Photoaffinity Labeling and Photoinactivation of the O_2^--Generating Oxidase of Neutrophils by an Azido Derivative of FAD†

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ABSTRACT: A photoactivable derivative of FAD, 4-[*N*-(4-azido-2-nitrophenyl)amino]butyryl-FAD (NAP_4-FAD), was synthesized in a tritiated form with tritium placed in the NAP_4 moiety of the photoprobe. [^{3}H]NAP_4-FAD was used to photolabel the putative flavin binding site of the O_2^--generating NADPH oxidase located in the plasma membrane of bovine neutrophils. Effective photolabeling required partial deflavination of membranes, which was achieved by mild treatment with ammonium sulfate added to 50% saturation and 0.05% Triton X-100 for 30 min at 2−4 °C. Under these conditions, 40−50% of the oxidase activity was lost, but it could be fully recovered by the addition of nanomolar amounts of FAD (K_M = 10−20 nM). Added FAD could be substituted by [^{3}H]NAP_4-FAD in photolabeling experiments. In the dark, [^{3}H]NAP_4-FAD bound reversibly with high affinity to deflavinated neutrophil plasma membranes (K_d = 50 nM), did not transport electrons, and efficiently inhibited the FAD-dependent restoration of oxidase activity (K_i = 60 nM). Upon photoirradiation of neutrophil plasma membranes in the presence of [^{3}H]NAP_4-FAD, the nitrene derivative formed bound covalently to a 80−120 kDa protein that was identified as the β-subunit of cytochrome b_{558} by immunodetection and enzymatic deglycosylation. The amount of [^{3}H]NAP_4-FAD covalently incorporated into the β-subunit of cytochrome b_{558} was 80−90% of the amount of photoprobe specifically bound to neutrophil plasma membranes. A linear relationship between the extent of specific photolabeling by [^{3}H]NAP_4-FAD and the percentage of NADPH oxidase inactivation was observed for percentages of inactivation of up to 70−80%, extrapolating to 0.5 mol of covalently bound [^{3}H]NAP_4-FAD per mol of heme b_{558}.

The O_2^--generating NADPH oxidase located in the plasma membrane of activated neutrophils consists of membrane-bound redox proteins and activation protein factors devoid of redox properties. These factors include two water soluble proteins, p47 and p67, and a prenylated monomeric G protein of the Rac type. Binding of the activation factors to the redox core of the NADPH oxidase results in the shift of the resting state of the enzyme to an active state (Cross & Jones, 1991; Morel et al., 1991; Babior et al., 1992; Segal & Abo, 1993). The first redox component of the oxidase complex to be identified was a low-potential *b* type cytochrome (Segal & Jones, 1978). This cytochrome is routinely referred to as cytochrome b_{558} because of the wavelength of the α reduced peak. After a number of unsuccessful attempts, a heterodimeric redox protein with spectral properties identical to those of cytochrome b_{558} was finally purified from neutrophil membranes (Parkos et al., 1987; Segal, 1987). The largest subunit, called the β-subunit, has a mass of 80−100 kDa and was characterized as a glycoprotein. The smaller subunit (α) of 21 kDa has some homology with the heme binding subunit of the mitochondrial cytochrome *c* oxidase (Parkos et al., 1988) and was tentatively assigned as the heme binding subunit of the heterodimer.

Recently, three articles appeared in close succession that drew attention to significant amino acid sequence homology between the β-subunit of cytochrome b_{558} and regions of ferredoxin $NADP^+$ reductase and related flavoproteins involved in the binding of NADPH and FAD (Segal et al., 1992; Rotrosen et al., 1992; Sumimoto et al., 1992). The authors postulated the presence of NADPH and FAD binding sites on cytochrome b_{558} and proposed that cytochrome b_{558} was in fact a flavocytochrome. The postulated NADPH binding site on the β-subunit of cytochrome b_{558} was demonstrated convincingly by photoaffinity labeling experiments carried out with two different photoactivable derivatives of NADPH, namely, 2-azido-NADPH (Segal et al., 1992) and 4-[*N*-(4-azido-2-nitrophenylamino]butyryl-NADPH (Doussière et al., 1993), and also by affinity labeling with pyridoxal phosphate−AMP (Ravel & Lederer, 1993).

The idea that FAD is a functional prosthetic group in the O_2^--generating neutrophil NADPH oxidase stems from early observations that, in neutrophil extracts, oxidase activity was enhanced by the addition of FAD (Babior & Kipnes, 1977) and, on the contrary, was inhibited by the addition of FAD analogs transporting electrons in pairs, such as deaza-FAD (Light et al., 1981). Later, a flavin semiquinone with a mean potential of −280 mV, intermediate between the potential of the $NADP^+$/NADPH couple (−320 mV) and that of the O_2/O_2^- couple (−160 mV), was found to accumulate in membranes of activated neutrophils upon the addition of NADPH (Kakinuma et al., 1986). It was also noted that there was often a parallel decrease in the amount of FAD and heme b_{558} in membranes of neutrophils from patients

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with the X-linked form of chronic granulomatous disease (Segal et al., 1992). Recently it was reported that a purified preparation of cytochrome b_{558} was able to catalyze the production of O_2^- in the presence of NADPH, provided that it was supplemented with lipids and FAD (Koshkin & Pick, 1993). All of these results were consistent with the presence of an FAD prosthetic group in the NADPH oxidase complex, although none of them afforded direct conclusive evidence.

The goal of the present work was to approach the problem of a specific FAD binding site in the NADPH oxidase by photolabeling and photoinactivation. A novel radiolabeled photoactivable derivative of FAD was synthesized, and its effect on the NADPH oxidase activity of purified plasma membranes from bovine neutrophils was tested. The photolabeled proteins in neutrophil plasma membranes were analyzed by SDS−PAGE.[1] The labeling pattern was compared to the position of the β-subunit of cytochrome b_{558} immunodetected by Western blot. Since the β-subunit of cytochrome b_{558} contains a large bulk of carbohydrate chains, we compared the effect of enzymatic deglycosylation on the electrophoretic migration of the β-subunit of cytochrome b_{558} and the photolabeled proteins of neutrophil membranes. Experiments were also carried out to determine whether a correlation between the covalent photolabeling by the azido derivative of FAD and the inactivation of the NADPH oxidase could be established. From these different lines of investigation, it appears that cytochrome b_{558} is a flavocytochrome with a functional FAD prosthetic group located on the β-subunit.

MATERIALS AND METHODS

Materials. The following materials were supplied by the companies indicated: GTP-γ-S, NADPH, alkaline phosophatase, soybean trypsin inhibitor, and endoglycosidaseF/N-glycosidase, F, Boehringer; FAD, FMN, DFP, arachidonic acid, pyrophosphatase, superoxide dismutase, ferricytochrome *c* (horse heart grade VI), carbodiimidazole, dimethyl sulfoxide, and dimethylformamide, Sigma; SDS and Coomassie Blue R250, Serva; iodoacetamide, 4-aminobutyric acid, and 4-fluoro-3-nitroaniline, Aldrich. [^{3}H]-4-Aminobutyric acid and reagents for enhanced chemiluminescent (ECL) detection of horse radish peroxidase-labeled goat antibodies were purchased from Amersham. Phosphate-buffered saline (PBS) consisted of 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, and 1.5 mM KH_2PO_4 (pH 7.4).

Biological Preparations. A particulate fraction enriched in plasma membranes was prepared from bovine neutrophils by a method differing in some details from that previously described (Morel et al., 1985). Neutrophils were suspended in PBS at a concentration of (1−2) × 10^8 cells/mL. The cell suspension was supplemented with 0.5 mM EDTA, 0.5 mM EGTA, 0.1 mM iodoacetamide, 1 mM DFP, and soybean trypsin inhibitor (50 μg/mL) as antiproteases and then subjected to four successive sonications of 15 s each, separated by 30 s intervals, using a Branson sonifier at 40 W output. During the course of sonication and all further operations, the temperature was maintained at 2−5 °C. The cell homogenate was centrifuged at 2000*g* for 10 min. The sediment made of nuclei and cell debris was discarded. Twenty milliliter aliquots of the postnuclear supernatant were placed on a discontinuous sucrose gradient made of one layer of 20 mL of 37% (w/v) sucrose and another layer of 15 mL of 10% (w/v) sucrose, both supplemented with 1 mM sodium azide and 0.5 mM EDTA. Centrifugation was carried out using an SW 25.2 rotor at 22 000 rpm for 90 min. The particulate fraction located between the two sucrose layers was enriched in plasma membranes, as shown by the increase in the specific activity of alkaline phosphatase (Morel et al., 1985). This plasma membrane-enriched fraction was collected by aspiration with a wide bore Pasteur pipet. Following dilution in PBS, membranes were sedimented by centrifugation in a 60 Ti rotor at 45 000 rpm for 40 min and finally resuspended in the same buffer at a concentration of 4−5 mg of protein/mL. The amount of cytochrome b_{558} in this membrane preparation was found to range between 240 and 280 pmol/mg membrane protein, using a differential absorption coefficient of 21.6 mM^{-1} cm^{-1} at 558 nm (Cross et al., 1982). The top of the gradient contained the cytosolic proteins. It was withdrawn with a Pasteur pipet and filtered through a 0.45 μm Millex filter; it was referred as cytosol.

Assay of Oxidase Activity. The assay was carried out in two steps (Ligeti et al., 1988). In the first step, oxidase was activated by mixing plasma membranes (35−40 μg of protein) and cytosol (350−400 μg of protein) from resting neutrophils supplemented with 2 mM $MgSO_4$, 20 μM GTP-γ-S, 200 μM ATP, and an optimal amount of arachidonic acid (1 μmol/mg protein) in a final volume of 50 μL of PBS. The activation reaction was allowed to proceed for 1 h at 2−4 °C. Full oxidase activation could also be achieved by preincubation at 20 °C for 7 min. The suspension was then transferred to a 2.5 mL photometric cuvette for measurement of the rate of O_2^- production using the SOD inhibitable reduction of ferricytochrome *c*, as described (Ligeti et al., 1988).

Synthesis of Tritiated 4-[N-(4-Azido-2-nitrophenyl)amino]-butyryl-FAD ([^{3}H]NAP$_4$-FAD). The method of synthesis of [^{3}H]NAP$_4$-FAD was derived from that described by Guillory and Jeng (1977) for the synthesis of 3-[*N*-(4-azido-2-nitrophenyl)amino]propionyl-ATP. The (azidoaryl)carboxylic acid, 4-[*N*-(4-azido-2-nitrophenyl)amino]-[^{3}H]butyric acid, referred to as [^{3}H]NAP$_4$, was synthesized and coupled by esterification to the ribose group of FAD, using carbodiimidazole as a catalyst of activation of the carboxylic group (Gottikh et al., 1970). The synthesis of [^{3}H]NAP$_4$ was carried out essentially as described by Guillory and Jeng (1977). In brief, 4-fluoro-3-nitroaniline was first diazotized in concentrated HCl medium in the presence of sodium nitrite. The diazonium salt was made to react with sodium azide in the dark, which led to the formation of 4-fluoro-3-nitrophenyl azide, and the azide was condensed with [^{3}H]-4-aminobutyric acid (10^5 cpm/nmol) at 60 °C for 18 h in a solution of DMSO and triethylamine (16/1, v/v) (Levy, 1973) to give [^{3}H]NAP$_4$. This product was purified by descending chromatography on 3MM Whatman paper using a mixture of ethanol, butanol, 1 M ammonium acetate, and 11.5 N ammonium hydroxide (40/20/40/1) as eluent. [^{3}H]NAP$_4$ migrated with an R_f of 0.8. The corresponding band was cut out and eluted with methanol, and the eluate was

[1] Abbreviations: CGD, chronic granulomatous disease; DMSO, dimethyl sulfoxide; DFP, diisopropyl phosphorofluoridate; ECL, enhanced chemiluminescence; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; SDS, sodium dodecyl sulfate; SOD, superoxide dismutase; PBS, phosphate-buffered saline; NAP$_4$-FAD, 4-[*N*-(4-azido-2-nitrophenyl)-amino]butyryl-FAD.

concentrated under vacuum. All further operations were conducted in the dark or in a dim light.

[^{3}H]NAP_4 (130 mg) and carbodiimidazole (90 mg) were dissolved in 2 mL of dimethylformamide (dried over molecular sieves) under stirring at room temperature for 15 min. To this mixture was added FAD (200 mg in 2 mL of distilled water) dropwise and allowed to react overnight. Then, solvent was evaporated under vacuum. The residue was washed with ether and dissolved in distilled water. The solution was applied as a band along the top of a piece of 20 cm wide 3MM Whatman paper and subjected to descending chromatography using *n*-butanol, acetic acid, and water (50/20/30) as eluent. A major radiolabeled orange band migrating with an R_f of 0.38 corresponded to [^{3}H]NAP_4-FAD (see Results section). This band was cut out, eluted with distilled water, and concentrated under vacuum. The aqueous solution of [^{3}H]NAP_4-FAD could be kept without noticeable degradation for several months at −20 °C. Two other bands, yellow and red in color, were observed. The yellow band (R_f = 0.12) was not radiolabeled; it corresponded to free FAD. The other band (R_f = 0.93) was labeled; it corresponded to unreacted [^{3}H]NAP_4. In some preparations, a radiolabeled orange material with an R_f of 0.52 accumulated to an extent of 2−5% of that with an R_f of 0.38. As shown in the Results section, this material was another azido derivative of FAD with two NAP_4 groupings branched on FAD.

Reversible Binding of FAD and [^{3}H]NAP_4-FAD to Neutrophil Membranes (Reaction Done in the Dark). Efficient and specific binding of [^{3}H]NAP_4-FAD to the flavin sites of neutrophil plasma membranes required treatment of the membranes with a detergent in saline solution to release endogenous flavin. Two methods were used concurrently. The first method consisted of partial deflavination followed by reflavination of empty sites with FAD or the FAD photoprobe. It is called the deflavination−reflavination method. In brief, a suspension of bovine neutrophil plasma membranes in PBS was supplemented with medium consisting of 50 mM sodium phosphate (pH 7.5), ammonium sulfate to 50% saturation, and 0.05% (w/v) Triton X-100 to reach a final concentration of 0.1 mg of protein/mL. The mixture was gently stirred for 20 min at 2−4 °C and centrifuged. The pellet was rinsed with PBS and resuspended in the same medium by slight sonication. This treatment resulted in a partial decrease of the NADPH oxidase activity, which could be fully restored by added FAD. Neutrophil plasma membranes deflavinated as described above were incubated with increasing concentrations of [^{3}H]NAP_4-FAD up to 1 μM in PBS (0.2 mg of protein in a 0.5 mL final volume) at 2−4 °C for 30 min in the dark and then sedimented by high-speed centrifugation. The membrane-bound radioactivity was measured by liquid scintillation counting. Nonspecific binding was assessed in the presence of a large excess of unlabeled FAD (20 μM).

The second method, referred to as the flavin exchange method, was based on facilitated exchange between membrane-bound FAD and the added FAD photoprobe in the salt/detergent medium. It consisted of incubating [^{3}H]NAP_4-FAD at increasing concentrations with the neutrophil plasma membranes in 50 mM sodium phosphate buffer (pH 7.5) (0.2 mg of protein in a 0.5 mL final volume) directly in the presence of ammonium sulfate added to 50% saturation and 0.05% Triton X-100. Incubation was carried out in the dark for 30 min at 2−4 °C, followed by sedimentation of the membranes and measurement of the membrane-bound radioactivity. For both methods, it was verified that full equilibrium between bound and free photoprobes was attained at the end of the incubation.

Covalent Photolabeling of Membrane Proteins with [^{3}H]-NAP_4-FAD. Neutrophil plasma membranes were incubated with [^{3}H]NAP_4-FAD, using either of the two methods described in the preceding paragraph. The membrane suspension treated with [^{3}H]NAP_4-FAD was introduced into a small glass flask that was placed 20 cm under a xenon XBO lamp of 1000 W (Muller GmbH, Moosinning, Germany). The glass flask was rotated horizontally in an ice bath and photoirradiated for periods of time ranging between 2 and 5 min. Since the glass wall behaved as a filter for UV light, photolysis of the probe was essentially due to its absorption in the visible region of the spectrum. Following photoirradiation, the membrane suspension was diluted 10 times with PBS. The membranes were collected by centrifugation and resuspended by slight sonication in PBS. When indicated, the remaining oxidase activity was measured.

SDS−PAGE and Immunoblot Analysis. The proteins of the photoirradiated neutrophil membranes were resolved by SDS−PAGE (Laemmli & Favre, 1973), as described (Doussière et al., 1993). In brief, for each protein sample, two tracks were used. After electrophoretic migration in 10% polyacrylamide gels, the gels were cut into strips. One of the strips was stained with Coomassie Blue and cut into 2 mm slices to localize radiolabeled proteins. The Coomassie Blue color of the slices was bleached by overnight incubation at 60−65 °C with 5% H_2O_2. Excess H_2O_2 was removed with catalase, and radioactivity was measured by liquid scintillation counting. The protein material of the other strip was electrotransferred to a nitrocellulose sheet, and the presence of the large β-subunit of cytochrome b_{558} was assessed by rabbit antibodies directed against a synthetic peptide corresponding to residues 552−559 of the C-ter sequence of the β-subunit, which was kindly provided by Dr. G. Brandolin, as previously described (Doussière et al., 1993). The rabbit antibodies−β-subunit complex was revealed by incubation with goat anti-rabbit IgG coupled to peroxidase, followed by detection of the bound peroxidase by a luminescence method using the ECL kit from Amersham. In some experiments, the photoirradiated membranes were subjected to endoglycosidase F/*N*-glycosidase F to deglycosylate the β-subunit of cytochrome b_{558} (Doussière et al., 1993).

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

RESULTS

Spectral Characteristics, Photochemical Reactivity, and Structural Elucidation of the Major Radiolabeled Azidoaryl Derivative of FAD with an R_f Value of 0.38. The spectrum of the orange-colored radiolabeled material with an R_f of 0.38, resulting from the esterification of FAD with the carboxylic group of [^{3}H]NAP_4 (see Materials and Methods), showed peaks at 262, 373, and 450 nm and a shoulder at about 476 nm (Figure 1A). The two maxima at 373 and 450 nm signified the contribution of FAD to the structure of this molecule. Both NAP_4 and FAD contributed to the

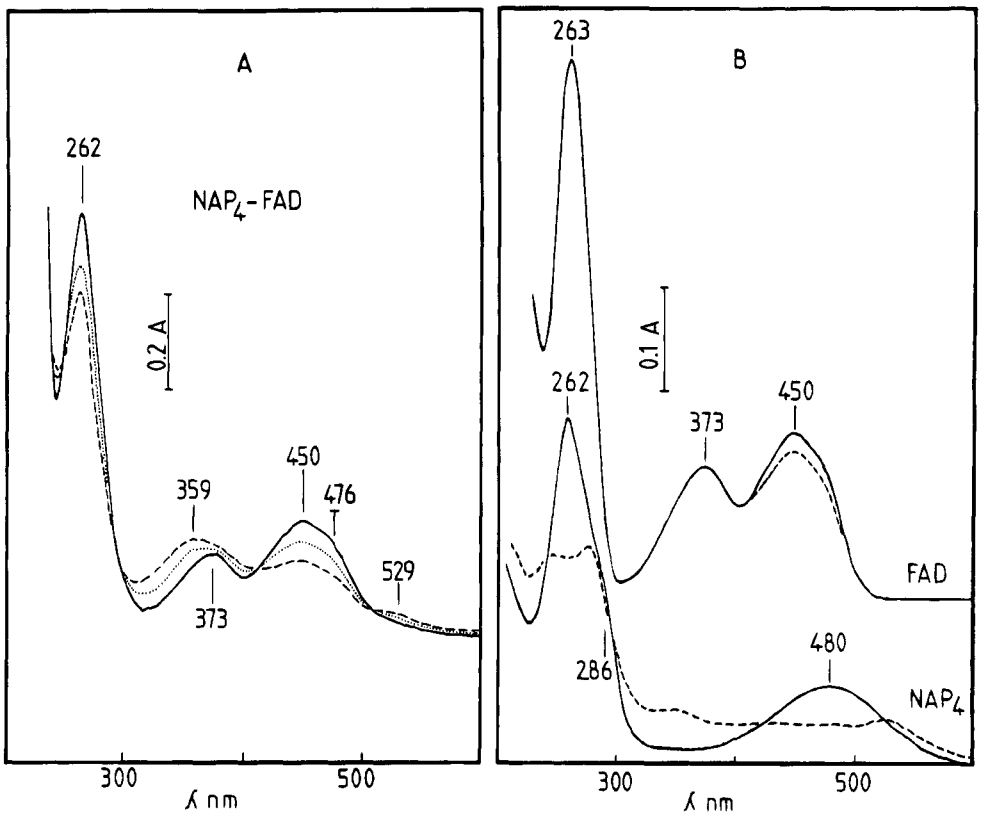

FIGURE 1: Absorption spectrum and photoreactivity of NAP_4-FAD. (A) A water solution of NAP_4-FAD in a 3 mL quartz cuvette of 1 cm pathway was subjected to photoirradiation by a 1000 W xenon lamp placed at a distance of 20 cm. The solid line corresponds to the spectrum of control (non-photoirradiated) NAP_4-FAD. The spectra of photolyzed NAP_4-FAD is indicated by the dotted line (20 s of photoirradiation) and by the dashed line (1 min of photoirradiation). Photoirradiation for more than 1 min did not change the shape of the curve. (B) Spectra of FAD and NAP_4 (solid lines). Dashed lines correspond to the spectra of FAD and NAP_4 photoirradiated for 10 and 1 min, respectively. Photoirradiation of NAP_4 for longer periods of time did not change the shape of the spectrum.

peak at 262 nm and to the shoulder between 474 and 478 nm. Upon photoirradiation with the xenon lamp for only 1 min, the peak at 450 nm was severely decreased, and there was an increase in absorbance between 300 and 410 nm, with a broad peak at 359 nm, and between 525 and 550 nm. The peak at 263 nm was diminished, but not as extensively as that at 450 nm. In contrast to NAP_4-FAD, the FAD spectrum was barely modified by photoirradiation, even after 10 min of exposure to light. Only a slight decrease in the peak at 450 nm was noted, probably due to photoreduction (Figure 1B). The NAP_4 spectrum showed peaks at 480 and 262 nm with a shoulder at 286 nm. The light effect consisted of a decrease in the peaks at 480 and 262 nm and of an increase in the absorptions between 300 and 410 nm and between 525 and 550 nm (Figure 1B). Taken together, the presence of peaks specific to FAD and spectral modifications due to photoirradiation could be explained by the selective photolysis of the NAP_4 moiety of a molecule resulting from the condensation of NAP_4 and FAD.

The next step in the elucidation of the structure of the radiolabeled material with the R_f of 0.38, tentatively identified as [^{3}H]NAP_4-FAD, was the determination of the molar ratio of [^{3}H]NAP_4 to FAD. Five potentially reactive hydroxyl groups within ribose and ribitol of the FAD molecule are candidates for esterification by the [^{3}H]NAP_4 group. Since the peak at 373 nm assigned to [^{3}H]NAP_4-FAD is specific to FAD with $\epsilon = 9$ mM^{-1} cm^{-1} (not corrected for the very small absorption due to the NAP_4 moiety), the absorbance at 373 nm was used to determine the amount of FAD in a given sample of [^{3}H]NAP_4-FAD. As shown in Table 1, the deduced amount of FAD in this sample was 0.29 μmol/mL. On the other hand, on the basis of the specific radioactivity of 10^5 cpm/nmol, the calculated amount of [^{3}H]NAP_4 in the same sample was 0.28 μmol/mL, indicating an [^{3}H]NAP_4 to FAD ratio of 1. A minor product of synthesis with an R_f of 0.52 and spectral features similar to those of the product with an R_f of 0.38 was analyzed by the method described earlier and found to contain 2 mol of NAP_4/mol of FAD (Table 1). Only the former product (R_f = 0.38) was analyzed to determine which of the five potentially reactive hydroxyl groups of FAD was esterified by NAP_4 and further used in photolabeling experiments.

Table 1: Determination of the Amounts of [^{3}H]NAP_4 and FAD Present in Two Azidoaryl Derivatives of FAD[a]

radiolabeled FAD derivative	R_f value	FAD (μM)	[^{3}H]NAP_4 (μM)	[^{3}H]NAP_4 to FAD ratio (mol/mol)
1	0.38	290	280	0.96
2	0.52	65	120	1.85

[a] The amount of FAD was determined from the absorption peak at 373 nm using an ϵ value of 9 mM^{-1} cm^{-1}. The amount of [^{3}H]NAP_4 present in [^{3}H]NAP_4-FAD was calculated from the specific radioactivity of [^{3}H]NAP_4 (10^5 cpm/mol). The R_f values of the two radiolabled FAD derivatives were determined after chromatography on silica plates (cf. Materials and Methods).

Determination of the site(s) of esterification in FAD was carried out through the use of pyrophosphatase and alkaline phosphatase. The cleavage products of [^{3}H]NAP_4-FAD were separated by chromatography on silica gel plates, using an *n*-butanol, acetic acid, and water (50/20/30) solvent, and

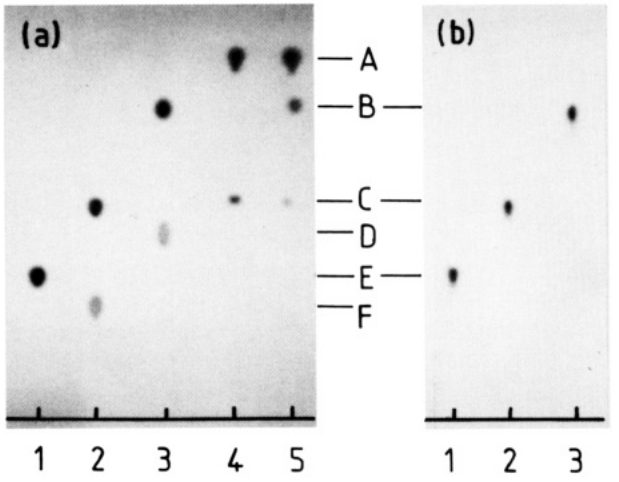

FIGURE 2: Separation of [^{3}H]NAP$_4$-FAD and its cleavage products by thin-layer silica gel chromatography. [^{3}H]NAP$_4$-FAD (10 nmol) was cleaved by pyrophosphatase (1 unit) and by the combined action of pyrophosphatase (1 unit) and alkaline phosphatase (1 unit). The plate was developed with *n*-butanol, acetic acid, and water (50/20/30). (a) Photography under visible light showing the location, after chromatography, of the azido derivatives and their enzymatic cleavage products: track 1, [^{3}H]NAP$_4$-FAD; track 2, cleavage products of [^{3}H]NAP$_4$-FAD by pyrophosphatase; track 3, cleavage products of [^{3}H]NAP$_4$-FAD by pyrophosphatase plus alkaline phosphatase; track 4, crude preparation of NAP$_4$-AMP; track 5, cleavage products of NAP$_4$-AMP by alkaline phosphatase. The identity and the color of the compounds were as follows: spots A-4 and A-5:NAP$_4$, red; spots B-3 and B-5:NAP$_4$-adenosine, red; spots C-2 and C-4:NAP$_4$-AMP, red; spot D-3:riboflavine, yellow; spot E-1:[^{3}H]NAP$_4$-FAD, orange; spot F-2:FMN, yellow. (b) Autoradiography of the silica gel plate showing the localization of control [^{3}H]NAP$_4$-FAD (track 1) and [^{3}H]NAP$_4$-FAD treated with pyrophosphatase (track 2) or alkaline phosphatase plus pyrophosphatase (track 3).

identified by reference to compounds of known structure (Figure 2a). Untreated [^{3}H]NAP$_4$-FAD migrated as a single orange-colored spot with an R_f of 0.38, coinciding with a radioactive spot detected by autoradiography (Figure 2b). After incubation with pyrophosphatase, two products of red and yellow color were formed, well separated by silica plate chromatography. The red-colored product had an R_f of 0.48, identical to that of NAP$_4$-AMP (Figure 2a), and was radiolabeled (Figure 2b). The yellow-colored product had an R_f of 0.27, similar to that of FMN (Figure 2a), and was unlabeled. When [^{3}H]NAP$_4$-FAD was treated with both pyrophosphatase and alkaline phosphatase, two new spots with R_f values of 0.70 and 0.42 were observed. The product with an R_f of 0.42, similar to that of riboflavin, was unlabeled and yellow-colored as expected. The other product with an R_f of 0.70 was radiolabeled and red-colored; its R_f was the same as that of NAP$_4$-adenosine, the product of hydrolysis of NAP$_4$-AMP by alkaline phosphatase. Taken together, these results led us to conclude that the photoactivatable adduct NAP$_4$ is branched on the ribose of the AMP moiety of FAD in the newly synthesized molecule. By analogy to the preferential esterification of the ribose of ATP at the 3′-position by an azidoaryl carboxylic acid in dimethylformamide (Guillory & Jeng, 1977), it is suggested that NAP$_4$ is branched on the 3′-OH of ribose associated with the adenine ring in the FAD molecule. The structure of [^{3}H]NAP$_4$-FAD with the sites of attack of the phosphate bonds by pyrophosphatase and alkaline phosphatase is illustrated in Figure 3. The ϵ values for the absorption peaks of NAP$_4$-FAD are as

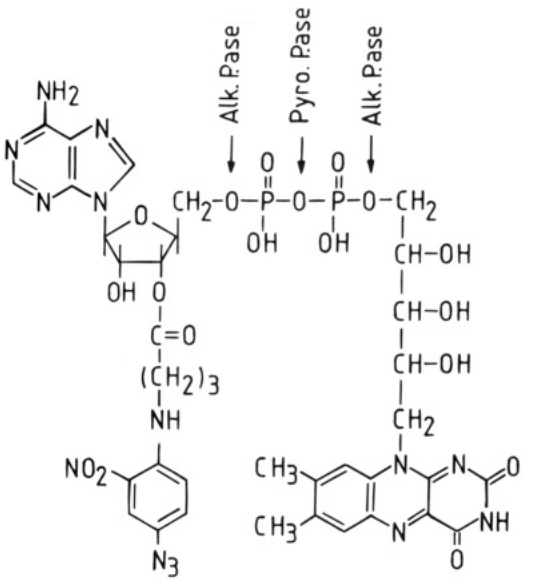

FIGURE 3: Structure of [^{3}H]NAP$_4$-FAD. Arrows indicate the bond cleaved by alkaline phosphatase (Alk.Pase) and pyrophosphatase (Pyro.Pase).

follows: $\epsilon_{262} = 48.6$ mM^{-1} cm^{-1}, $\epsilon_{373} = 9.5$ mM^{-1} cm^{-1}, and $\epsilon_{450} = 13.3$ mM^{-1} cm^{-1}.

The absence of esterification of ribitol by NAP$_4$ in the FMN moiety of FAD was puzzling. A complementary experiment therefore was carried out in an attempt to compare the efficiency of branching [^{3}H]NAP$_4$ on the ribitol of FMN and on the ribose of AMP, using the same method as that described for the synthesis of [^{3}H]NAP$_4$-FAD. It was found that the esterification of ribitol in FMN was barely feasible, whereas the esterification of ribose in AMP was very efficient.

Optimal Conditions for FAD Access to the Flavin Sites of the NADPH Oxidase of Neutrophil Plasma Membranes. The potential oxidase activity of the purified plasma membrane preparation from resting bovine neutrophils was elicited in a cell-free system in the presence of neutrophil cytosol, GTP-γ-S, and arachidonic acid and assayed as described (Ligeti et al., 1988). The elicited oxidase activity attained values as high as 2−3 μmol of O_2^- formed/min/mg of membrane protein due to the high concentration of plasma membrane in the membrane preparation. Upon the addition of 1 μM FAD, only a slight enhancement of oxidase activity (less than 10%) could be seen (Figure 4A), suggesting that if the redox component of the oxidase complex contains a functional flavin group, this group is firmly attached. Treatment of neutrophil plasma membranes in PBS with ammonium sulfate added to 50% saturation and 0.05% Triton X-100 (see Materials and Methods) led to a partial decrease in oxidase activity (40−50%), which was fully reversed by the addition of 1 μM FAD (Figure 4A). FAD was apparently specific since the addition of FMN even at concentrations as high as 10 μM was ineffective. A likely explanation was that FAD attached to the flavin site of the NADPH oxidase was partly released by the salt/detergent medium and that added FAD was able to bind to the empty flavin site, reinstating the catalytic potency of the oxidase. A more drastic treatment of the neutrophil membranes with concentrations of Triton X-100 higher than 0.05% resulted in a larger loss of oxidase activity, which was not fully recovered by the addition of FAD, probably due to alteration of

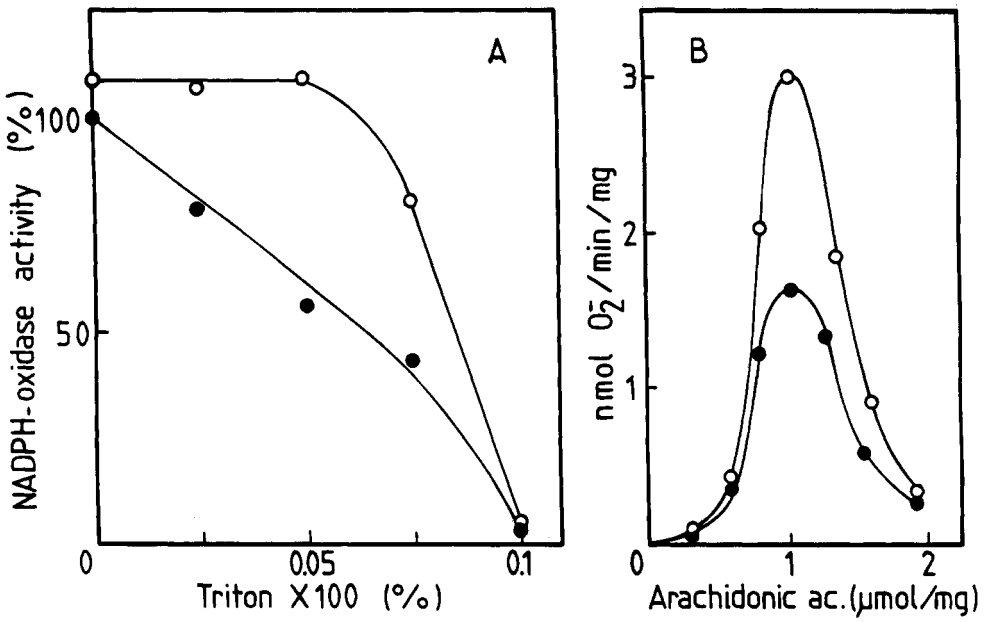

FIGURE 4: Loss of elicited oxidase activity of neutrophil plasma membranes after salt/detergent treatment and restoration of oxidase activity by added FAD. (A) Effect of increasing concentrations of Triton X-100 on the loss of oxidase activity. Purified plasma membranes from bovine neutrophils (0.2 mg/mL) were incubated for 20 min at 2 °C in 50 mM P_i buffer (pH 7.5) supplemented with ammonium sulfate added to 50% saturation and increasing concentrations of Triton X-100 up to 0.1%. Membranes were sedimented by centrifugation in a TL100 ultracentrifuge at 90 000 rpm for 20 min. The pellet was rinsed with PBS, and membranes were resuspended by slight sonication. An aliquot of the suspension (20 μg of protein) was used for assay of the oxidase activity after activation in a cell-free system for 1 h at 2−4 °C (●). The figure shows the effect of 1 μM FAD added to the extracted membranes in the cell-free medium of oxidase activity (○). 100% oxidase activity corresponds to 2.9 μmol of O_2^- formed/min/mg of membrane protein. (B) Effect of the amount of arachidonic acid added to the cell-free system of oxidase activation. Same conditions as in panel A. Triton-extracted membranes (●) and extracted membranes supplemented with 1 μM FAD (○).

membrane components of the oxidase complex. The reflavination-dependent recovery of the oxidase potency of neutrophil membranes did not interfere with the activating effect of arachidonic acid, as shown by the same optimal concentration of arachidonic acid required for maximal oxidase activation (Figure 4B). These experiments strongly suggested that FAD is a redox prosthetic group attached to NADPH oxidase with high affinity. Therefore, we carried out an exploration of the putative FAD binding site of the neutrophil oxidase with the photoactivatable derivative of FAD, [^{3}H]NAP_4-FAD.

FAD-Dependent Restoration of the NADPH Oxidase Activity of Deflavinated Neutrophil Membranes; Inhibitory Effect of NAP_4-FAD. In the experiment illustrated in Figure 5, the elicited oxidase activity of neutrophil membranes corresponded to 2.8 μmol of O_2^- formed/min/mg of membrane protein. After extraction for 30 min at 2−4 °C with 0.05% Triton X-100 in the presence of ammonium sulfate to 50% saturation, the oxidase activity dropped to 55% of the initial value (Figure 5A, bar 2 vs bar 1). Upon addition of only 0.1 μM FAD to the oxidase activation medium, the oxidase activity was fully restored and even slightly increased with respect to the initial value (3.1 vs 2.8 μmol of O_2^-/min/mg of membrane protein) (Figure 5A, bar 3 vs bar 1). The rates of O_2^- production, *vf*, plotted in Figure 5B,C, were obtained by subtracting the values calculated with extracted membranes (Figure 5A, bar 2) from those measured after reflavination with FAD (Figure 5A, bar 3). Half-maximal restoration of the oxidase activity of extracted membranes required only 10−20 nM FAD, pointing to a high affinity of FAD for the flavin binding site of NADPH oxidase.

NAP_4-FAD neither restored the lost oxidase activity of neutrophil membranes partly deflavinated by the salt/detergent treatment nor decreased the remaining oxidase activity of these membranes (Figure 5A, bar 4 vs bar 2). However, it inhibited the FAD-dependent restoration of oxidase activity of deflavinated membranes. These results indicate that, in the absence of detergent, NAP_4-FAD does not exchange with bound FAD in native oxidase and that after treatment by detergent it may load the stripped flavin sites.

The inhibitory effect of NAP_4-FAD on the FAD-dependent restoration of oxidase activity was determined at increasing concentrations of FAD up to 100 nM and at different fixed concentrations of the photoprobe (Figure 5B). The Eadie−Hofstee plots of the kinetic data (Figure 5C) revealed the presence of an apparent cooperativity. Up to 50 nM, NAP_4-FAD essentially lowered the V_{max} of the elicited oxidase behaving as a noncompetitive inhibitor. Above 50 nM, NAP_4-FAD was able to substitute for added FAD and to competitively inhibit the oxidase with a K_i of 60 nM.

Reversible Binding of [^{3}H]NAP_4-FAD to Neutrophil Plasma Membranes (Reaction Done in the Dark). The two procedures described to load flavin sites of neutrophil plasma membranes with flavin compounds, namely deflavination followed by reflavination and flavin exchange (cf. Materials and Methods), were used to measure the reversible binding of [^{3}H]NAP_4-FAD. The binding experiment illustrated in Figure 6A was carried out using the deflavination−reflavination method. Partial deflavination by salt/detergent treatment, namely, addition of ammonium sulfate to 50% saturation and 0.05% Triton X-100, resulted in a 38% loss of the elicited oxidase activity. The extracted membranes were then supplemented with increasing concentrations of [^{3}H]NAP_4-FAD. Specifically bound [^{3}H]NAP_4-FAD was determined after the subtraction of bound photoprobe in the

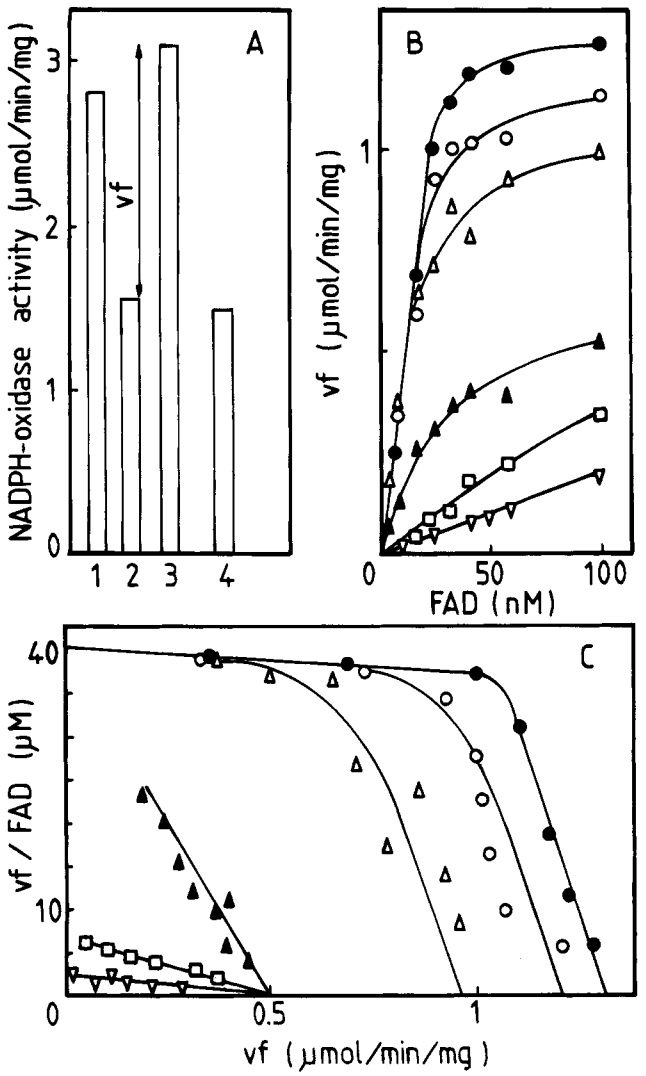

FIGURE 5: Inhibitory effect of NAP_4-FAD on the FAD-dependent restoration of NADPH oxidase in neutrophil plasma membranes. Neutrophil plasma membranes were treated with the ammonium sulfate/Triton medium as in Figure 4, using 0.05% Triton X-100, and then washed and assayed for oxidase activity after activation in a cell-free system. Data are expressed in micromoles of O_2^- formed/minute/milligram of membrane protein. (A) The NADPH oxidase activity of control membranes (2.8 μmol of O_2^- formed/min/mg of membrane protein) (bar 1) was decreased to an extent of 45% (1.5 μmol of O_2^-/min/mg membrane of protein) (bar 2). Upon the addition of 0.2 μM FAD to the oxidase activation medium, oxidase activity was restored and attained a value of 3.1 μmol of O_2^-/min/mg of membrane protein (bar 3). When added FAD was replaced by NAP_4-FAD, oxidase activity was not restored (bar 4). The rates of O_2^- formation following reflavination (*vf*) correspond to the difference between bar 3 and bar 2 and are used in panels B and C. (B) Direct plots of the rate of O_2^- production corresponding to *vf* (see panel A) at increasing concentrations of FAD and the following fixed concentrations of NAP_4-FAD: ●, none; ○, 10 nM; △, 20 nM; ▲, 50 nM; □, 100 nM; ▽, 200 nM. (C) Eadie–Hofstee plots of the data from panel B.

presence of excess (20 μM) unlabeled FAD. The corresponding K_d was 50 nM, a value very close to the K_i (60 nM) relative to the inhibition of NADPH oxidase by NAP_4-FAD (Figure 5) and on the same order of magnitude as the K_M for FAD (10–20 nM). The maximal amount of bound [^{3}H]NAP_4-FAD was 48 pmol/mg of membrane protein. The flavin binding sites labeled by [^{3}H]NAP_4-FAD belonged not only to the NADPH oxidase but also to other flavoproteins. However, as shown in photolabeling experiments to be described in the next section, NADPH oxidase was a major labeled flavoprotein of the neutrophil plasma membrane, representing 80–90% of the labeled flavoproteins located in this membrane. Since partial deflavination resulted in a 38% loss of oxidase activity, and a maximal amount of 48 pmol of flavin sites could be reflavinated with [^{3}H]NAP_4-FAD per milligram of membrane protein, it ensued that reflavination of membranes totally stripped of their flavin sites would theoretically correspond to the binding of 126 pmol of [^{3}H]NAP_4-FAD/mg of membrane protein.

In another experiment (Figure 6B), the reversible binding of [^{3}H]NAP_4-FAD to neutrophil plasma membranes was explored by the flavin exchange method. This method differed from the former one in that [^{3}H]NAP_4-FAD was directly added to the membranes in the salt/detergent medium and therefore had access to the flavin sites during the release of bound FAD. A K_d of 170 nM for the binding of [^{3}H]-NAP_4-FAD was determined. This value was significantly higher than that found by the deflavination–reflavination method, indicating a lower affinity of the probe, probably due to the presence of detergent in the exchange medium; on the other hand, the total number of flavin binding sites, 135 pmol/mg of membrane protein, was close to the theoretical value, 126 pmol, calculated in the deflavination–reflavination experiment.

Combined Photolabeling of Neutrophil Plasma Membranes with [^{3}H]NAP_4-FAD and Immunodetection of the β-Subunit of Cytochrome b_{558}. The two methods used for the reversible binding of [^{3}H]NAP_4-FAD to neutrophil plasma membranes, namely, reflavination with [^{3}H]NAP_4-FAD after deflavination and exchange between bound FAD and added [^{3}H]NAP_4-FAD (see the preceding section), were used for covalent photolabeling. The results of a typical photolabeling experiment in which membranes were loaded with [^{3}H]NAP_4-FAD by deflavination–reflavination are illustrated in Figure 7A. Following preincubation of deflavinated membranes in the presence of 1 μM [^{3}H]NAP_4-FAD in the dark for 20 min, the neutrophil membrane suspension was photoirradiated for 2 min at 2–4 °C. The membranes were diluted 10 times in PBS and then collected by centrifugation. The pellet was rinsed with PBS, resuspended in Laemmli buffer, and heated at 100 °C for 1 min. Two aliquots of the soluble membrane extract were subjected to SDS–PAGE in two parallel tracks, as described in Materials and Methods. Following electrophoresis, the gel was cut into strips. One strip was used for the localization of photolabeled proteins. The other strip was electroblotted on a nitrocellulose sheet, and the localization of the β-subunit of cytochrome b_{558} was immunodetected with anti-β-subunit antibodies. Appropriate controls were performed as described (Doussière et al., 1993) to ensure the specificity of the immunodetection. Assays were carried out in parallel with 20 μM FAD or 20 μM NAP_4 added to the membranes prior to [^{3}H]NAP_4-FAD to ascertain the specificity of the recognition of the FAD moiety of [^{3}H]NAP_4-FAD by the flavin site. The data in Figure 7A show the presence of a predominant broad radiolabeled band in the range of 80–120 kDa and a few minor radiolabeled bands with lower molecular masses. Addition of 20 μM unlabeled FAD prior to [^{3}H]NAP_4-FAD led to a decrease in bound radioactivity, particularly in the region of 80–115 kDa. Radioactivity located in the narrow region of 115–120 kDa was less influenced by FAD, indicating the presence of a distinct

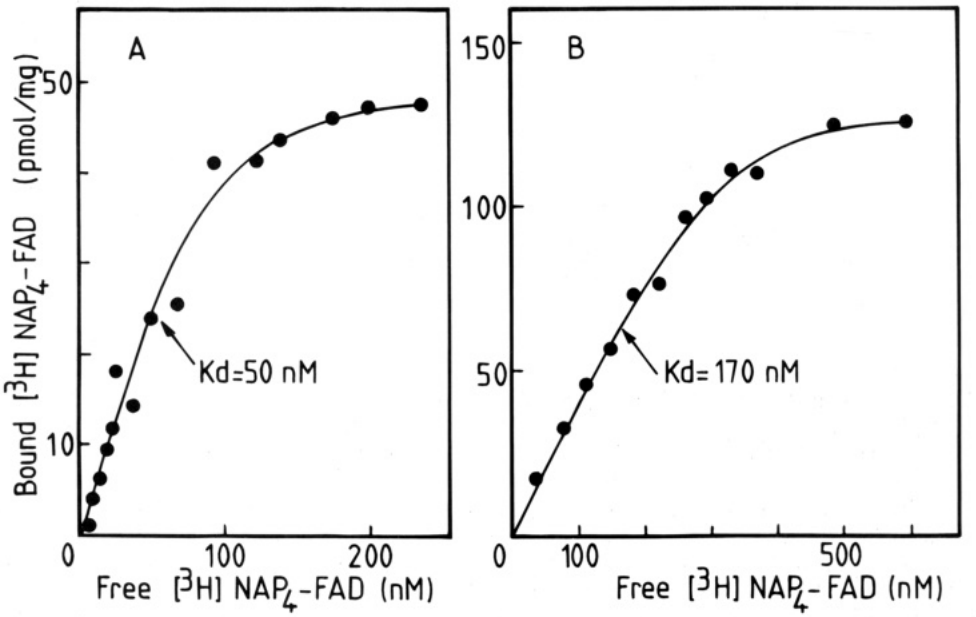

FIGURE 6: Reversible specific binding of [^{3}H]NAP$_4$-FAD to neutrophil plasma membranes (dark reaction). [^{3}H]NAP$_4$-FAD was added at increasing concentrations to neutrophil plasma membranes in the absence or presence of 20 μM FAD and left to incubate in the dark for 30 min. Labeled membranes were recovered by centrifugation. Specific binding of [^{3}H]NAP$_4$-FAD was determined by the difference between the amounts of bound photoprobe in the absence and in the presence of FAD. Data are expressed in picomoles of specifically bound [^{3}H]NAP$_4$-FAD/milligram of membrane protein. (A) Reversible specific binding of [^{3}H]NAP$_4$-FAD assayed for the deflavination−reflavination method. (B) Reversible specific binding of [^{3}H]NAP$_4$-FAD assayed by the flavin exchange method.

protein. On the other hand, unlabeled NAP$_4$ added prior to [^{3}H]NAP$_4$-FAD did not modify the amount of bound photoprobe (Figure 7B).

FAD and NAP$_4$ are colored compounds and may behave as light filters, hampering the efficiency of photolabeling. This apparently was not the case since under our conditions of photoirradiation the added NAP$_4$ did not quench the covalent binding of [^{3}H]NAP$_4$-FAD (Figure 7B). In the immunoblot assay carried out in parallel, the immunoreactive β-subunit of cytochrome b_{558} coincided with the major protein band of 80−120 kDa specifically labeled by [^{3}H]-NAP$_4$-FAD. Furthermore, enzymatic deglycosylation shifted the molecular mass of both the radiolabeled and immunoreacted bands ascribed to the β-subunit of cytochrome b_{558} from 80−120 to 50−60 kDa (Figure 8). This finding is in agreement with the fact that the β-subunit of cytochrome b_{558} is a glycoprotein with one-third of its mass consisting of carbohydrate chains (Parkos et al., 1987), and it strongly suggests that the photolabeled protein of 80−120 kDa is the β-subunit of cytochrome b_{558}. From the distribution pattern of photolabeled proteins resolved by SDS−PAGE, the specifically labeled β-subunit of cytochrome b_{558} represented 80−90% of all specifically labeled proteins, indicating that cytochrome b_{558} is a major flavoprotein in the plasma membrane of neutrophils.

Photolabeling of neutrophil plasma membranes with [^{3}H]-NAP$_4$-FAD using the flavin exchange method to load the flavin sites resulted in a similar pattern of distribution of bound radioactivity, with a predominant peak at 80−120 kDa, coinciding with the immunodetected β-subunit of cytochrome b_{558}, and two minor peaks of lower molecular masses at 53 and 38 kDa (Figure 9). The extent of photolabeling with the flavin exchange method was about 3 times higher than that with the deflavination−reflavination method.

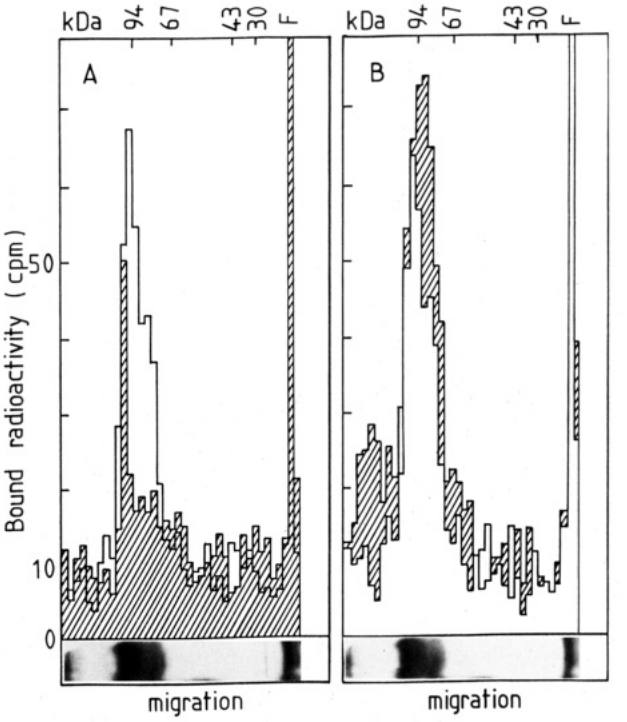

FIGURE 7: Comparison of migrations by SDS−PAGE of deflavinated neutrophil plasma membrane proteins photolabeled by [^{3}H]-NAP$_4$-FAD and the β-subunit of cytochrome b_{558} immunodetected by specific antibodies. Deflavinated neutrophil plasma membranes in PBS (250 μg of protein) were incubated in the dark for 30 min at 2−4 °C with 1 μM [^{3}H]NAP$_4$-FAD (final volume 200 μL) (same conditions as in Figure 6A) and then photoirradiated for 2 min. Proteins were resolved by SDS−PAGE using two tracks, as described in Materials and Methods. The upper part of panels A and B shows the distribution pattern of bound radioactivity in the gel. The hatched bars correspond to photolabeling in the presence of 20 μM FAD (A) and 20 μm NAP$_4$ (B). The lower part in panels A and B shows Western blots corresponding to immunodetection of the β-subunit of cytochrome b_{558} by specific rabbit antibodies.

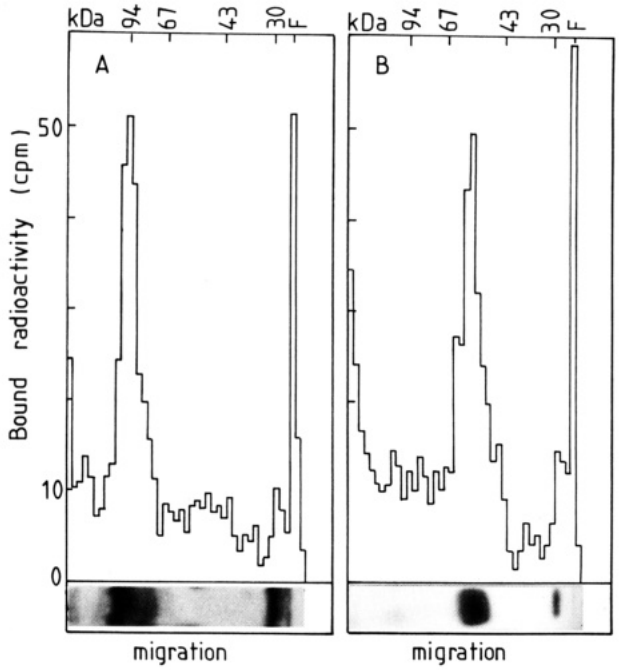

FIGURE 8: Effect of *N*-glycosidase treatment on the molecular masses of the major protein of 80−120 kDa photolabeled by [^{3}H]-NAP_4-FAD and the immunodetected β-subunit of cytochrome b_{558} revealed by specific antibodies. (A) Control neutrophil plasma membranes. (B) Neutrophil plasma membranes treated with *N*-glycosidase. Photolabeling of the neutrophil plasma membranes was performed as described in Figure 7. The membranes were solubilized in 0.2% SDS and 1% Triton X-100 and treated at 100 °C for 2 min. The soluble extract was supplemented with 10 units of *N*-glycosidase and allowed to incubate for 3 h at 37 °C. Following deglycosylation, proteins were precipitated by acetone, solubilized in Laemmli buffer, and then subjected to SDS−PAGE on two tracks, as in Figure 7. The upper part of the panels shows the distribution of the bound radioactivity, and the lower part shows the immunodetection of the β-subunit of cytochrome b_{558}.

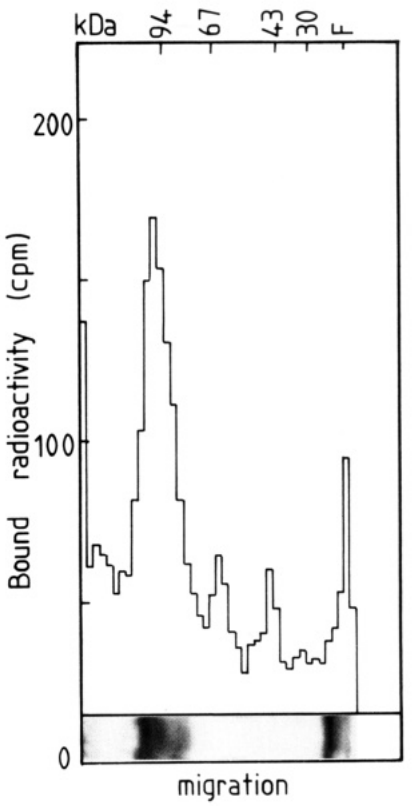

FIGURE 9: Migration by SDS−PAGE of neutrophil plasma membrane proteins photolabeled by [^{3}H]NAP_4-FAD, after loading the flavin sites with the photoprobe by the flavin exchange method. Membranes were first incubated in the dark in the presence of 1 μM [^{3}H]NAP_4-FAD, directly in the presence of ammonium sulfate added to 50% saturation, and 0.05% Triton X-100 (same conditions as in Figure 6B) and then photoirradiated and subjected to SDS−PAGE.

Relation between Photolabeling of Neutrophil Plasma Membranes by [^{3}H]NAP_4-FAD and Photoinactivation of NADPH Oxidase. In this experiment, the flavin exchange method was used extensively to load the flavin sites of neutrophil plasma membranes with [^{3}H]NAP_4-FAD. Membranes were photolabeled with increasing concentrations of the photoprobe and then washed. This was followed by the assay of both the specifically bound [^{3}H]NAP_4-FAD and the oxidase activity after elicitation of oxidase activition in the cell-free system. The amount of covalently bound radioactivity was linearly related to the loss of oxidase activity to approximately 70−80% inactivation. Above 70−80% inactivation, the curve was not longer linear (Figure 10). Extrapolation of the linear portion of the curve to 100% inactivation corresponded to 130 pmol of [^{3}H]NAP_4-FAD covalently bound per milligram of membrane protein. On the basis of the fact that the labeling of the 80−120 kDa protein identified with the β-subunit of cytochrome b_{558} was largely predominant and corresponded to 80−90% of the total specific photolabeling, it was readily calculated that NADPH oxidase was fully inactivated when 105−120 pmol of β-subunit/mg of membrane protein was photolabeled by [^{3}H]NAP_4-FAD. The amount of heme b_{558} determined in this experiment was 250 pmol/mg of membrane protein, i.e., virtually twice the amount of oxidase-bound FAD. In three other experiments, the molar ratios of FAD to heme *b* were in the range of 0.42−0.48. These values are in agreement with those calculated from experiments dealing with the reversible binding of [^{3}NAP_4-FAD.

DISCUSSION

The photolabeling experiments reported here were based on the use of a radiolabeled azidoaryl derivative of FAD. They provide convincing evidence that FAD is a prosthetic group of the O_2^--generating NADPH oxidase and add a new facet to the understanding of the function of FAD as an electron carrier between NADPH and the heme prosthetic group of the oxidase.

[^{3}H]NAP_4-FAD as a Probe of the Flavin Binding Site of Neutrophil NADPH Oxidase. The synthesis and some properties of a novel radiolabeled photoactivatable derivative of FAD are described in this paper. In [^{3}H]NAP_4-FAD, the [^{3}H]NAP_4 adduct was linked by an ester bond to one of the two available hydroxyl groups of the ribose ring of FAD. Because of the lability of the ester bond, the radioactive label was placed on the NAP_4 group rather than on the FAD moiety of the photoprobe.

To determine whether NAP_4-FAD could be used as a probe of the putative flavin binding site of NADPH oxidase, neutrophil plasma membranes that contain the redox core of the oxidase complex were treated with a mild detergent, Triton X-100, in a salt solution to free flavin sites from bound FAD. Under carefully controlled conditions, part of the oxidase activity was lost, but it could be totally recovered by the addition of nanomolar amounts of FAD. In photo-

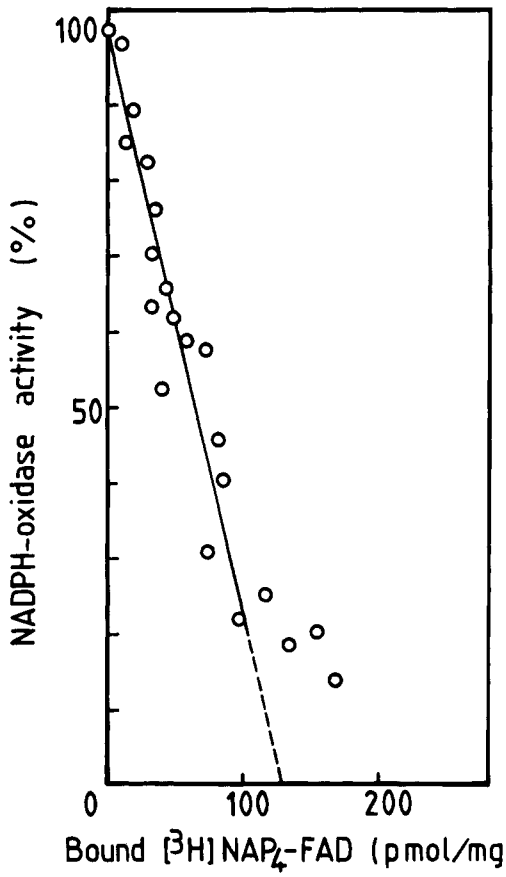

FIGURE 10: Correlation between the extent of covalent binding of [^{3}H]NAP$_4$-FAD to membranes by photolabeling and the percentage of photoinactivation of membrane-bound NADPH oxidase. Neutrophil plasma membranes (200 μg of protein) were incubated for 30 min at 2−4 °C in the dark with increasing concentrations of [^{3}H]NAP$_4$-FAD (up to 700 nM), directly on the presence of ammonium sulfate added to 50% saturation, and 0.05% Triton X-100 (flavin exchange method). The mixture was photoirradiated for 2 min at 2−4 °C as described in Materials and Methods. After a PBS rinse, the suspension was divided into three aliquots. One aliquot was used for protein determination, another for the determination of covalently bound [^{3}H]NAP$_4$-FAD, and the third for assay of oxidase activity after activation of the oxidase in cell-free medium. The specific covalent binding of [^{3}H]NAP$_4$-FAD (expressed in picomoles of bound photoprobe per milligram of membrane protein) was obtained by subtraction of the nonspecific binding that was determined with neutrophil membranes first preincubated with 20 μM FAD for 5 min and then photoirradiated in the presence of [^{3}H]NAP$_4$-FAD. A control was carried out in the absence of the photoprobe to assess the sensitivity of the oxidase to light; the loss of oxidase activity was less than 5%.

labeling experiments, the FAD analogue, [^{3}H]NAP$_4$-FAD, was substituted for FAD for reflavination of the stripped flavin sites. Different lines of evidence indicate that [^{3}H]-NAP$_4$-FAD specifically interacts with the FAD binding site of the neutrophil oxidase. (1) In reversible binding experiments (absence of photoirradiation), the FAD-dependent restoration of oxidase activity in deflavinated neutrophil membranes was inhibited by NAP$_4$-FAD with a K_i of 60 nM, which is very close to the K_d of 50 nM for the specific binding of [^{3}H]NAP$_4$-FAD and on the same order of magnitude as the K_M for FAD (10−20 nM). (2) Photoirradiation of neutrophil plasma membranes in the presence of [^{3}H]NAP$_4$-FAD led to the predominant covalent labeling of a protein of 80−120 kDa that was identified with the β-subunit of cytochrome b_{558} on the basis of two criteria. The β-subunit detected by antibodies and the photolabeled protein of 80−120 kDa showed the same migration in SDS−PAGE. Both were characterized as glycoproteins with the same percentage of carbohydrate chains since, upon incubation with *N*-glycosidase, their molecular masses were shifted similarly from 80−120 to 50−60 kDa. (3) NADPH oxidase inactivation linearly increased with the covalent specific binding of [^{3}H]NAP$_4$-FAD to neutrophil plasma membranes.

FAD Stoichiometry of Neutrophil NADPH Oxidase. The release of bound FAD during cytochrome b_{558} purification makes it difficult to evaluate the FAD/heme stoichiometry in the purified molecule. This evaluation can, however, be approximated by indirect approach. In a recent paper, Segal et al. (1992) showed that by subtracting the FAD measured in neutrophil membranes from X-CGD patients from that present in neutrophil membranes of healthy subjects the molar amount of FAD bound to the NADPH oxidase was one-half the molar amount of heme b_{558}. A FAD to heme ratio of 1/2 was also determined by studying O_2^- production by a relipidated purified cytochrome b_{558} supplemented with variable amounts of FAD (Koshkin & Pick, 1994). This 1/2 stoichiometry is supported by the results of experiments described in the present study, in which the extent of covalently bound [^{3}H]NAP$_4$-FAD was assessed as a function of the percentage of oxidase inactivation. By extrapolation to 100% inactivation, the amount of oxidase-bound photoprobe was approximated to be 105−120 pmol/mg of membrane protein, which corresponds to about one-half the amount of heme b_{558} in the plasma membrane preparation used. A similar amount of FAD binding sites was found by reversible binding of [^{3}H]NAP$_4$-FAD, which was consistent with the finding that flavocytochrome b_{558} is a major flavoprotein in the plasma membrane of neutrophils. Altogether, our results demonstrate that FAD is required for the activity of the NADPH oxidase. The FAD to heme stoichiometry of 1/2 appears to be in accordance with the postulate that $FADH_2$ delivers electrons one by one to the heme component of the oxidase, with the formation of the transitory semiquinone $FADH^\bullet$ (Kakinuma et al., 1985). However, these stoichiometric data do not provide information concerning the percentage of heme *b* effectively involved as an electron carrier in the activated NADPH oxidase. Furthermore, the heme localization, either in the α-subunit or (and) at the boundary between the α- and β-subunits of NADPH oxidase, is still a debated problem.

Although the redox core of the NADPH oxidase complex contains two essential prosthetic groups, FAD and heme, for efficient electron transport from NADPH to O_2, it remains possible that the heme component of the flavocytochrome *b* may accept electrons from other redox carriers. This appears to be the case for cytochrome b_{558} purified from pig neutrophils, which requires the addition of NADPH cytochrome *c* reductase to carry electrons from NADPH to O_2 (Isogai et al., 1991).

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REFERENCES

Babior, B. (1992) in *Advances in Enzymology* (Meister, A., Ed.) Vol. 65, pp 49−95, Wiley, New York.

Babior, B., & Kipnes, R. S. (1977) *Blood 50*, 517−524.

Cross, A. R., & Jones, O. T. G. (1991) *Biochim. Biophys. Acta 1057*, 281−298.

Cross, A. R., Higson, F. K., Jones, O. T. G., Harper, A. M., & Segal, A. W. (1982) *Biochem. J. 204*, 479−485.

Doussière, J., Brandolin, G., Derrien, V., & Vignais, P. V. (1992) *Biochemistry 32*, 8880−8887.

Gottikh, B. P., Krayevsky, A. A., Tarussova, N. B., Puyagin, P. P., & Tsilvich, T. L. (1970) *Tetrahedron 26*, 4419−4433.

Guillory, R. J., & Jeng, S. J. (1977) *Methods Enzymol. 46*, 259−289.

Isogai, Y., Shiro, Y., Nasuda-Kouyama, A., & Iizuka, T. (1991) *J. Biol. Chem. 266*, 13481−13484.

Kakinuma, K., Kaneda, M., Chiba, T., & Onishi, T. (1986) *J. Biol. Chem. 261*, 9426−9432.

Koshkin, V., & Pick, E. (1994) *FEBS Lett. 338*, 285−289.

Laemmli, U. K., & Favre, M. *Nature 227*, 680−685.

Levy, D. (1973) *Biochim. Biophys. Acta 322*, 329−336.

Ligeti, E., Doussière, J., & Vignais, P. V. (1988) *Biochemistry 27*, 193−200.

Light, D. R., Walsh, C., O'Callaghan, A. M., Goetzl, E. J., & Tauber, A. T. (1981) *Biochemistry 20*, 1468−1476.

Morel, F., Doussière, J., & Vignais, P. V. (1991) *Eur. J. Biochem. 201*, 523−546.

Parkos, C. A., Allen, R. A., Cochrane, C, G., & Jesaitis, A. J. (1987) *J. Clin. Invest. 80*, 732−742.

Parkos, C. A., Dinauer, M. C., Walker, L. E., Allen, R. A., Jesaitis, A. J., & Orkin, S. H. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 3319−3323.

Ravel, P., & Lederer, F. (1993) *Biochem. Biophys. Res. Commun. 196*, 543−552.

Rotrosen, D., Yeung, C. L., Leto, T., Maleck, H. L., & Kuong, C. H. (1992) *Science 256*, 1459−1462.

Segal, A. W. (1987) *Nature 326*, 88−91.

Segal, A. W., & Jones, O. T. G. (1978) *Nature 276*, 515−517.

Segal, A. W., & Abo, A. (1993) *Trends Biochem. Sci. 18*, 43−47.

Segal, A. W., West, I., Wientjes, F., Nugent, J. H. A., Chavan, A. J., Haley, B., Garcia, R. C., Rosen, H., & Scarce, G. (1992) *Biochem. J. 284*, 781−788.

Sumimoto, H., Sakamoto, N., Nozaki, M., Sakali, Y., Takeshige, K., & Minakami, S. (1992) *Biochem. Biophys. Res. Commun. 186*, 1368−1374.

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