The Heme Component of the Neutrophil NADPH Oxidase Complex Is a Target for Aryliodonium Compounds†

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Received October 1, 1998; Revised Manuscript Received January 5, 1999

ABSTRACT: The redox core of the neutrophil NADPH oxidase complex is a membrane-bound flavocytochrome *b* in which FAD and heme *b* are the two prosthetic redox groups. Both FAD and heme *b* are able to react with diphenylene iodonium (DPI) and iodonium biphenyl (IBP), two inhibitors of NADPH oxidase activity. In this study, we show that the iodonium modification of heme *b* contributes predominantly to the inhibition of NADPH oxidase. This conclusion is based on the finding that both iodonium compounds decreased the absorbance of the Soret peak of flavocytochrome *b* in neutrophil membranes incubated with NADPH, and that this decrease was strictly correlated with the loss of oxidase activity. Furthermore, the heme component of purified flavocytochrome *b* reduced to no more than 95% by a limited amount of sodium dithionite could be oxidized by DPI or IBP. Butylisocyanide which binds to heme iron precludes heme *b* oxidation. In activated neutrophil membranes, competitive inhibition of O_2 uptake by DPI or IBP occurred transiently and was followed by a noncompetitive inhibition. These results, together with those of EPR spectroscopy experiments, lead us to postulate that DPI or IBP first captures an electron from the reduced heme iron of flavocytochrome *b* to generate a free radical. Then, the binding of this radical to the proximate environment of the heme iron, most probably on the porphyrin ring, results in inhibition of oxidase activity. In the presence of an excess of sodium dithionite, DPI and IBP produced a biphasic decrease of the Soret band of flavocytochrome *b*, with a break in the dose effect curve occurring at 50% of the absorbance loss. This was consistent with the presence of two hemes in flavocytochrome *b* that differ by their sensitivity to DPI or IBP.

Flavocytochrome b_{558}, the redox core of the NADPH oxidase complex in phagocytic cells, is a membrane-bound protein consisting of two subunits, a glycoprotein of 91 kDa (subunit β) and a protein of 22 kDa (subunit α) with redox centers corresponding to the prosthetic groups FAD and heme *b* (for review, see *1*−*3*). The redox potentials of these centers of −280 mV (4) and −245 mV, respectively (5), are close to that of the O_2/O_2^- couple (−160 mV) (6), enabling them to transfer electrons from NADPH to O_2 which is reduced to the superoxide anion O_2^-. All redox centers are located in the β subunit (7−10). Over the past few years, considerable attention has been paid to the role of strategic amino acid residues in each of the two subunits, α and β, of the neutrophil flavocytochrome *b*, using a genetic approach. The results led the authors to draw a number of conclusions relative to specific interactions between the cytosolic factors of oxidase activation and well-delineated regions of the α and β subunits (for review, see *3*), and also to postulate specific amino acid environments for the redox centers, for example, the existence of two histidine bridges to the heme groups in the β subunit (*11*, *12*). The genetic approach has been complemented by the use of inhibitors of electron transfer. The bis-aryliodonium salts diphenylene iodonium (DPI)[1] and iodonium biphenyl (IBP), which are typical arylating agents (for review, see *13*), proved to be particularly potent inhibitors of neutrophil NADPH oxidase (*14*−*16*) and of macrophages (*17*). In a recent study, DPI was shown to form an adduct with free FAD reduced by sodium dithionite, and it was postulated that inhibition of NADPH oxidase by DPI results from the binding of DPI to the FAD prosthetic group of flavocytochrome *b* (*18*). There are, however, other data which show that the heme *b* component of flavocytochrome *b* is also reactive to DPI and IBP (*19*, *20*). In the present work, we have pursued our exploration of the effect of IBP and DPI on neutrophil NADPH oxidase. We have analyzed by optical and EPR spectroscopy the modifications induced in heme *b* upon addition of the two iodonium salts, and we have correlated the spectral modifications of heme *b* with the inhibition of the NADPH oxidase activity. We have also compared the effects of DPI and IBP on optical modifications of free hemin and free FAD. The results show that the heme component of neutrophil flavocytochrome *b* is modified by IBP and DPI as efficiently as the FAD

† 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 (4034) and the Direction des Systèmes de Forces et de Prospectives (DGA 96/2558A).

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[1] Abbreviations: BICN, *tert*-butylisocyanide; DPI, diphenylene iodonium; IBP, iodonium biphenyl.

10.1021/bi9823481 CCC: $18.00
Published on Web 03/03/1999

prosthetic group and that the chemical modification of heme *b* by the two iodonium salts is correlated with the inhibition of NADPH oxidase activity.

EXPERIMENTAL PROCEDURES

Materials. NADPH, ATP, FAD, and GTPγS were from Boehringer; horse heart cytochrome *c* type III, arachidonic acid, and dimethyl sulfoxide were from Sigma; butylisocyanide (BICN) and *N-tert*-butyl-α-phenylnitrone were from Fluka; hemin (chloroferriprotoporphyrin) and iodonium biphenyl (IBP) were from Aldrich; diphenylene iodonium (DPI) was synthesized as described (*20*).

Biological Preparations. Neutrophil plasma membranes and cytosol were prepared from bovine neutrophils 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, supplemented with 1 mM diisopropyl fluorophosphate and 1 mM EDTA. Flavocytochrome *b* was purified as previously described (*21*). Protein concentration was assayed with the BCA reagent using bovine serum albumin as standard. Hemin was solubilized in dimethyl sulfoxide. Azurophil granules, as a source of myeloperoxidase, were prepared by centrifugation in a discontinuous sucrose gradient (15−40%) at 100000*g* for 1 h. The resulting pellet was highly enriched in azurophil granules.

Spectrophotometric Experiments. Spectra were recorded at room temperature with an Uvikon 930 spectrophotometer or with a double-beam Perkin-Elmer 557 spectrophotometer. In some experiments, spectra were recorded in an anaerobic chamber with a Hewlett-Packard 8452A diode array spectrophotometer. Fluorescence measurements were carried out with a Perkin-Elmer MPF2A fluorimeter.

EPR Spectroscopy. 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 were transferred to EPR quartz tubes. The tubes were frozen, and the EPR spectra were recorded.

Assay of Oxidase Activity in the Cell-Free System. NADPH oxidase activity was assayed either by measurement of the production of the superoxide O_2^- or by measurement of O_2 uptake. In both cases, the assay of oxidase activity was preceded by an activation step (*22*) which consisted of mixing neutrophil plasma membranes and cytosol prepared from resting neutrophils, using a cytosol to membrane protein ratio of about 5:1. The mixture was supplemented with 2 mM $MgSO_4$, 20 μM GTPγS, 200 μM ATP, and an optimal amount of arachidonic acid (between 1 and 2 μmol/mg of membrane protein). After 7 min of activation at room temperature, the elicited oxidase activity was measured. When the oxidase activity was assessed by the rate of production of the superoxide anion O_2^-, a 20 μL sample of the suspension of activated particles corresponding to 10−30 μg of membrane protein was transferred to a photometric cuvette containing 200 μM NADPH and 100 μM cytochrome *c* in 2 mL of PBS at room temperature. Cytochrome *c* reduction was recorded at 550 nm, then 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 production of O_2^- was calculated from the difference between the two slopes. In the assay of oxidase activity by O_2 uptake, the suspension of activated particles (200−300 μg of membrane protein in 100 μL) was transferred to an oxygraphic cuvette containing 200 μM NADPH in 1.5 mL of PBS. The rate of O_2 uptake was measured at 25 °C polarographically with a Clark electrode at a voltage of 0.8 V.

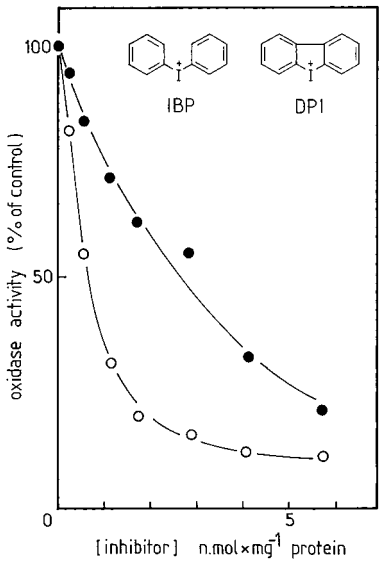

FIGURE 1: Inhibition of NADPH oxidase activity of activated neutrophil membranes by DPI and IBP. The membrane-bound oxidase was activated in a cell-free system consisting of purified plasma membranes, cytosol, arachidonic acid, GTPγS, ATP, and $MgSO_4$ in PBS (volume 50 μL) as described under Experimental Procedures. After incubation for 7 min at 25 °C, the suspension was supplemented with 0.3 mM NADPH and IBP (●) or DPI (○) at the indicated concentrations. After 3 min, the suspension was added to 2 mL of PBS containing 200 μM NADPH and 100 μM cytochrome *c* in a photometric cuvette. The rate of O_2^- production 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%, indicating that the rate of cytochrome *c* reduction reflected the NADPH oxidase activity of neutrophil membranes.

RESULTS

IBP and DPI as Inhibitors of the NADPH Oxidase Activity Elicited in a Cell-Free System of Oxidase Activation. In earlier studies, it was found that IBP and DPI inhibit the production of O_2^- by activated neutrophil membranes, provided that a reducing agent is present together with the iodonium compounds (*19*, *20*). In the experiment of Figure 1, neutrophil membranes activated in a cell-free system (cf. Experimental Procedures) were preincubated with NADPH as reducing agent and either DPI or IBP for a few minutes before the assay of oxidase activity. DPI proved to be a more potent inhibitor than IBP, half-inhibition of oxidase activity requiring 4−5 times less DPI than IBP, i.e., 0.6 nmol vs 2.8 nmol/mg of membrane protein. The higher inhibitory potency of DPI, compared to IBP, might be due to the more hydrophobic nature of DPI, which allows it to reach the redox components of the oxidase complex embedded in the membrane, or to a better chemical reactivity toward specific redox targets. In the following experiments, IBP and DPI were used concurrently.

Aryliodonium-Dependent Redox Modifications of the Heme b Component of Flavocytochrome b_{558}. In the experiment

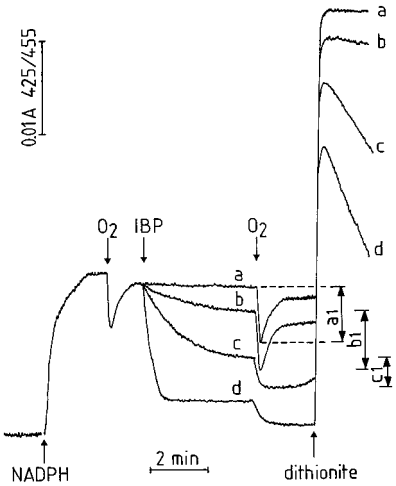

FIGURE 2: Effect of IBP on the height of the Soret peak of membrane-bound flavocytochrome *b* as a function of redox conditions. The photometric cuvette contained 2 mg of neutrophil membrane protein in PBS pregassed with argon, final volume 1 mL. Membrane-bound NADPH oxidase was activated by preincubation with 8 mg of cytosol protein, 4 μmol of arachidonic acid, 0.5 mM ATP, 2.5 mM $MgSO_4$, and 20 μM GTPγS. The photometric cuvette was hermetically sealed with a gastight rubber stopper in which three needles were inserted. One of the needles was used for flushing with argon, another for gas outflow, and the third for additions. Membranes were kept in suspension with a magnetic stirrer. The absorbance of the Soret peak of the membrane-bound flavocytochrome *b* was measured between 425 and 455 nm with a double-beam spectrophotometer. Additions were in the following order: NADPH, 250 μM; O_2, 5 nmol (brought with 20 μL of air-saturated PBS); IBP, 0.5, 2.5, or 5 nmol/mg of membrane protein; O_2, 5 nmol (second addition); and finally sodium dithionite, in excess. Addition of O_2 as air-saturated PBS resulted in some dilution of the membrane suspension, which explains why, after return to the reduced state, the absorbance was slightly decreased, compared to that before addition of O_2. Maximal reoxidation of IBP-treated particles following addition of O_2, was denoted a1, b1, and c1.

illustrated in Figure 2, neutrophil membranes were activated in a cell-free system, as in Figure 1. The redox state of the heme component of flavocytochrome *b* was monitored under anaerobic conditions by recording variations in the height of the reduced Soret peak of heme *b*, using the difference in absorbance between 425 and 455 nm. In the absence of a reducing substrate, heme *b* was almost fully oxidized. NADPH, added at the saturating concentration of 250 μM, reduced no more than 40% of heme *b*. The subsequent addition of 5 nmol of O_2, delivered in an aliquot of aerated PBS medium, led to an abrupt and limited reoxidation of heme *b*; this was followed by a slow recovery of the initial reduced state, which required about 50 s for completion. This transient cycle of reoxidation and reduction of heme *b* could be repeated at least 10 times. At this stage of the experiment, four photometric cuvettes were used, in which neutrophil membranes were first subjected to addition of NADPH and O_2. Three of the four cuvettes were supplemented with different concentrations of IBP, namely, 0.5, 2.5, and 5.0 nmol of IBP/mg of membrane protein, the fourth cuvette corresponding to the control. Upon addition of IBP, the absorbance at 425 nm was decreased and did not return to its initial value. The rate and the extent of the absorbance decrease depended on the concentration of IBP. For the three concentrations of IBP used, the absorbance decrease reached a limit that was time-dependent. With 0.5 nmol of IBP added per milligram of membrane protein, it did not go beyond 16%, and this required a period of time of 4 min. With 2.5 and 5 nmol of IBP/mg of membrane protein, the absorbance was decreased by 49% and 79%, in a lapse of time of 3.3 and 0.8 min, respectively.

In parallel to the spectrophotometric assay, we tested the effect of IBP on the NADPH oxidase activity of activated neutrophil membranes. In the presence of 0.5, 2.5, and 5.0 nmol of IBP/mg of membrane protein, the percentages of inhibition of the rate of O_2^- production were 15%, 48%, and 75%, respectively (see Figure 1). The striking parallel between the effect of IBP on the absorbance decrease at 425 nm and the extent of inhibition of the NADPH oxidase activity suggests that heme *b* might be a rate-limiting component in the production of O_2^- by NADPH oxidase. Similar results were obtained with DPI, the only difference being that the absorbance decrease at 425 nm was obtained with a 5-fold lower concentration of inhibitor.

After the IBP-dependent decrease in absorbance had reached completion, a small amount of O_2 was injected into the medium (Figure 2). In the control assay, oxygen addition resulted in rapid reoxidation of reduced heme *b*, followed by a slow return to the reduced state, similar to that encountered after the first addition of oxygen (trace a). With the lower amount of IBP (0.5 nmol/mg of membrane protein), there was no significant modification of the redox cycle (trace b). In contrast, with 2.5 and 5.0 nmol of IBP/mg of membrane protein, the redox cycle was strikingly perturbed; the decrease in absorbance, reflecting oxidation of heme *b*, was slowed (traces c and d), and after the decrease has reached completion, there was either a slow return (trace c) or no return to the initial absorbance value (trace d). The percentages of maximal reoxidation corresponding to traces a, b, and c, denoted a1, b1, and c1 in Figure 2, ranged between 42 and 48%. In other words, in the presence of 0.5 and 2.5 nmol IBP/ mg of membrane protein, the percentage of reoxidized heme *b* was virtually the same as in the control. If the primary site of action of IBP was on FAD, i.e., upstream of heme *b*, reoxidation of heme *b* would increase with the amount of IBP used. Thus, the experimental data support the view that the heme component of flavocytochrome *b* is the predominant target of IBP.

The final addition was that of an excess of sodium dithionite, which led to full reduction of heme *b* in the control membranes. Unexpectedly, the increase in absorbance was smaller in the membrane samples that had been treated with IBP or DPI. Furthermore, at high concentrations of IBP (2.5 and 5 nmol/mg of protein), after reaching a maximum, the absorbance decreased in a concentration- and time-dependent manner, becoming stable within 4−5 min. This suggested the occurrence of a chemical modification of heme *b*, as was previously reported for the inactivation of horseradish peroxidase by phenylhydrazine (*23*, *24*).

Aryliodonium-Dependent Optical Changes of Heme b in Neutrophil Membranes Reduced with an Excess of Sodium Dithionite. To explore in more detail the absorbance modifications at 425 nm observed in the experiment of Figure 2, sequential difference spectra were recorded using neutrophil membranes treated with an excess of sodium dithionite and then with increasing concentrations of IBP (Figure 3, panel A). The absorbance of the reduced Soret peak at 425 nm was progressively lost, and concomitantly the α band of the

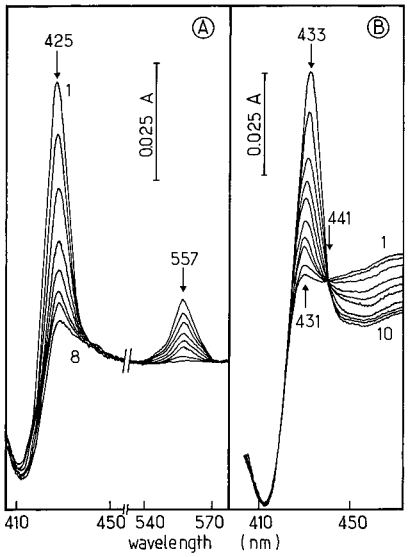

FIGURE 3: Effect of sequential additions of IBP on the difference spectra (dithionite-reduced minus oxidized) of membrane-bound flavocytochrome *b* in the absence or presence of BICN. Panel A. The membrane-bound NADPH oxidase was activated in a cell-free system (cf. Experimental Procedures). The difference spectrum relative to the Soret band and the α band was recorded between 380 and 450 nm and between 520 and 600 nm. After each addition of IBP, membranes were left in contact with the iodonium salt for 5 min, and then the spectrum was recorded. Traces 2−8 correspond to concentrations of IBP of 50, 100, 150, 200, 250, 300, and 400 μM, respectively. Trace 1 corresponds to the control. Panel B: Same protocol as in (A), except that the reduced membranes were treated by 30 mM BICN prior to addition of IBP.

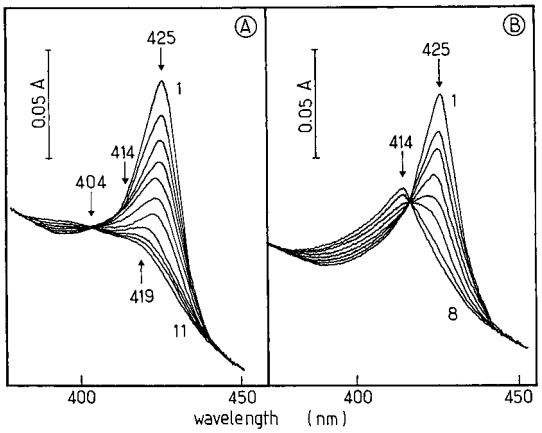

FIGURE 4: Effect of reduction conditions on the modification of absolute spectra of purified flavocytochrome *b* by increasing concentrations of IBP. The effect of IBP on the spectrum of purified flavocytochrome *b* (1.2 nmol/mL of PBS supplemented with 0.1% sucrose monolaurate) was tested after reduction with an excess of sodium dithionite (panel A), and by limiting amounts of sodium dithionite just sufficient to reduce about 95% of heme *b* (panel B). In the latter case, determination of the end point of sodium dithionite reduction was controlled by observing the absence of increase in absorbance at 316 nm. In both cases, IBP was added at increasing concentrations up to 300 μM (trace 8). Trace 1 corresponds to the control. Two minutes after each IBP addition, the spectra were recorded at 20 °C with a Hewlett-Packard 8452A diode array spectrophotometer in an anaerobic chamber.

reduced spectrum disappeared. In a parallel experiment, prior to adding IBP, dithionite-reduced neutrophil membranes were treated by butylisocyanide (BICN), a ligand of the heme iron (Figure 3, panel B). Addition of BICN resulted in a red shift of the Soret peak from 425 to 433 nm and in the disappearance of the α peak at 557 nm, two events which were ascribed to the binding of BICN to the reduced heme iron. In the presence of sodium dithionite, sequential additions of IBP to membranes pretreated with BICN led to a decrease in absorbance at 433 nm. There was, however, no shift of the peak at 433 nm, except at very high concentrations of IBP, indicating that IBP does not displace bound BICN from the heme iron in sodium dithionite-reduced flavocytochrome *b*. The loss of absorbance at 433 nm due to IBP suggested modification of the geometry of the porphyrin ring, presumably due to the binding of IBP to heme *b* at a site other than BICN. Similar results were obtained using DPI as inhibitor.

Effect of Modulating the Redox Conditions on the Aryliodonium-Dependent Spectral Changes of the Heme b Component of Purified Neutrophil Flavocytochrome b. In the experiment of Figure 3, changes in the optical spectrum of flavocytochrome *b* upon addition of IBP or DPI were measured as difference spectra (dithionite-reduced minus oxidized) using a turbid suspension of neutrophil membranes. Whereas gross spectral modifications are readily revealed under these conditions, some minor changes may remain undetected. A complementary experiment was therefore carried out with a clarified preparation of purified flavocytochrome *b* solubilized in detergent, which allowed us to record absolute spectra. The experiment was conducted in an anaerobic chamber (O_2 < 2 ppm). All solutions used were deprived of O_2 by flushing for a few minutes with oxygen-free argon, and they were kept for 1 h in the anaerobic chamber before recording spectra. In the first assay (Figure 4A), sodium dithionite was added in excess to the solution of flavocytochrome *b* for full reduction of the redox centers, and optical spectra were recorded after sequential additions of IBP. The first additions of IBP led to a progressive loss of the Soret peak at 425 nm without noticeable shift of the maximum. At concentrations of IBP higher than 5−10 μM, which fully inhibit oxidase activity, the Soret peak at 425 nm was both decreased and shifted to the lower wavelengths. At concentrations of IBP higher than 50 μM, the shift attained a limit value of 8−10 nm. Despite the shift, all the spectra crossed at an isosbestic point corresponding to 404 nm. Similar spectral modifications of heme *b* were obtained upon sequential additions of DPI to dithionite-reduced flavocytochrome *b*, with the difference that the blue shift started at a concentration of 2−5 μM.

The second IBP titration assay (Figure 4B) differed from the first in that sodium dithionite was added to purified flavocytochrome *b* in the deoxygenated medium in an amount just sufficient to keep heme *b* reduced to no more than 95%. Besides the attenuation of the reduced Soret peak at 425 nm resulting from IBP addition, a peak at 414 nm emerged progressively, reflecting the accumulation of oxidized heme *b*. Definite assignment of the 414 nm peak to oxidized heme *b* was ascertained by its shift to 425 nm upon addition of an excess of sodium dithionite (not shown). Thus, under these specific conditions, it was possible to show that reduced heme *b* delivers electrons directly to IBP.

The spectral changes of heme *b* elicited by IBP in Figure 4B, as well as those observed in a parallel experiment upon addition of DPI (not shown), were quantified in terms of percentages of the initial amount of heme *b* in the fully reduced state (Figure 5). Concentrations of oxidized heme *b*, [b_{ox}], and reduced heme *b*, [b_{red}], were calculated from

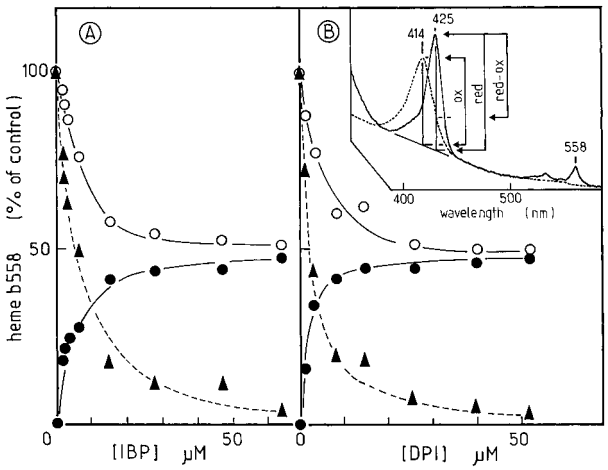

FIGURE 5: Quantification of the effect of IBP (panel A) and DPI (panel B) on the loss of absorbance of the reduced Soret band of purified flavocytochrome *b* and the appearance of oxidized heme *b* under conditions of threshold reduction with sodium dithionite. Concentrations of oxidized heme *b* (●) and reduced heme *b* (▲) in Figure 4 were calculated from the absorbances of absolute spectra measured at 414 and 425 nm, using molar extinction coefficients of 110 and 154 $mM^{-1}\cdot cm^{-1}$, respectively. The following relationships were used (see text): [heme b_{ox}] = $10.87(A_{414} - 0.35A_{425})$ and [heme b_{red}] = $7.75(A_{425} - 0.46A_{414})$, which take into account the respective contribution of the reduced form of heme *b* in the oxidized Soret peak and that of the oxidized form of heme *b* in the reduced Soret peak (see insert). The curve (○), which corresponds to the sum of oxidized heme *b*, and reduced heme *b*, indicates a loss of spectroscopically detectable heme *b*.

the absorbance values (*A*) measured at 414 and 425 nm, using the equations:

$$A_{414} = [b_{ox}]\epsilon_{ox\ 414} + [b_{red}]\epsilon_{red\ 414} \quad (1)$$

$$A_{425} = [b_{ox}]\epsilon_{ox\ 425} + [b_{red}]\epsilon_{red\ 425} \quad (2)$$

The molar extinction coefficients, $\epsilon_{ox\ 414}$, $\epsilon_{ox\ 425}$, $\epsilon_{red\ 414}$, and $\epsilon_{red\ 425}$ were determined from the fully oxidized heme *b* and the fully reduced heme *b*, using as reference the ϵ value of 106 $mM^{-1}\cdot cm^{-1}$ of the Soret band in the difference red/ox spectrum of heme *b* (cf. insert of Figure 5). As shown in Figure 5, upon addition of IBP or DPI, the amount of reduced heme *b* decreased rapidly, and this decrease was accompanied by a rise in oxidized heme *b* which finally amounted to roughly 50% of the initial reduced heme *b*. If the combined amounts of reduced heme *b* (425 nm) and oxidized heme *b* (414 nm) are compared with the initial amount of heme *b*, a loss of spectrally detectable heme *b* of about 50% can be estimated, which probably reflects the formation of an iodonium derivative of heme *b* (see Discussion).

The effect of IBP in the presence of BICN on the reduced Soret band of purified flavocytochrome *b* was different depending on conditions of reduction, namely, reduction with an excess of sodium dithionite or use of a limited amount of sodium dithionite just sufficient to reduce 90−95% of heme *b*. In the first case, sodium dithionite was the main electron donor for IBP or DPI, whereas in the second case, only reduced heme *b* serves as the electron donor. As shown in Figure 6, in the two cases, added BICN shifted the 425 nm reduced Soret peak of heme *b* to 433 nm. With an excess of sodium dithionite, the absorbance at 433 nm was progressively lost upon addition of IBP at increasing concentrations. Above 10 μM IBP, the absorbance loss was accompanied by a blue shift. However, when sodium dithionite was used in limiting amounts, and reduced heme *b* was the only electron donor for IBP, the loss of absorbance at 433 nm due to IBP was markedly reduced, and no blue shift was observed. This implies that when reduced heme *b* is the only electron donor, binding of BICN to the reduced heme iron prevents the electron transfer to IBP and thereby the formation of a reactive radical.

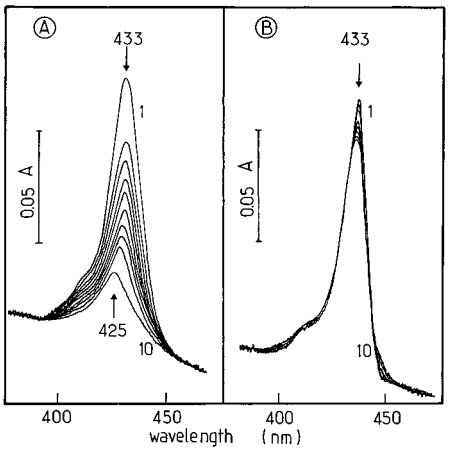

FIGURE 6: Effect of IBP on the absolute spectra of purified flavocytochrome *b* in the presence of BICN, depending on conditions of reduction. Experimental conditions were the same as in Figure 4, except that in the present experiment BICN was added to give a final concentration of 30 mM, following reduction by sodium dithionite, but prior to IBP additions (3, 6, 10, 13, 16, 24, 33, 50, and 100 μM). Curve 1 corresponds to the control. Panel A: Reduction with an excess of sodium dithionite. Panel B: sodium dithionite was added in a limited amount, just sufficient to reduce 95% of heme *b*.

Aryliodonium-Dependent Inhibition of O_2^- Uptake by Activated Neutrophil Membranes. Comparison of the optical spectra shown in Figure 4A,B and also in Figure 6 suggested that, prior to forming a stable heme adduct, IBP or DPI interacts transitorily with the heme iron. This explanation is supported by the oxygraphic experiment illustrated in Figure 7. Neutrophil membranes activated in a cell-free system (see Experimental Procedures) were mixed with IBP and then added to PBS in the oxygraphic cuvette. To test the effect of O_2 concentration on IBP inhibition, the O_2 concentration in the medium of the oxygraphic cuvette was lowered to 40−50 μM by bubbling with N_2. O_2 uptake was then initiated with NADPH added at a saturating concentration, and recorded until anaerobiosis was attained. In a parallel experiment, the activated neutrophil membranes were preincubated for 2 min with IBP in the presence of NADPH, and then the oxidase activity was assayed as described above. The double reciprocal plots in Figure 7 illustrate the effect of IBP on O_2 uptake by activated neutrophil membranes as a function of the O_2 concentration under the two described conditions. In the absence of preincubation, the control curve and the curves obtained at low concentrations of IBP were straight lines that intersected the ordinate at the same point, indicating competition between IBP and O_2 (Figure 7, panel A). However, at higher concentations of IBP, the curves departed from linearity. On the other hand, when IBP was preincubated for 2 min with the neutrophil membranes in the presence of NADPH, linear plots were obtained, which

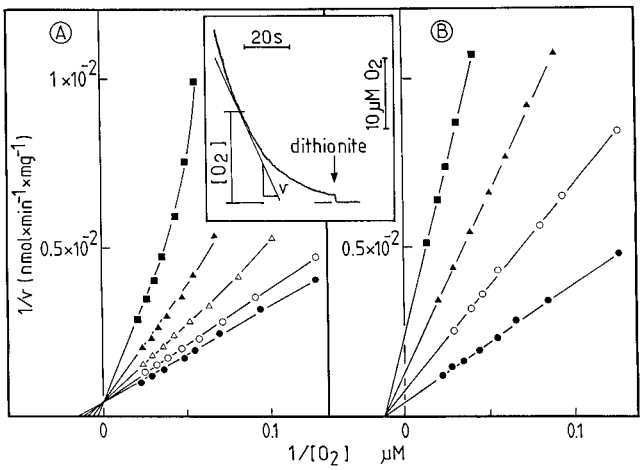

FIGURE 7: Effect of IBP on the affinity of NADPH oxidase for O_2. Neutrophil membranes (300 μg of protein per sample) were activated in a cell-free system of NADPH oxidase activation (cf. Experimental Procedures), and their oxidase activity in PBS was assayed by measuring the rate of O_2 uptake with a Clark electrode at 25 °C. The O_2 concentration of the medium was lowered to 40−50 μM by controlled bubbling of nitrogen, and O_2 uptake was initiated by addition of NADPH at the saturating concentration of 250 μM. The rate of O_2 uptake was deduced from the slopes of the tangents to the oxygraphic traces, and the contact point of the tangent with the curve was used to determine the mean average of O_2 concentration at which O_2 uptake proceeds (see insert). The amount of activated neutrophil membranes was adjusted so that anaerobiosis was achieved in about 1 min. Panel A: IBP was added to the membrane suspension at the following concentrations (nmol/mg of membrane protein): none (●), 0.8 (○), 1.5 (△), 2.3 (▲), 3.8 (■), and then O_2 uptake was immediately recorded. Panel B: at difference with the experiment of panel A, membranes were preincubated with IBP for 2 min at 25 °C, in the presence of 250 μM NADPH before measurement of O_2 uptake. Insert: Record of O_2 uptake between 40 and 50 μM O_2 and anaerobiosis.

intercepted the abscissa at the same point, indicating a noncompetitive inhibition of O_2 uptake by IBP (Figure 7, panel B). Similar inhibition kinetics were found when IBP was replaced by DPI, with the difference that the effective concentrations of DPI were 4−5 times smaller than those of IBP. Thus, competition between IBP or DPI and O_2 for the activated NADPH oxidase is a transient phenomenon which is experienced only at low concentrations of aryliodonium salts and in the absence of preincubation with an electron donor. These results are consistent with a sequential mechanism of action of IBP or DPI which consists first of the transitory formation of an aryliodonium radical by capture of an electron from reduced heme *b* iron, followed by the covalent binding of the generated radical to a reactive residue in the close neighborhood, most probably on the porphyrin ring (see Discussion).

Aryliodonium-Dependent Changes of EPR Spectra of Heme b in Activated Neutrophil Membranes. The effect of IBP and DPI, in parallel with that of BICN, on the coordination state of the heme iron was monitored by EPR spectroscopy (Figure 8). The EPR spectrum of activated neutrophil plasma membranes in the oxidized state (Figure 8, trace a) shows a number of signals that belong to flavocytochrome *b* and myeloperoxidase. Myeloperoxidase, which is a minor contaminant in the plasma membrane preparation and is present in high amount in an azurophil granule extract, was typically characterized by high-spin signals at $g = 6.7$ and $g = 5.0$ (Figure 8, trace d). The signal

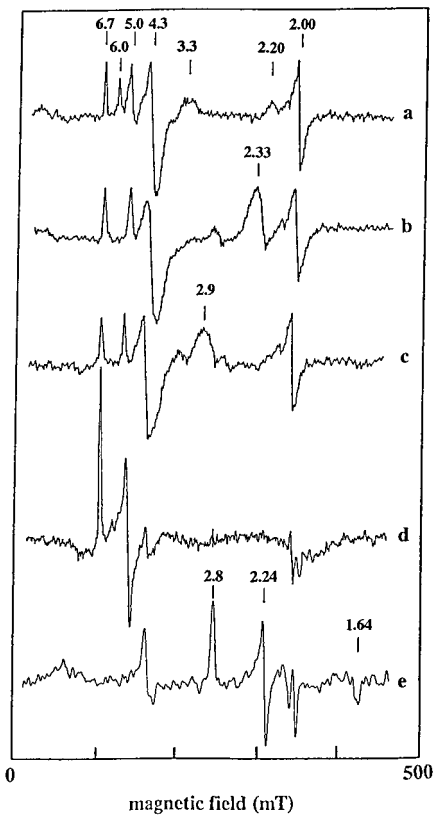

FIGURE 8: Effect of DPI and BICN on the EPR spectrum of neutrophil membranes. Traces a−c are EPR spectra of neutrophil plasma membranes recorded in the following conditions. Trace a corresponds to control plasma membranes (9 mg of protein) activated in a cell-free system (cf. Experimental Procedures), final volume 4 mL. The activated membranes were pelleted by centrifugation and resuspended in 200 μL of PBS supplemented with 1 mM 2′,5′-ADP. Trace b was obtained after addition of 30 mM BICN to the same membrane suspension as that used for trace a. Trace c corresponds to a sample in which 30 mM BICN was added after treatment of membranes with DPI (see text for details). Traces d and e are EPR spectra of a soluble extract of azurophil granules from neutrophils obtained by sonication of the granules in PBS supplemented with 1 M KCl and centrifugation at 100000*g* for 1 h. The extract was enriched in myeloperoxidase (6 μM) and devoid of flavocytochrome *b*. Trace d corresponds to the control and trace e to the granule extract treated by 1 mM KCN. The spectra were recorded under the following conditions: microwave power, 10 mW; modulation frequency, 100 kHz; modulation amplitude, 1.25 mT; microwave frequency, 9.228 GHz; temperature, 10 K.

at $g = 4.3$ was most probably due to adventitious ferric species. In agreement with an early study carried out with purified flavocytochrome *b* (*21*), the low-spin signal at $g = 3.3$, typical of nonactivated neutrophil membranes, disappeared upon oxidase activation, whereas a high-spin signal at $g = 6.0$ which was negligible in resting flavocytochrome *b* was markedly increased as was the signal at $g = 2.20$ (Figure 8, trace a). The hypothesis that the high-spin signal at $g = 6.0$ reflects a pentacoordinated state of the heme iron was corroborated by the effect of BICN, a ligand of the pentacoordinated high-spin heme iron; its effect was to quench the high spin signal at $g = 6.0$ and to promote the emergence of a low-spin signal at $g = 2.33$ (Figure 8, trace b). As shown in Figure 8, trace e, addition of KCN to the granule extract enriched in myeloperoxidase resulted in the disappearance of the signals at $g = 6.7$ and $g = 5.0$, and in the emergence of rhombic low-spin ferric signals with g values of 2.8, 2.24, and 1.64. In contrast to myeloperoxidase,

flavocytochrome *b* was not sensitive to KCN (not shown). Conversely, BICN which readily reacts with flavocytochrome *b* was much less reactive than KCN on myeloperoxidase (not shown). The differential effects of BICN and KCN on the EPR spectrum of neutrophil plasma membranes allow the discrimination of flavocytochrome *b* from myeloperoxidase. As binding of DPI to membrane-bound flavocytochrome *b* requires the presence of a reducing agent, NADPH was added together with DPI to the activated membranes, and the mixture was incubated at room temperature for 2 min. It was checked that under these conditions NADPH oxidase activity was fully inhibited. Then the membranes were sedimented, washed with PBS, and resuspended in PBS supplemented with 2′,5′-ADP, a competitive inhibitor of NADPH oxidase, the role of which is to prevent the residual reduction of flavocytochrome *b*. Finally the suspension was reoxidized by O_2 bubbling. The recorded EPR spectrum did not show any striking difference from that of activated membranes, except for a slight decrease in the high-spin signal at $g = 6.0$ (not shown). Upon addition of BICN to membranes pretreated by DPI (Figure 8, trace c), the signal at $g = 6.0$ was totally suppressed, due to the binding of BICN to the heme iron, and the low-spin signal at $g = 2.33$ was replaced by a signal at $g = 2.9$, suggesting changes in the geometry of heme *b* imposed by the dual binding of DPI and BICN. Similar results were obtained with IBP. They indicate that DPI and IBP form a stable adduct with a functional group in the neighborhood of the heme iron.

Comparison of the Effect of DPI and IBP on the Optical Absorbance Spectra of Free Hemin and Purified Flavocytochrome b. Hemin was reduced by an excess of sodium dithionite, and spectral changes were recorded after sequential additions of DPI or IBP, following the same procedure as that described in Figure 3 in the case of neutrophil membranes. In Figure 9, the log value of the height of the Soret peak at 414 nm was plotted against the concentrations of DPI and IBP. A parallel experiment was carried out with purified flavocytochrome *b* from neutrophil membranes, and likewise the log value of the Soret peak at 425 nm was plotted against the concentrations of the two aryl iodonium salts. Comparison of the two series of plots clearly shows different reactivities of hemin and heme *b* in flavocytochrome *b* toward both DPI and IBP. In the case of hemin, the absorbance value of the Soret peak decreased linearly upon addition of DPI and IBP until a concentration of 8−10 μM was attained, resulting in a loss of absorbance of 75−80%. Above this concentration, the absorbance decrease departs from linearity, and concomitantly the decrease of the residual Soret peak was accompanied by a progressive blue shift to a limit value of 404 nm. In the case of purified flavocytochrome *b* reduced with sodium dithionite (see Figure 4A), a break in the linear plot of the absorbance of the Soret peak of heme *b* as a function of DPI and IBP was observed when the absorbance had decreased by about 50% with respect to the initial absorbance value. The break occurred at a concentration of DPI significantly lower than for IBP (4 μM vs 15 μM). The lower reactivity of heme *b* in flavocytochrome *b* toward IBP compared to DPI, which contrasts with the equal reactivity of hemin toward the two iodonium salts, suggests that the access of IBP to heme *b* in flavocytochrome *b* may be limited by the protein environment of heme *b*. The fact that the break in the linear decrease of the Soret absorbance of flavocytochrome *b* occurred after a loss of absorbance of 50% supports the view that flavocytochrome *b* contains two hemes (25) and that the two hemes differ in their sensitivity to iodonium salts.

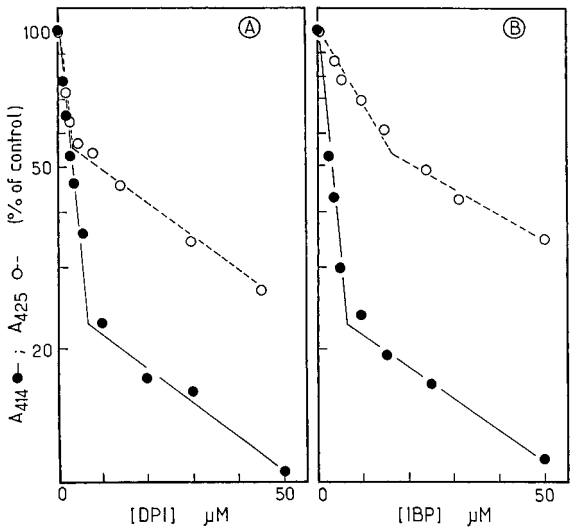

FIGURE 9: Effect of DPI and IBP on the absorption spectrum of free hemin and heme *b* in purified flavocytochrome *b*. A solution of 1 μM hemin in dimethyl sulfoxide was reduced by addition of sodium dithionite, and the spectrum was recorded after sequential additions of DPI and IBP. The percent absorbance of the reduced Soret peak at 414 nm as a function of DPI and IBP concentrations is plotted in a semilogarithmic scale in panels A and B (●). Purified flavocytochrome *b* (1 μM heme b) in PBS and 0.1% sucrose monolaurate was subjected to the same treatment as hemin, and, likewise, the percent absorbance of the Soret peak at 425 nm was plotted in a semilogarithmic scale (○).

Effect of Aryliodonium Salts on the Fluorescence Emission Spectrum of Free FAD. The above data all support the view that the heme *b* moiety of flavocytochrome *b* is a target for aryliodonium salts. It has, however, been argued that DPI binds preferentially to FAD, the other redox prosthetic group of flavocytochrome *b*, by analogy with the well-known reactivity of DPI with flavoproteins (18). Additional experiments were therefore carried out with free FAD, and the binding efficiency of IBP and DPI to FAD was compared to that found in the case of free hemin and purified flavocytochrome *b* (cf. Figure 9).

Aryliodonium-dependent modification of free FAD was analyzed by fluorescence spectrophotometry (Figure 10). The insert of Figure 10 illustrates the protocol of the titration of reduced FAD by IBP. First it was checked that the reduction and reoxidation cycles did not perturb the fluorescence emission of oxidized FAD at 520 nm. FAD was reduced by sodium dithionite, and reoxidized by bubbling O_2. Addition of IBP to oxidized FAD did not modify the fluorescence emission (not shown). In contrast, when IBP was added to dithionite-reduced FAD, followed by reoxidation of the medium, the fluorescence emission of the reoxidized FAD was lower than that of the control without IBP. Sequential additions of dithionite, IBP, and O_2 allowed us to titrate the chemical modification of FAD by IBP. The same experiment was carried out with DPI with similar results. The half-maximum decrease of fluorescence emission was obtained with 5 μM DPI and 15 μM IBP, i.e., concentrations slightly higher than those found to promote loss of absorbance of the Soret peak of hemin in the absence of blue shift. It is

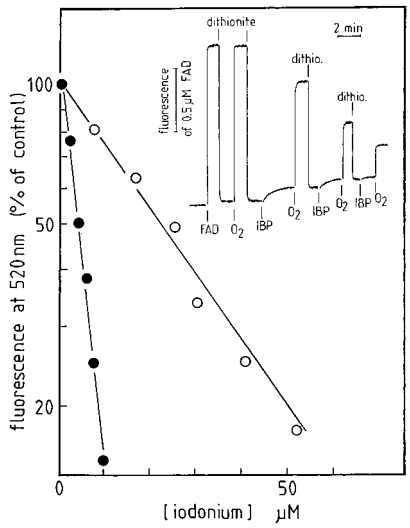

FIGURE 10: Effect of IBP and DPI on the fluorescence emission of free FAD. The insert shows the experimental protocol to analyze modifications of FAD by IBP. The emission fluorescence of oxidized FAD was recorded at 520 nm, using an excitation wavelength of 450 nm. A solution of FAD in PBS (1.5 μM) was used. For reaction with IBP, FAD was first fully reduced by sodium dithionite, and then IBP was added. After a 2 min incubation at room temperature, O_2 was bubbled in the medium to reoxidize the nonmodified FAD, and fluorescence emission was recorded (○). This sequence of additions was repeated until full loss of emitted fluorescence occurred. The same protocol was used for the modification of FAD by DPI (●). The data are plotted in terms of the percentages of emitted fluorescence with respect to iodonium salt concentrations.

noteworthy that a concentration of DPI of 5 μM which fully inhibits activated NADPH oxidase is required to modify half of free FAD (Figure 10), but is sufficient to modify the totality of the highly sensitive fraction of heme *b* in purified flavocytochrome *b* (Figure 9).

DISCUSSION

DPI and IBP are potent arylating agents which react with nucleophiles to form covalent phenylated adducts (*13*). Interest in their use for the study of cellular redox proteins started with the finding that DPI inhibits NADH oxidation by bovine heart submitochondrial particles, and that the extent of inhibition is correlated with the binding of DPI to a component of Complex I of the mitochondrial respiratory chain (*26*). Later, it was reported that iodonium salts react with a number of redox systems including the O_2^- generating NADPH oxidase in phagocytic cells (*14*−*20*), macrophage NO° synthase (*27*), bacterial nicotine oxidase (*28*), xanthine oxidase (*20*), and hepatic cytochrome P450 reductase (*29*). On the basis that aryliodonium compounds inhibit the activity of a variety of flavoproteins, it was accepted that aryliodonium radicals target preferentially flavin prosthetic groups in redox systems. Less attention was paid to the reactivity of iodonium salts toward metal-bound porphyrins which may be encountered in biological systems. In a pioneer work, Battioni et al. (*30*) described the formation of aryl ferric complexes by reaction of a number of diaryliodonium salts with a synthetic ferrotetraphenyl porphyrin and hepatic cytochrome P450 in the presence of sodium dithionite or NADPH as reducing agents. Consistent with this report was our finding that the reduced optical spectrum of the heme *b* component of the neutrophil NADPH oxidase complex is modified upon incubation of neutrophil membranes with IBP (*19*) and DPI (*20*). It is recalled that the two redox components of flavocytochrome *b*, FAD and heme *b*, are potential sites of generation of O_2^-. However, the following data militate in favor of heme *b* as the only site of O_2^- production in neutrophil NADPH oxidase: (1) BICN, which combines selectively with the heme iron of flavocytochrome *b*, fully inhibited O_2^- production (*21*); (2) as a consequence of the mutation in the β subunit of flavocytochrome *b*, no O_2^- was produced by activated neutrophils, and yet electrons were transferred from NADPH via FAD to iodonitrotetrazolium violet used as acceptor (*31*), confirming the occurrence of a cryptic diaphorase activity in flavocytochrome *b* (*20*).

The present work is a follow-up study undertaken to elucidate whether and how heme *b* modification by either IBP or DPI is responsible for inhibition of NADPH oxidase. To answer the question of whether DPI and IBP are potential redox probes of the heme component of the neutrophil NADPH oxidase, a number of experiments described in this paper were carried out under conditions compatible with the functioning of the oxidase, to establish some correlation between the heme modification and the inhibition of the oxidase. As shown in Figures 1, 2, and 3, the loss of the Soret peak absorbance of heme *b* reduced with NADPH in neutrophil membranes treated by IBP or DPI increased in parallel with the NADPH oxidase inhibition. This led us to postulate that the heme component of flavocytochrome *b* regulates the electron flow from NADPH to O_2. A similar loss of absorbance of the Soret peak of protein-bound heme was reported in the case of the reaction of phenylhydrazine with catalase (*32*) and peroxidase (*23*). For both metalloenzymes, the loss of absorbance was due to the formation of a phenyl adduct on the porphyrin ring and was correlated with enzyme inactivation.

A peculiar optical spectral modification of heme *b* by IBP or DPI was revealed under anaerobic conditions when the iodonium salts were added to purified flavocytochrome *b* in the presence of an amount of sodium dithionite just sufficient to reduce no more than 95% of heme *b* (Figures 4 and 5). The reduced heme *b* was converted into its oxidized form with a Soret peak at 414 nm. Analysis of the optical spectra showed a loss of about half the initial amount of heme *b*. We suspected that this peculiar behavior of heme *b* was the result of iodonium-dependent reactions proceeding by a mechanism involving transient free radical species. In an EPR spectroscopy experiment (not shown), we checked that a free radical species was generated from IBP or DPI, using a limited amount of sodium dithionite as a reducing agent as in Figure 4 and *N-tert*-butyl-α-phenylnitrone as a trapping agent; the signal at $g = 2.0$ was roughly 10 times higher in the presence of IBP or DPI than in their absence. Taking into account (1) the ability of IBP and DPI to react with target molecules by a free radical mechanism, (2) the formation of iron porphyrin complexes with aryliodonium compounds (*30*) and (3) the presence of two interacting hemes in the β subunit of flavocytochrome *b* (*10*, *11*, *25*), we interpret the results of Figures 4 and 5 by the reactions described in the scheme of Figure 11. Reaction 1 corresponds to the capture of an electron from the first reduced heme (heme 1) by IBP which is split to give rise to a phenyl radical and iodophenyl. The second reduced heme (heme 2) delivers

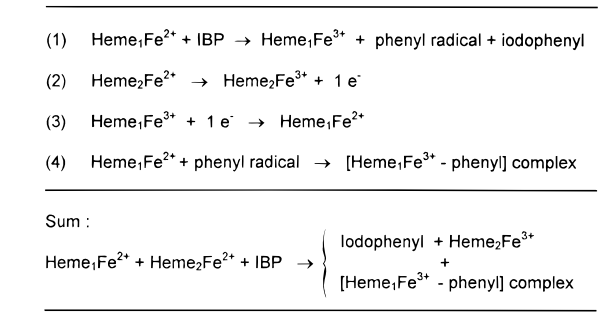

FIGURE 11: Postulated mechanism for reaction of IBP with the heme component of flavocytochrome *b*. Sequential reactions involving IBP and the two hemes of flavocytochrome *b* result in oxidation of one of the two hemes and in accumulation of a complex between the other heme and the phenyl radical derived from IBP (see Discussion for details).

an electron to the first oxidized heme (heme 1) (reactions 2 and 3). The newly reduced heme 1 reacts with the phenyl radical to form a complex (reaction 4). This complex would be responsible for the loss of absorbance of the Soret peak (Figures 4 and 5). The blue shift observed at high IBP concentrations in the presence of an excess of dithionite might correspond to the binding of more than one iodonium radical to the porphyrin ring, probably resulting in some gross perturbation of its geometry. This scheme holds true for interaction of DPI with flavocytochrome *b*, except that in this case DPI is not split and a iodobiphenyl radical is formed by electron capture.

The possibility that the initial binding of iodonium to heme *b* occurs at the level of the heme iron is deduced not only from the capacity of iodonium to oxidize heme *b*, a reaction prevented by BICN, but also from the transient competitive inhibition of O_2 uptake by IBP or DPI (Figure 7), which implies that iodonium and O_2 interact transitorily at the same site. In a lapse of time of less than 2 min, the competitive inhibition of O_2^- production by IBP was converted into noncompetitive inhibition, indicating the formation of a stable inhibitory heme *b* adduct. With DPI, noncompetitive inhibition was attained more rapidly than with IBP, but was also a time-dependent process. EPR spectrometry experiments conducted with neutrophil membranes (Figure 8) showed that DPI and IBP both modified the geometry of the iron environment of heme *b* and that their binding could occur together with that of BICN, corroborating the conclusions deduced from the experiments illustrated in Figures 3 and 6. Since BICN binds to the heme iron, it was inferred that DPI and IBP are able to bind covalently to a functional group in the close environment of the heme iron, possibly to the edge of the porphyrin ring, as was reported for the reaction of phenylhydrazine with peroxidase (*23*).

To elucidate in more detail the modifications encountered by heme *b* upon addition of DPI and IBP, additional experiments were carried out in which excess sodium dithionite was present as a reducing agent. The results obtained with purified flavocytochrome *b* from neutrophils were compared to those obtained with hemin used as a model system (Figure 9). The spectral modifications encountered by purified flavocytochrome *b*, characterized by a break occurring at 50% of the decrease of the Soret peak absorbance, supported the contention that two hemes are present in flavocytochrome *b* (25) and that both hemes differ by their sensitivity to iodonium salts.

Iodonium salts appeared to react with a roughly similar efficiency with reduced free hemin and free FAD (Figure 10), suggesting that both the aryliodonium-modified FAD and heme *b* redox centers of flavocytochrome *b* might contribute to inhibition of the neutrophil NADPH oxidase. Yet the correlation between spectral modifications of heme *b* and inhibition of NADPH oxidase in neutrophil membranes (Figures 1 and 2) as well as the transient competition between O_2 and IBP or DPI (Figure 7) strongly suggests that the iodonium-dependent modification of the heme component of flavocytochrome *b* is a major factor contributing to inhibition of the neutrophil NADPH oxidase.

ACKNOWLEDGMENT

We thank Dr. J. Willison for careful reading of the manuscript and Mrs. J. Bournet-Cauci for excellent secretarial assistance and F. Bouzidi for assistance in some of the experiments.

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BI9823481