this reagent, the neutrophils were able to recover their full capacity to reduce O_2 to O_2^-.

The effect of butyl isocyanide on the dithionite-reduced minus oxidized spectrum of the membrane-bound flavocytochrome *b* in whole neutrophils was explored with resting and PMA-activated cells. In the case of resting neutrophils, butyl isocyanide did not modify the difference spectrum (Figure 7, trace a). In contrast, addition of butyl isocyanide to PMA-activated cells resulted in significant changes in the reduced heme *b* peaks at 426, 529, and 558 nm as well as in the peak at 472 nm typical of myeloperoxidase (Figure 7, trace b). Specifically, the appearance of a shoulder at 434 nm on the Soret band and a decrease of the size of the peaks at 558 and 529 nm pointed to the binding of butyl isocyanide to a fraction of membrane-bound flavocytochrome *b* in activated neutrophils. Taking the decrease in the height of the peak at 558 nm as an index of the amount of modified heme *b*, we calculated that about 15% of the flavocytochrome *b* in activated neutrophils had combined with butyl isocya-

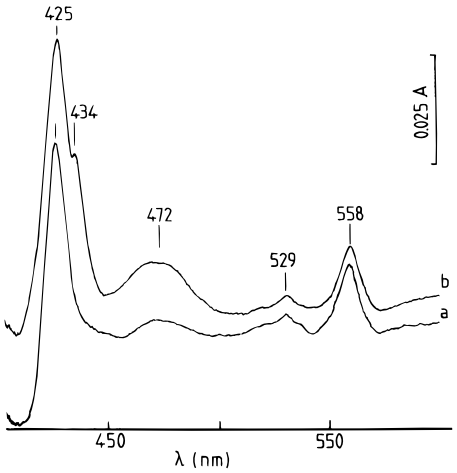

FIGURE 7: Effect of butyl isocyanide on the dithionite-reduced spectrum of PMA-activated and resting neutrophils. A suspension of neutrophils (16.5 mg of protein/mL) in aerated PBS was divided into two samples. The first sample (a) corresponded to resting neutrophils. The second sample (b) was supplemented with 0.1 μM PMA. The two samples were incubated for 5 min at 22 °C, and then butyl isocyanide was added to each sample to a final concentration 30 mM. After 10 min, the difference spectra (dithionite reduced minus oxidized) were recorded at room temperature.

nide to form a complex. In bovine neutrophils, 15%−20% of flavocytochrome *b* is located in the plasma membrane, and the rest in the membrane of specific granules (Morel et al., 1985). The parallel between the percentage of neutrophil flavocytochrome *b* located in the plasma membrane and that ligated by butyl isocyanide might be fortuitous; however, it is tempting to speculate that the flavocytochrome *b* bound to the plasma membrane is preferentially activated upon addition of PMA to the cells (Morel et al., 1985). Nevertheless, the hydrophobic butyl isocyanide diffuses rapidly to all compartments of the cell, and in particular to the azurophil granules where it reacts with myeloperoxidase, as revealed by an increase of the band at 472 nm and the appearance of a shoulder at 463 nm.

In the above spectrophotometric experiment, the bovine neutrophils were reduced with sodium dithionite prior to the addition of butyl isocyanide, which prevented any competition of O_2 for the binding of butyl isocyanide. A complementary experiment was performed with a suspension of bovine neutrophils activated by PMA in an aerated medium supplemented with glucose, and difference spectra of PMA-activated neutrophils *vs* resting neutrophils were recorded. In the absence of butyl isocyanide (Figure 8, trace a), the reduction of the heme component of the flavocytochrome *b* in the steady state was only 12% of that corresponding to full reduction by Na dithionite. Upon addition of butyl isocyanide, a peak at 434 nm appeared, which was accompanied by the decrease of the peaks at 529 nm and 558 nm (Figure 8, trace b). This spectral change reflected the binding of butyl isocyanide to the heme component of the flavocytochrome *b*, whereas the peak at 454 nm most likely reflected the binding of butyl isocyanide to myeloperoxidase. To summarize, the *in vivo* experiments illustrated in Figures 7 and 8 show that activation of neutrophils is required for the binding of butyl isocyanide to flavocytochrome *b*.

EPR Study of the Effect of Arachidonic Acid and Butyl Isocyanide on Air-Oxidized Flavocytochrome b in Neutrophil Plasma Membranes. Figure 9, trace a, shows the EPR

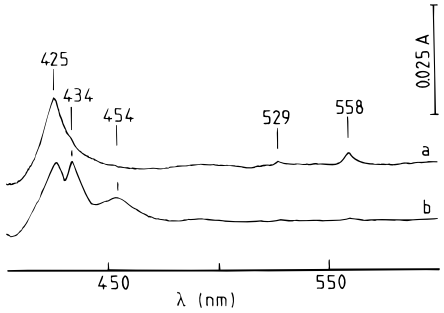

FIGURE 8: Effect of butyl isocyanide on the optical spectrum of PMA-activated neutrophils in stationary phase of respiration. A suspension of neutrophils in aerated PBS (16.5 mg of protein/mL) was divided into three samples, the first one being used as a control. The second sample was supplemented with 10 mM glucose and 0.1 μM PMA. The third sample was supplemented with 10 mM glucose, 0.1 μM PMA, and 30 mM butyl isocyanide. All samples were incubated for 5 min at 22 °C with gentle stirring in order for respiration to reach the stationary phase. The spectra were then recorded at room temperature. Trace a corresponds to the difference spectrum of PMA-activated neutrophils *vs* resting neutrophils. Trace b corresponds to the difference spectrum of PMA-activated neutrophils incubated with butyl isocyanide *vs* resting neutrophils.

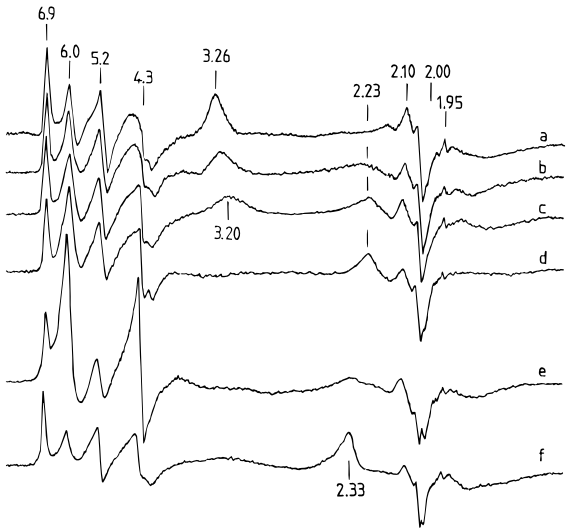

FIGURE 9: Effect of arachidonic acid on the EPR spectrum of neutrophil membranes. Trace a corresponds to neutrophil membranes (20 mg of protein/mL) without added arachidonic acid (control). Traces b−e correspond to spectra of neutrophil membranes incubated for 5 min at 22 °C with increasing concentrations of arachidonic acid. Trace b, 1 μmol/mg of protein; trace c, 2 μmol/mg of protein; trace d, 3 μmol/mg of protein; trace e, 10 μmol/mg of protein; trace f, same conditions as in trace d plus 30 mM butyl isocyanide. After incubation, 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, 9.228 GHz; temperature, 10 K.

spectrum of oxidized hemoproteins in a neutrophil particulate preparation enriched in plasma membranes suspended in aerated PBS supplemented with antiproteases (cf. Materials and Methods). Two heme species were detected, namely, the O_2^- generating flavocytochrome *b* and myeloperoxidase present as a contaminant hemoprotein. Myeloperoxidase was easily distinguished by the typical high-spin resonance lines with g at 6.9 and 5.2 (Wever & Bakkenist, 1980; Ikeda-Saito & Prince, 1985). The signal at $g = 4.3$ was due to adventitious ferric species (Gadsby & Thomson, 1986). The low-field component of the low-spin signal at $g = 3.26$ was considered to be specific of functional flavocytochrome *b*

(Fujii, 1992, 1995c; Miki et al., 1992). We show here that flavocytochrome *b* contributed, at least in part, to the high-spin signal a $g = 6.0$ (see below). Signals in the $g = 2.0$ region resulted from the overlapping of several components and will not be considered for discussion.

After a 5-min incubation of the neutrophil membranes with arachidonic acid added at concentrations ranging between 1 and 3 μmol/mg of protein, which are the effective concentrations for oxidase activation in a cell-free system, the low-spin signal at $g = 3.26$ broadened and then vanished whereas the high-spin signal at $g = 6.0$ steadily increased and a low-spin signal at $g = 2.23$ appeared and increased in parallel with the signal at $g = 6.0$. The decrease of the $g = 3.26$ signal was accompanied by a small shift of the signal toward higher field. The maximal increase in the $g = 2.23$ signal coincided with the loss of the $g = 3.26$ signal and was attained for a concentration of arachidonic acid which enhanced maximally oxidase activation in a cell-free system.

When the concentration of arachidonic acid was increased to 10 μmol/mg of membrane protein, well beyond the optimal value, the elicited oxidase activity in the cell-free system was inhibited (see Figure 2), and, concomitantly, the signal at $g = 2.23$ virtually disappeared whereas the signals at $g = 6.0$ and $g = 4.3$ were markedly increased. The increase of the signal at $g = 4.3$ due to unbound Fe(III) suggests that beyond a critical threshold amount of arachidonic acid, neutrophil flavocytochrome *b* is denatured and part of the structurally-bound iron is released. From these results, it appears that arachidonic acid at concentrations of 1−3 μmol/mg of membrane protein which are not deleterious for flavocytochrome *b* promotes the transition of the heme iron from a low-spin state with a *g* value of 3.26 to two other spin states in equilibrium, namely, a low-spin state at $g = 2.23$ and a high-spin state at $g = 6.0$. This interpretation is corroborated by the EPR spectra of neutrophil membranes treated with 30 mM butyl isocyanide in the presence of arachidonic acid (Figure 9, trace f). Whereas butyl isocyanide added alone to neutrophil membranes did not modify the optical spectrum of the heme component of bound flavocytochrome *b* (cf. Figure 1), it promoted significant changes when its addition was preceded (or followed) by that of arachidonic acid at 2 μmol/mg of protein. Under this condition, the signal at $g = 2.23$ disappeared, the signal at $g = 6.0$ was strongly diminished, and a low-spin signal at $g = 2.33$ emerged. The $g = 2.33$ signal is attributed to the butyl isocyanide liganded form of flavocytochrome *b*. Most probably, butyl isocyanide reacts with the open sixth coordination site of the high-spin heme component of flavocytochrome *b*. It can be concluded that specific experimental conditions, originating in the present case from interactions of arachidonic acid with neutrophil membranes, may induce a reversible transition between different spin states of the heme iron of the membrane-bound flavocytochrome *b*. In this context, butyl isocyanide proves to be a valuable probe to detect the presence of the heme iron of flavocytochrome *b* in a pentacoordinated state. It is inferred that the butyl isocyanide-dependent red shift of the optical spectrum of flavocytochrome *b* reflects the binding of butyl isocyanide to the pentacoordinated state of the heme iron of flavocytochrome *b*. The increase of the signal at $g = 6.0$ observed by Fujii et al. (1995c) upon lowering or raising the medium pH from the value of 7 to values close to 4 or 12 could not explain the effect of arachidonic acid described here, since addition of arachidonic acid at concentrations up to 3 μmol/mg of protein did not result in significant modification of the pH of the medium.

Since the data of the EPR spectroscopy experiments afford evidence for an arachidonic acid-induced transition of the heme iron of flavocytochrome *b* from the hexacoordinated state to the pentacoordinated state, it was interesting to determine whether this transition could be reversed upon the removal of arachidonic acid. For this purpose, bovine serum albumin was used to trap arachidonic acid as previously described (Ligeti et al., 1989), and the reversal of the transition was followed by optical spectroscopy, using butyl isocyanide as a probe of the coordination state. Dithionite-reduced neutrophil membranes treated by arachidonic acid and then by butyl isocyanide showed the typical red shift of the Soret band from 425 to 434 nm, indicating that butyl isocyanide had bound to the pentacoordinated heme iron. In contrast, neutrophil membranes treated first by arachidonic acid and a few minutes later by serum albumin displayed, after dithionite reduction, a Soret band at 425 nm, that was no longer sensitive to butyl isocyanide. It is concluded that removal of arachidonic acid results in the reversal of the pentacoordinated state of the heme iron to a hexacoordinated state. The combined results of EPR spectroscopy and optical spectroscopy experiments clearly show that arachidonic acid modifies the heme environment of flavocytochrome *b* in a reversible manner. This modification is required for the access and the binding of butyl isocyanide to the heme iron of the flavocytochrome.

Compared Analysis by EPR Spectroscopy and Optical Spectroscopy of the Effect of Arachidonic Acid and Butyl Isocyanide on Air-Oxidized Samples of Purified Flavocytochrome b. The results of EPR experiments carried out with neutrophil membranes (preceding Section) have shown substantial modifications of the low- and high-spin components of the heme of flavocytochrome *b* upon addition of arachidonic acid and butyl isocyanide. These modifications were clearly identified by the low-field components of the EPR signals, and we focused our attention on these components in a further analysis of a sample of purified flavocytochrome *b* in Triton X-100. Purified flavocytochrome *b* used in EPR experiments was fully competent for activation by cytosolic factors and production of O_2^-. Using similar conditions as those described by Escriou et al. (1996), the rate of formation of O_2^- by the activated flavocytochrome *b* at optimal arachidonic acid concentrations amounted to 30 mol/mol of heme *b*/s, a value which compares favorably with those reported by others (Rotrosen et al., 1992; Sumimoto et al., 1992; Koshkin & Pick, 1993, 1994; Escriou et al., 1996).

The EPR spectrum of purified flavocytochrome *b* solubilized in 0.1% Triton X-100 exhibited the same high-spin and low-spin signals, at $g = 6.0$ and $g = 3.26$ respectively, as those found with neutrophil membranes (Figure 10, trace a). Although the optical spectrum of purified flavocytochrome *b* did not show the typical peak of myeloperoxidase at 472 nm (Figure 3, insert), small signals at $g = 6.9$ and $g = 5.2$ indicated the presence of traces of myeloperoxidase. Upon incubation of the purified flavocytochrome *b* with increasing concentrations of arachidonic acid, a new signal at $g = 2.23$ appeared and increased in parallel with the signal at $g = 6.0$, whereas the $g = 3.26$ signal was virtually erased (Figure 10, traces b−d). The arachidonic acid-dependent

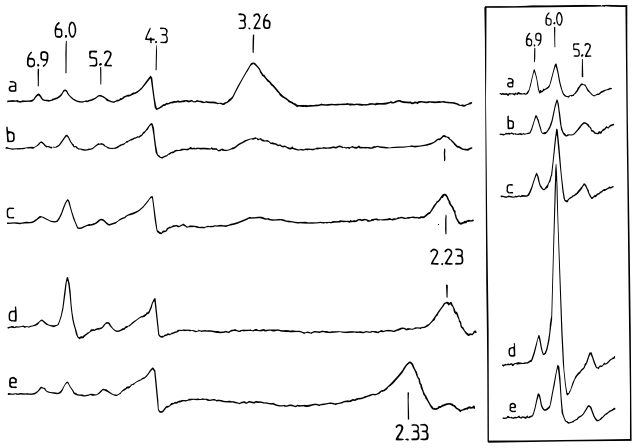

FIGURE 10: Effect of arachidonic acid and butyl isocyanide on the EPR spectrum of purified neutrophil flavocytochrome *b*. The concentration of heme b_{558} in the preparation of the purified flavocytochrome *b* was 22 μM. Traces a−e correspond to EPR spectra of purified flavocytochrome *b* under various conditions. Trace a, Control flavocytochrome *b*; traces b−d, flavocytochrome *b* incubated for 2 min at 22 °C with 0.5, 1.2, and 2 μmol of arachidonic acid per nmol of heme *b*, respectively; trace e, flavocytochrome *b* incubated for 2 min at 22 °C with 2 μmol of arachidonic acid per nmol of heme *b* plus 12 mM butyl isocyanide. Same conditions of recording as in Figure 10. Insert. Temperature was kept at 4 K instead of 10 K.

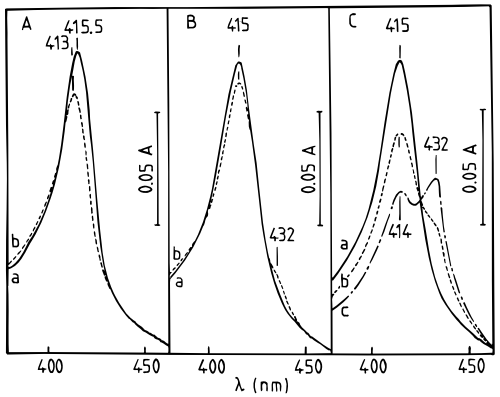

FIGURE 11: Effect of arachidonic acid and butyl isocyanide on oxidized absorption spectra of purified flavocytochrome *b* in 0.1% Triton X-100 in PBS. (A) Spectrum recorded at 77 K: (—) contorl; (- - -) incubation with arachidonic acid (2 μmol/nmol of heme *b*) for 5 min at 22 °C. (B) Spectrum recorded at 22 °C: (—) control; (- - -) incubation with 30 mM butyl isocyanide for 10 min at 22 °C. (C) Spectrum recorded at 22 °C: (—) control; (- - -) incubation with arachidonic acid (2 μmol/nmol of heme *b*) plus 30 mM butyl isocyanide for 5 min at 22 °C; (—•—) incubation with arachidonic acid plus butyl isocyanide for 10 min.

transition between the two low-spin states at $g = 3.26$ and $g = 2.23$ might be interpreted as being due to a constraint change induced by the binding of arachidonic acid to the heme *b*. Even at a concentration of arachidonic acid of 2 μmol/nmol of heme *b*, which markedly amplified the signal at $g = 6.0$, neither the signal at $g = 4.3$ assigned to unbound Fe(III) was modified nor were the signals of myeloperoxidase at $g = 6.9$ and 5.2. Butyl isocyanide reversed the increase of the $g = 6.0$ signal due to arachidonic acid and suppressed the signal at $g = 2.23$ at the expense of a new low-spin signal at $g = 2.33$, an effect similar to that described for the membrane-bound flavocytochrome *b* (Figure 10, trace e). It is noteworthy that the small signals at $g = 6.9$ and at $g = 5.2$, which reflect the presence of minute amounts of myeloperoxidase, are not modified by addition of arachidonic acid.

For comparison with EPR data, analysis of the effect of arachidonic acid and butyl isocyanide on the optical properties of the purified flavocytochrome *b* was carried out on an air-oxidized solution of this flavocytochrome *b*. Three different sets of conditions were used (Figure 11): (1) addition of arachidonic acid (panel A); (2) addition of butyl isocyanide (panel B); (3) addition of arachidonic acid plus butyl isocyanide (panel C). The spectrum presented in Figure 11, panel A, was recorded at 77 K. It shows that arachidonic acid added to oxidized flavocytochrome *b* induced a blue shift of 2−3 nm of the Soret peak at 415.5 nm. The blue shift took place in 2−3 min at room temperature. It suggested the emergence of a high-spin pentacoordinated configuration of the heme iron in flavocytochrome *b* (Brill & Williams, 1961), an hypothesis consistent with the results of the EPR experiment carried out with the air-oxidized flavocytochrome, presented above. The blue shift was not generated by arachidonic acid methyl ester. Spectra in panels B and C of Figure 11 recorded at room temperature show the effect of butyl isocyanide on the Soret peak of the oxidized flavocytochrome *b* in the absence or presence of arachidonic acid, respectively. In the absence of arachidonic acid, the effect of added butyl isocyanide was restricted to a slight decrease of the 415 nm peak and the emergence of a very small shoulder localized essentially in the 430−432 nm region (Figure 11, panel B). This shoulder most likely reflected the binding of butyl isocyanide to less than 10% of the heme *b*. It was stabilized in 2−3 min and was markedly amplified upon the subsequent addition of arachidonic acid (Figure 11, panel C). The butyl isocyanide-induced shifts of the signals in EPR spectroscopy and of the Soret band in optical spectroscopy support the conclusion that butyl isocyanide directly reacts with the heme iron of flavocytochrome *b*. It is noteworthy that the spectral changes of the purified flavocytochrome in its oxidized state in the presence of arachidonic acid plus butyl isocyanide are much slower (20 times at least) than those of the purified flavocytochrome *b* in its reduced state. No significant modification of the oxidized spectrum was observed when arachidonic acid methyl ester was added first, followed by butyl isocyanide, which confirms the central role played by the carboxylic group of arachidonic acid.

DISCUSSION

Paradox of the Reactivity of Flavocytochrome b to O_2. Results accumulated during the past few years have permitted a significant advance in the understanding of the steps leading to the assembly of the components of the neutrophil NADPH oxidase complex. Yet, the mechanism by which flavocytochrome *b*, which is the redox core of NADPH oxidase, undergoes a transition from the resting state to the activated state remains to be elucidated. In particular, although it is generally accepted that the heme group of flavocytochrome *b* reacts directly and rapidly with O_2 to generate the superoxide anion O_2^-, the mechanism of electron transfer from the heme to O_2 remains a matter of debate. For example, both cytochrome oxidase and cytochrome P450 have five of the heme iron coordination sites occupied by intrinsic ligands, the sixth coordination site being opened for the binding of O_2 or other extrinsic ligands. In contrast, neutrophil flavocytochrome *b*, either purified (Miki et al.,

1992; Isogai et al., 1993) or membrane-bound (Ueno et al., 1991; Fujii & Kakinuma, 1992), apparently behaves in EPR as a low-spin hexacoordinated hemoprotein with two histidine residues (Fujii et al., 1995b). To explain this paradox, Isogai et al. (1995) hypothesized that electrons are transferred from the heme of flavocytochrome *b* to O_2 at (or near) the heme edge without formation of an heme iron−O_2 complex. The above rationale does not, however, explain the following results: (1) although butyl isocyanide reacts slowly with flavocytochrome *b*, its inhibitory effect on the respiratory burst of activated neutrophils [Cross et al. (1984) and this paper] is as effective as that of cyanide on cytochrome *c* oxidase; (2) pyridine, which is a reversible inhibitor of NADPH oxidase, modifies the EPR spectrum of neutrophil flavocytochrome *b* in such a way that the new EPR spectrum strikingly resembles that of cytochrome P450 (Fujii et al., 1995a).

Another paradox is the high reactivity of flavocytochrome *b* to O_2 which contrasts with its inability to react with CO. It has been shown that O_2 uptake and production of O_2^- by PMA-activated neutrophils are strictly insensitive to CO (Morel & Vignais, 1984; Morel et al., 1985). This may appear surprising since CO mimicks O_2 for binding to a number of hemoproteins. There are, however, exceptions, and in addition to neutrophil flavocytochrome *b*, some bacterial cytochromes *o* which function as oxidase have been found to be insensitive to CO (Cypionka & Meyer, 1983).

Anionic Amphophils Which Are Required for Oxidase Activation in a Cell-Free System Increase the Reactivity of Flavocytochrome b toward Butyl Isocyanide. The present study originated from the finding and arachidonic acid, an anionic amphophil which elicits oxidase activation in a cell-free system (Bromberg & Pick, 1984; Heyneman & Vercauteren, 1984) and which was reported to be a physiological activator of the oxidase in stimulated neutrophils (Henderson et al., 1993), drastically enhanced the optical spectral modifications of the heme component of flavocytochrome *b* induced by butyl isocyanide. In this study, butyl isocyanide was used as a probe of the coordination state of the heme iron of flavocytochrome *b*.

The effect of arachidonic acid was clearly exhibited with dithionite-reduced flavocytochrome *b* either membrane-bound or purified. It was also found with oxidized flavocytochrome *b* although the reactivity of butyl isocyanide toward the oxidized form of the heme iron was markedly lower than toward the reduced form. The effect of arachidonic acid was counteracted by its methyl ester derivative, suggesting that the carboxylic group of arachidonic acid interacted with a strategic region in the vicinity of the heme iron. Similar optical changes were observed when arachidonic acid was replaced by another anionic amphophil, sodium dodecyl sulfate. The possibility that the anionic group of the two amphophilic reagents interacted with the flavocytochrome *b* component of the oxidase complex was consistent with the report that sodium dodecyl sulfate increases by more than 2-fold the rate of O_2^- production in purified relipidated flavocytochrome *b* in the absence of cytosolic factors (Koshkin & Pick, 1993). Although the structural modifications induced by amphophilic anions resulted in effects similar to those induced by detergents, or changes of pH, they were probably not the same. Presumably, alkalinization or acidification and possibly anionic amphophils modified the ionization state of amino acid residues in the close neighborhood of heme *b*. However, in addition to their ionizable group, anionic amphophils contain a long hydrophobic chain which may facilitate their access to heme *b* and consequently promote the binding of butyl isocyanide to the heme iron. Even if arachidonic acid and Triton X-100 have in common a hydrophobic tail, arachidonic acid has *per se* some specific properties probably due to its ionizable carboxylic group, as illustrated in the experiment of Figure 11. In this experiment, flavocytochrome *b* solubilized in Triton X-100 reacted in its oxidized state with butyl isocyanide exclusively in the presence of arachidonic acid; only then was butyl isocyanide capable of binding to the heme iron of flavocytochrome *b*.

Butyl isocyanide is known to react with carboxylic acids (Passerini reaction), and therefore one may wonder whether a mixture of butyl isocyanide and arachidonic acid might have potential for nonspecific effects. This does not appear to be the case. In fact, arachidonic acid *per se* was shown to have specific effects in modifying, in the absence of butyl isocyanide, the spin state of the heme iron of flavocytochrome *b* (Figures 9 and 10). Further, the red shift in the optical spectrum of reduced neutrophil membranes observed after addition of arachidonic acid and butyl isocyanide was also observed after addition of butyl isocyanide, in the absence of arachidonic acid, to activated neutrophils (Figure 8). This red shift was not observed with resting neutrophils. Finally, purified flavocytochrome *b* in Triton X-100 was able to react in its reduced state with butyl isocyanide, in the absence of arachidonic acid, to make a heme *b* butyl isocyanide complex (Figure 3).

The physical state of the anionic amphophils used in this study, namely arachidonic acid and sodium dodecyl sulfate, depends on a number of parameters including the ionic strength and the pH of the medium, the concentration of the amphophils, and temperature. The efficient concentrations of sodium dodecyl sulfate used were below the critical micellar concentration. On the other hand, arachidonic acid in aqueous solutions is supposed to assume different physical states, depending on the pH of the medium, as this has been shown for long-chain saturated, and unsaturated, fatty acids (Cistola et la., 1988). Briefly, at physiological pH, a lamellar phase is formed, and at pH higher than 9 a micellar phase takes place. Therefore, the relationship between the physical states of the anionic amphophils, sodium dodecyl sulfate and arachidonic acid, and their effects on oxidase activation and the promotion of a pentacoordinated state of the heme iron do not appear to be unequivocal.

Optical Absorption Spectroscopy vs EPR Spectroscopy for Analysis of the Coordination State of the Oxidized Heme Iron of Flavocytochrome b. Because EPR spectroscopy experiments are conducted with aerated samples of flavocytochrome *b*, the comparison with optical spectroscopy experiments required an oxidized state of the flavocytochrome *b*. In both cases, although the purified flavocytochrome was solubilized in Triton X-100, added arachidonic acid has a decisive effect. In the EPR spectrum of Figure 10, addition of arachidonic acid resulted in the appearance of a low-spin signal at $g = 2.23$ and a high-spin signal at $g = 6.0$. The high-spin signal at $g = 6.0$ was consistent with the blue shift of the Soret peak of the oxidized flavocytochrome *b* found in the optical spectrum recorded at 77 K (Figure 11, panel A). The two EPR signals at $g = 2.23$ and $g = 6.0$ were lost upon the subsequent addition of butyl isocyanide and a new

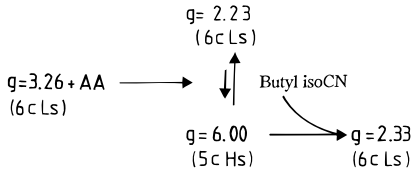

FIGURE 12: Scheme depicting the arachidonic acid- and butyl isocyanide-dependent changes of the spin state of the heme component of neutrophil flavocytochrome *b*. The initial heme configuration of the flavocytochrome with a signal at $g = 3.26$ (hexacoordinated heme iron, 6cLs) is postulated to be modified by arachidonic acid (AA) to provide at least two distinct heme species in equilibrium, characterized by a high-spin signal at $g = 6.0$ (pentacoordinated heme iron, 5cHs) and a low-spin signal at $g = 2.23$ (hexacoordinated heme iron, 6cLs), respectively. The $g = 6.0$ high-spin species is supposed to react with butyl isocyanide to form a hexacoordinated complex as shown by the appearance of the signal at $g = 2.33$ and the optical spectral modification. On the basis of available data, it is not possible to elaborate a more precise pathway for the transition from the $g = 3.26$ species to the $g = 2.23$ and $g = 6.0$ species.

signal emerged at $g = 2.33$. The most likely explanation is that butyl isocyanide binds to the pentacoordinated high-spin heme iron with a $g = 6.0$ signal to generate a butyl isocyanide−heme *b* complex in which the heme iron is characterized by a signal at $g = 2.33$. The butyl isocyanide oxidized heme *b* complex was clearly identified in optical spectra by a peak at 432 nm (Figure 11, panel C). These results and their interpretation in terms of transitions between different coordinated forms of the heme *b* iron are summarized in the scheme of Figure 12. The likelihood that the transitions depicted in Figure 12 occur in intact activated neutrophils is based on the finding that added butyl isocyanide binds to the heme iron of the flavocytochrome *b* only after the cells have been activated (Figures 7 and 8).

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