Critical Assessment of the Presence of an NADPH Binding Site on Neutrophil Cytochrome b_{558} by Photoaffinity and Immunochemical Labeling†

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ABSTRACT: The presumed NADPH dehydrogenase function of the heterodimeric cytochrome b_{558} in the neutrophil oxidase complex has been investigated by combined photoaffinity labeling and immunoblot analysis of membrane proteins from bovine neutrophils. The photoaffinity probe was a radiolabeled analog of NADPH, [4-[*N*-(4-azido-2-nitrophenyl)[^{3}H]amino]butyryl]NADPH ([^{3}H]azido-NADPH), and the antibodies were directed against the C-terminal regions of the two subunits of cytochrome b_{558}. Plasma membrane vesicles obtained by differential centrifugation of bovine neutrophil homogenates were routinely used as a source of NADPH oxidase. They were permeabilized by sodium deoxycholate to facilitate the access of NADPH or its azido analog to the totality of the specific binding sites. In the absence of light, azido-NADPH behaved as a competitive inhibitor of NADPH oxidase with a K_i of 6 μM, and was able to bind to high-affinity specific binding sites with a K_d of 5–6 μM, indicating a higher affinity of the oxidase for the photoprobe than for the substrate NADPH (K_M = 30–40 μM). Upon photolabeling, the oxidase was fully inactivated. Following resolution of the membrane proteins by SDS–PAGE, a predominant photolabeled protein band of 80–100 kDa was revealed, which coincided with the large subunit (β) of cytochrome b_{558} identified by immunoblot in a parallel gel. The enzymatic deglycosylation of photolabeled neutrophil membranes shifted the masses of both the photolabeled band and the immunoreactive β subunit from 80–100 to 55–65 kDa in accordance with the glycoprotein nature of the β subunit. Omission of sodium deoxycholate in the photolabeling medium or in the oxidase assay medium resulted in a 3–5-fold decrease of the extent of photolabeling and the rate of O_2^- production, indicating that, in the preparation of plasma membrane vesicles utilized, the NADPH binding site of the oxidase as well as the site recognized by [^{3}H]azido-NADPH was predominantly oriented to the inside. With phagosomal vesicles obtained after phagocytosis of phorbol ester coated latex beads, the extent of photolabeling and the oxidase activity were independent of the presence of sodium deoxycholate, which means that both the NADPH binding site of the oxidase and the [^{3}H]azido-NADPH site were exposed to the outside. Aging of plasma membranes resulted in the gradual loss of both photolabeling of the 80–100-kDa band and O_2^- production. Taken together, these results indicate that the β subunit of the plasma membrane-bound cytochrome b_{558} contains an NADPH binding site that appears to be catalytically competent in the functioning of the oxidase complex. With a bovine neutrophil granule fraction containing 2–3 times more cytochrome b_{558} per milligram of protein than the plasma membrane fraction, photoirradiation in the presence of [^{3}H]azido-NADPH resulted in a rather low photolabeling of the 80–100-kDa protein band compared to that of the plasma membrane-enriched fraction.

A controversial problem about the O_2^--generating oxidase complex of neutrophils concerns the putative NADPH dehydrogenase function of the heterodimeric cytochrome b_{558}, a redox component of the phagocytic oxidase complex [for reviews, see Morel et al. (1991) and Babior (1992)]. Early studies have led to the conclusion that cytochrome b_{558} purified from human neutrophils (Parkos et al., 1987; Segal, 1987) is a dimeric protein consisting of a large (β) subunit (90 kDa) of glycoprotein nature with a protein moiety of about 64 kDa, and a small (α) subunit of 21 kDa which is thought to be the heme-bearing component capable of reacting with O_2 (Nugent et al., 1989). In the case of cytochrome b_{558} purified from bovine neutrophils (Morel et al., 1987), two main bands were detected by SDS–PAGE,[1] with molecular masses of 64 and 20 kDa. The 64-kDa band could have been the deglycosylated form or a degradation product of the β subunit. Prior to purification of cytochrome b_{558}, attempts by different groups to purify the dehydrogenase component of the neutrophil oxidase complex had resulted in the isolation of a 65-kDa protein with an affinity for NADPH similar to that of the oxidase complex (Markert et al., 1985; Doussière and Vignais, 1985; Kakinuma et al., 1987). The dilemma concerning the nature of the 65-kDa protein, either a degradation product of the β subunit of cytochrome b_{558} or a genuine dehydrogenase, could not be resolved at that time. Interest in cytochrome b_{558} was recently revived with the report that a purified membrane oxidase preparation considerably enriched in cytochrome b_{558} was able, after activation by cytosolic factors in a cell-free system, to generate O_2^- in the presence of NADPH (Knoller et al., 1991). This was followed by reports describing the ability of cytochrome b_{558} to bind FAD (Segal et al., 1992; Rotrosen et al., 1992; Sumimoto et al., 1992) and 2-azido-

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[1] Abbreviations: PBS, phosphate-buffered saline; azido-NADPH, [4-[*N*-(4-azido-2-nitrophenyl)amino]butyryl]NADPH; SOD, superoxide dismutase; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; TLCK, *Nα-p*-tosyl-L-lysine chloromethyl ketone; DFP, diisopropyl fluorophosphate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PMA, phorbol myristate acetate.

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$NADP^+$ (Segal et al., 1992). In these reports, it was pointed out that a limited homology (up to 30%) existed between scattered sequences in the β subunit of cytochrome b_{558} and a number of NADPH binding sites in several flavodehydrogenases. Although these reports converged toward the same conclusion, each of them afforded incomplete evidence for the β subunit of cytochrome b_{558} being an NADPH dehydrogenase.

In the present study, bovine neutrophil membranes were covalently photolabeled with a radioactive azido derivative of NADPH and the proteins were separated by SDS–PAGE. The distribution pattern of the photolabeled proteins in the gels was compared with the position of protein bands detected on Western blots by reactivity to antibodies raised against the α and β subunits of cytochrome b_{558}. Experiments were also performed with photolabeled membranes treated with a glycosidase preparation to remove the carbohydrate chains from the β subunit of cytochrome b_{558}. Furthermore, we have assessed the orientation of the NADPH binding site of the oxidase complex in crude plasma membrane vesicles and in phagosomal vesicles obtained from neutrophils after phagocytosis of latex beads, through the use of sodium deoxycholate and its permeabilizing effect on both the extent of photolabeling and the oxidase activity. The effects of aging of the membrane fraction on oxidase activity and photolabeling were also determined. Similar experiments were carried out with a granule fraction particularly rich in cytochrome b_{558}. The results support the view that the β subunit of cytochrome b of the plasma membrane fraction contains a potentially functional NADPH binding site.

MATERIALS AND METHODS

Materials. NADPH and endoglycosidase F/*N*-glycosidase F were purchased from Boehringer; superoxide dismutase, ferricytochrome *c* (horse heart grade VI), leupeptin, DFP, trypsin inhibitor, and phorbol myristate acetate from Sigma; and TPCK, TLCK, β-mercaptoethanol, SDS, and Coomassie blue R250 from Serva. [γ-[*N*-(4-Azido-2-nitrophenyl)[^{3}H]-amino]butyryl]$NADP^+$ ([^{3}H]azido-$NADP^+$) (Figure 2, inset) was synthesized as described (Doussière et al., 1986) with a specific radioactivity of (50–60) $\times 10^6$ dpm/μmol. Reagents for enhanced chemiluminescent (ECL) detection of horse radish peroxidase-labeled antibodies were purchased as a kit 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. Neutrophils were obtained from bovine blood and were used as resting cells or after activation with phorbol myristate acetate (Morel et al., 1985). Membrane fractions were isolated from bovine neutrophil homogenates by differential centrifugation (Doussière & Vignais, 1985) in the presence of a mixture of antiproteases consisting of 1 mM diisopropyl fluorophosphate, 0.2 mM EDTA, 0.2 mM EGTA, E_{64} (3 μg/mL), leupeptin (3 μg/mL), TPCK (100 μg/mL), trypsin inhibitor (100 μg/mL), and 1 mM iodoacetamide. Nondisrupted cells, nuclei, and a large portion of the granule material were eliminated by centrifugation at 10000*g* for 10 min. The supernatant was spun at 130000*g* for 1 h. The resulting fraction, enriched in plasma membrane, was resuspended in PBS supplemented with antiproteases. Phagocytic vacuoles (phagosomes) were obtained from bovine neutrophils left in contact for 10 min at 37 °C with PMA-coated latex particles. They were recovered by centrifugation of the neutrophil homogenate in a sucrose gradient as described (Morel et al., 1985). This centrifugation also allowed the recovery of a granule fraction consisting of azurophil and specific granules particularly rich in cytochrome b_{558} (Morel et al., 1985). In all cases, membrane fractions were supplemented with antiproteases.

Synthesis of Peptides and Preparation of Antiserums. Two types of antibodies were used, corresponding to the C-terminal (C-ter) region of the α subunit of cytochrome b_{558} (anti-α-C-ter (b_{558}) antibodies) and to the C-ter region of the β subunit (anti-β-C-ter (b_{558}) antibodies). Peptides corresponding to residues 184–195 of the C-ter sequence of the α subunit of cytochrome b_{558} (Gln-Val-Asn-Pro-Ile-Pro-Val-Thr-Asp-Glu-Val-Val) and to residues 552–559 of the C-ter sequence of the β subunit (Phe-Ile-Phe-Asn-Lys-Glu-Asn-Phe) were synthesized. A tyrosyl residue was added at the N-ter extremity of these peptides to facilitate the chemical coupling to ovalbumin by bisdiazotized benzidine (Brandolin et al., 1989). The conjugates were used to immunize male New Zealand white rabbits. For the primary immunization, samples corresponding to 100 μg of coupled peptide in 0.5 mL of PBS were emulsified with 0.5 mL of complete Freund's adjuvant. This was followed by two series of intradermal injections at 2-week intervals. Serums were decomplemented by treatment at 56 °C for 30 min. Preimmune serums were used as the control.

Assay of Oxidase Activity. The production of O_2^- by membranes of PMA-activated neutrophils was monitored spectrophotometrically at 550 nm by the superoxide dismutase inhibitable reduction of cytochrome *c* in the presence of NADPH (Doussière & Vignais, 1985). The assay was carried out at 25 °C in PBS supplemented with 200 μM NADPH and 100 μM cytochrome *c*. As a control, SOD (50 μg) was added after a few minutes of reduction of cytochrome *c* to quench O_2^-.

Photolabeling Experiments. In routine experiments, membranes isolated from bovine neutrophils were preincubated for 2 min at 20–22 °C with 20 μM [^{3}H]azido-$NADP^+$, 1 mM glucose 6-phosphate, and 5 μL of glucose-6-phosphate dehydrogenase in a medium consisting of PBS supplemented with 20 mM Tris–HCl (pH 7.4) and 0.05% sodium deoxycholate. Azido-$NADP^+$ is a substrate for the glucose-6-phosphate dehydrogenase and was therefore reduced into azido-NADPH in the above medium. The Tris buffer decreased the nonspecific labeling, and sodium deoxycholate at low concentration permeabilized the membrane vesicles and facilitated the access of azido-NADPH to the NADPH binding sites. Photoirradiation was performed with a xenon XBO lamp of 1000 W (Müller GmbH Moosinning, Germany) placed 20 cm above the glass flask containing the membrane suspension diluted at 1 mg/mL. The flask was rotated horizontally in an ice bath, and irradiation lasted routinely for 5 min. This period, much larger than that required for photolysis of [^{3}H]azido-$NADP^+$ (less than 10 s), was imposed by the turbidity of the suspended membranes. The glass wall behaved as a filter for UV radiation, and therefore photolysis was predominantly due to the absorption peak of the probe at 480 nm. This point is of importance for competition experiments conducted with NADPH which has peaks of absorption at 340 and 260 nm.

SDS–PAGE and Immunoblot Analysis. Following photoirradiation, the membrane suspension was diluted 10-fold in PBS supplemented with antiproteases and then centrifuged for 1 h at 100000*g*. The pellet was rinsed with PBS, dissolved in 5% SDS, 1% β-mercaptoethanol, and 20% sucrose, and heated for 2 min at 100 °C. The solubilized extract was subjected to SDS–PAGE [Laemmli & Favre, 1973) in parallel runs on slab gels, using a 4% stacking gel and running gels

with acrylamide concentrations of 12%, 10%, or 8%. The gels were cut into strips. One of the strips was stained with Coomassie blue for determination of the molecular masses of the separated proteins by comparison with standards, and cut into slices of 2 mm for localization of protein-bound radioactivity. Each slice was incubated overnight at 60–65 °C with 5% H_2O_2. The remaining H_2O_2 was removed by treatment with catalase, and radioactivity was measured by liquid scintillation. The proteins of the other strips were electrotransferred to nitrocellulose sheets (Towbin et al., 1979) for immunodetection by specific antiserums. In brief, the electrotransferred proteins were reacted with antipeptide antibodies directed against the C-terminal regions of the α and β subunits of cytochrome b_{558}, followed by incubation with goat antirabbit antibodies conjugated with horse radish peroxidase. The peroxidase activity was revealed by luminescence, using the kit from Amersham and following the instructions of the manufacturer. Controls were carried out with preimmune serums. Molecular mass markers were phosphorylase *b*, 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; and trypsin inhibitor, 20 kDa. Photolabeling experiments were performed at least three times with essentially similar results. The data presented are from representative experiments.

Immunoprecipitation of a Photolabeled Cytochrome b_{558}–Antibody Complex on Protein A–Agarose Beads. Photolabeled neutrophil membranes (5–6 mg of protein) in 5 mL of PBS supplemented with antiproteases were solubilized in 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris (pH 7.8) and then heated for 2 min at 100 °C. Following centrifugation at 130000*g* for 1 h, the clear supernatant was withdrawn, diluted 3-fold with PBS, and divided into two fractions of 7 mL. The two fractions were supplemented with 20 μL of antiserum raised against the C-ter region of the β subunit of cytochrome b_{558}; in one of them, the C-ter peptide of the β subunit (400 μg) was added. After a 1-h incubation at 22 °C, protein A–agarose beads (36 mg) were added to the two fractions, and incubation was continued overnight at 4 °C with gentle stirring. The agarose beads coated with the immunoprecipitated material were recovered by centrifugation at 6000*g* for 10 min and then washed three times with 0.5 M NaCl, 50 mM Tris (pH 7.8), 0.8% Triton X100, and 0.1% SDS, once with 0.5 M NaCl and 50 mM Tris (pH 7.8), and finally once with PBS. The immunoprecipitate was eluted from the agarose beads by heating with Laemmli sample buffer for 2 min at 100 °C. The radioactivity of the two fractions was determined by scintillation counting. The difference in radioactivity corresponded to the specific labeling of the β subunit of cytochrome b_{558} by [^{3}H]azido-NADPH.

Deglycosylation of Cytochrome b_{558}. The β subunit of neutrophil cytochrome b_{558} is a glycoprotein with about 30% of its mass corresponding to the carbohydrate part. In some photolabeling experiments, deglycosylation of the membrane-bound cytochrome b_{558} was performed using plasma membranes treated as follows. Membranes photolabeled with [^{3}H]azido-NADPH (1.5–2.0 mg of protein) in 5 mL of PBS were precipitated with 0.2 M perchloric acid for 1 h at 0 °C. The precipitate was collected by centrifugation, washed twice with 5 mL of acetone at –20 °C, and then dried under vacuum. It was taken up in 250 μL of PBS supplemented with 0.1% Tween 20 and subjected to ultrasonication for 4 × 20 s with a Branson sonicator at 40-W output. After heating at 100 °C for 2 min, the protein material was incubated at 37 °C for 2 h with 15 units of endoglycosidase F/*N*-glycosidase F, in the presence of the antiprotease cocktail described above. This was followed by addition of 5 mL of acetone at –20 °C. The precipitate was recovered by centrifugation and dissolved in a mixture of 5% SDS, 10% β-mercaptoethanol, and 10% glycerol. An aliquot corresponding to 600 μg of protein was subjected to SDS–PAGE. A parallel control was run, in which the glycosidase preparation was absent.

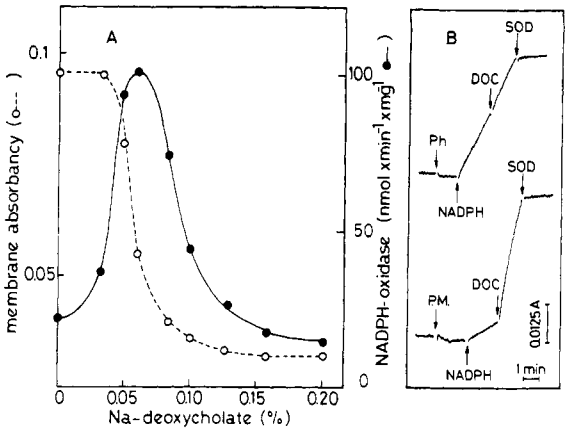

FIGURE 1: Permeabilization of bovine neutrophil plasma membrane vesicles by sodium deoxycholate (DOC). (A) Effect of increasing concentrations of sodium deoxycholate on light absorbancy and oxidase activity of crude plasma membrane vesicles from PMA-activated bovine neutrophils. An aliquot of the suspension of plasma membranes (100 μg of protein in 2 mL of PBS) was supplemented with increasing concentrations of sodium deoxycholate. The absorbancy at 520 nm (O) was measured. After reaching a stable value (2–3 min), the rate of reduction of cytochrome *c* (100 μM) in the presence of 200 μM NADPH, inhibitable by SOD (50 μg) (O_2^--generating oxidase), was determined at 550 nm (●), as described in the Materials and Methods. (B) Comparison of oxidase activity in vesicles of crude plasma membranes (PM) (50 μg of protein) and phagocytic vacuoles (Ph) (20 μg of protein) obtained from neutrophils after endocytosis of latex beads coated with PMA (cf. Materials and Methods). Conditions are the same as in (A).

Protein Determination. The protein concentration in membranes was determined by the Biuret method as previously described (Morel et al., 1985).

RESULTS

[4-[N-(4-Azido-2-nitrophenyl)[^{3}H]amino]butyryl]-NADPH ([^{3}H]Azido-NADPH) Is an Efficient Probe of the Dehydrogenase Component of the Oxidase Complex of Bovine Neutrophils: Optimal Conditions for Access of the Azido Probe to the NADPH Site of Neutrophil Oxidase. The specificity and affinity of azido-NADPH for the NADPH binding site of the bovine neutrophil oxidase complex were assayed in the absence of light, i.e., under conditions of reversible binding. A membrane fraction, enriched in plasma membrane, obtained by differential centrifugation at 100000*g* of homogenates of PMA-activated neutrophils or resting neutrophils (Morel et al., 1985), was routinely used as the biological material for photolabeling experiments. Azido-$NADP^+$ was reduced to azido-NADPH by addition of glucose 6-phosphate and glucose-6-phosphate dehydrogenase.

As membranes form closed vesicles, and that the NADPH binding sites of the NADPH oxidase can be oriented to the inside or to the outside, it was of interest to test the permeabilizing effect of sodium deoxycholate on the access of NADPH (or azido-NADPH) to specific binding sites. The effect of increasing concentrations of sodium deoxycholate on the absorbancy at 520 nm and NADPH oxidase activity is illustrated in Figure 1A. At 0.05% sodium deoxycholate, a 3–4-fold increase in the rate of O_2^- production was observed, whereas absorbancy was only slightly decreased. This denoted

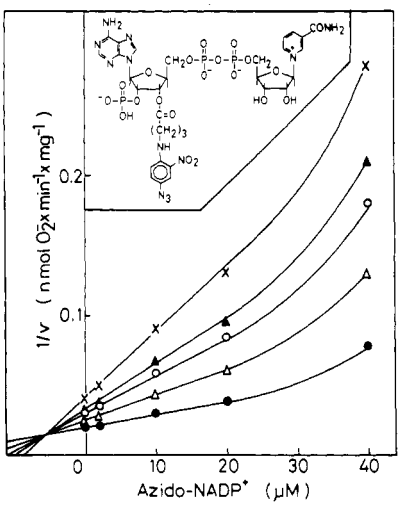

FIGURE 2: Reversible inhibition of membrane-bound NADPH oxidase by azido-NADPH in the absence of light. The photometric cuvette contained PBS, plasma membranes from PMA-activated bovine neutrophils (100 μg of protein), 0.05% sodium deoxycholate, 100 μM cytochrome *c*, 0.5 mM sodium azide, 1.5 mM glucose 6-phosphate, 5 μL of glucose-6-phosphate dehydrogenase, NADPH at different fixed concentrations, and azido-NADPH added as azido-NADP$^+$ at increasing concentrations. The final volume was 2 mL. The graph corresponds to a Dixon plot of the rates of O_2^- production (NADPH cytochrome *c* reductase activity inhibitable by SOD) obtained with the following concentrations of NADPH: 10 μM (×); 14 μM (▲); 17 μM (○); 25 μM (△); and 50 μM (●). Note that, above 20 μM azido-NADPH, the plots become curvilinear. Insert: Structure of azido-NADPH.

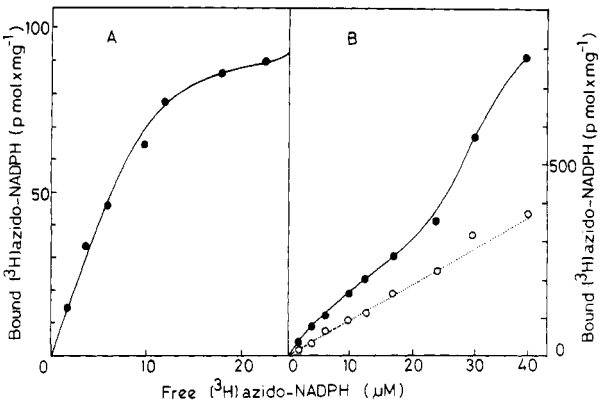

FIGURE 3: Reversible binding of [^{3}H]azido-NADPH to bovine neutrophil plasma membranes. The incubation medium used was the same as that of the experiment of Figure 2. [^{3}H]Azido-NADPH was added at increasing concentrations with aliquots of plasma membrane (1 mg of protein) and allowed to incubate for 15 min at 4 °C in the dark. Under these conditions equilibrium between free and bound photoprobe was fully reached. Following incubation, the membranes were sedimented by centrifugation at 140000*g* for 30 min. The pellets were rinsed with cold PBS, and then solubilized in 0.4 mL of 10% SDS. The radioactivity of each digest was measured by liquid scintillation. A parallel experiment was performed with an excess of NADPH (1 mM) added to the test tubes to determine the nonspecific bound [^{3}H]azido-NADPH. The two binding curves are shown on the right-hand side (B). The portion of the specific [^{3}H]-azido-NADPH binding curve corresponding to the high-affinity NADPH binding sites is shown on the left-hand side (A).

an efficient permeabilization of the plasma membrane vesicles without marked lysis and facilitation of the access of NADPH to the NADPH binding site of the oxidase. Above 0.06–0.07% sodium deoxycholate, there was a drastic decrease in absorbancy accompanied by a decrease in oxidase activity, due to lysis of the vesicles.

Permeabilization of crude plasma membrane vesicles by the optimal concentration of sodium deoxycholate, 0.05%, was compared to that of phagocytic vacuoles (phagosomes) obtained from bovine neutrophils after endocytosis of latex beads coated with PMA (Figure 1B). In the case of plasma membrane vesicles, sodium deoxycholate markedly increased the rate of NADPH cytochrome *c* reductase activity, and the reduction was totally sensitive to SOD, showing that it was due essentially to O_2^- (Figure 1B, lower trace). In the case of phagosomes, sodium deoxycholate did not enhance the rate of O_2^- production (Figure 1B, upper trace). Thus, in phagosomes the NADPH site of the oxidase complex is exposed to the outside, whereas in the case of plasma membrane vesicles, it is predominantly exposed to the inside. All further experiments dealing with plasma membrane vesicles were performed, unless indicated, in the presence of 0.05% sodium deoxycholate.

Not only was azido-NADPH not oxidized by bovine neutrophil plasma membranes, but it inhibited the O_2^- production linked to NADPH oxidation. At concentrations lower than 20 μM, the inhibition was essentially competitive. The K_i value determined by the method of Dixon was approximately 6 μM, which is 5–7 times lower than the K_M of the oxidase for NADPH (30–40 μM) (Figure 2). Upon photoirradiation in the presence of azido-NADPH, the O_2^--generating oxidase activity of the membrane was irreversibly inhibited, due to the covalent photolabeling of the NADPH binding component of the oxidase. It was checked that, in the absence of azido-NADPH, exposure of plasma membranes to light did not perturb oxidase activity.

Binding of [^{3}H]azido-NADPH (up to 40 μM) to neutrophil membranes in the absence of light is illustrated in Figure 3. The binding curve was biphasic with an inflection for probe concentrations around 20 μM (Figure 3B). The nonspecific binding of [^{3}H]azido-NADPH measured in the presence of an excess amount of NADPH (1 mM) increased linearly with the photoprobe concentration. The specific binding was calculated from the difference between the two curves. Figure 3A shows the portion of the specific binding curve corresponding to the high-affinity specific binding sites for the photoprobe. From the transitory plateau of saturation which characterizes this portion of the curve, it is possible to calculate an amount of high-affinity [^{3}H]azido-NADPH binding sites of about 90 pmol/mg of membrane protein and to deduce a K_d of 5–6 μM, a value similar to the K_i (6 μM) calculated for the inhibition of oxidase activity by the photoprobe (Figure 2). The higher affinity of the oxidase complex for a substrate analog, namely, azido-NADPH, than for the natural ligand NADPH has precedent. For example, the photoprobe [4-[*N*-(4-azido-2-nitrophenyl)amino]butyryl]AMP has been reported to bind to the regulatory site of the alternative oxidase complex of mitochondrial membranes of *Paramecium aurelia* with a much higher affinity than the natural ligand AMP (Doussière & Vignais, 1984). This is possibly due to the occurrence of hydrophobic interactions of the phenyl ring of the photoprobe with membranes, resulting in a local increase in probe concentration.

At concentrations higher than 20 μM, the amount of the reversibly bound [^{3}H]azido-NADPH increased significantly, following the pseudoplateau of high-affinity binding (Figure 3B). A substantial percentage of the low-affinity binding sites was displaced by NADPH. For these different classes of NADPH binding sites in the neutrophil plasma membranes, there are several possible explanations. (1) There may exist several membrane-bound NADPH dehydrogenases differing by their affinity for NADPH. (2) The dehydrogenase component of the oxidase complex may contain two NADPH binding sites with different affinities for NADPH, for example,

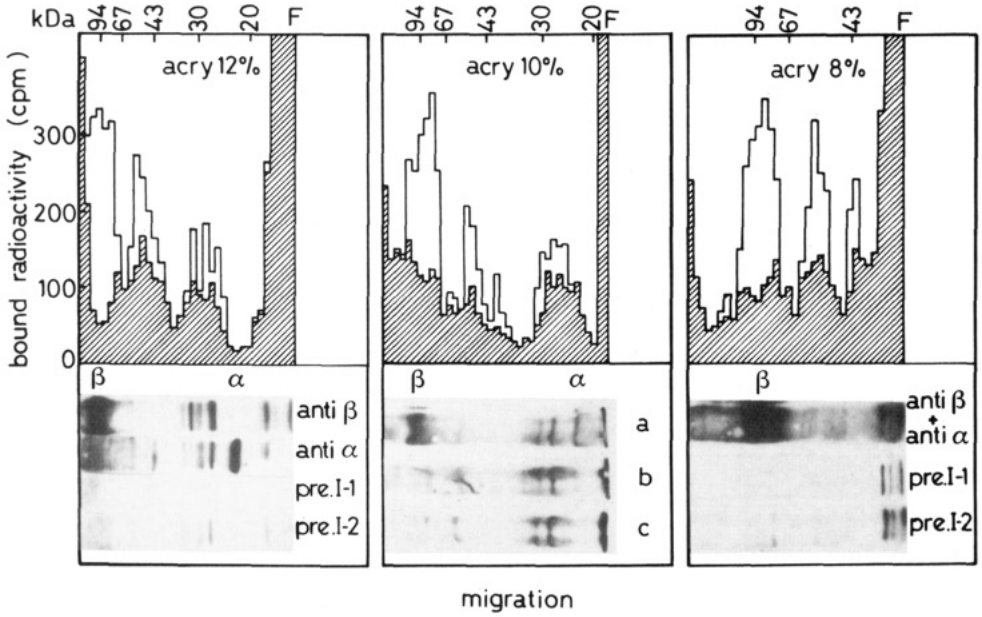

FIGURE 4: Comparison of the migration of [^{3}H]azido-NADPH-labeled proteins from bovine neutrophil plasma membranes and the α and β subunits of cytochrome b_{558} revealed by specific antibodies (SDS–PAGE). The upper part shows photolabeling. Photoirradiation of the plasma membrane-enriched fraction in the presence of [^{3}H]azido-NADPH was conducted as described in the Materials and Methods. The bar graphs show the distribution of radioactivity in the gels (600 μg of protein/lane). The shaded bars show the photolabeling in the presence of 0.5 mM NADPH. Apparent molecular masses of protein markers are given in kilodaltons. F represents the front of migration. (Panel A) 12% acrylamide. (Panel B) 10% acrylamide. (Panel C) 8% acrylamide. The lower part shows Western blots with antibodies directed to the C-ter regions of the α and β subunits of cytochrome b_{558} (experiment run in parallel). The antiserums and the preimmune serums were diluted 2000-fold. (Panel A) The immunoblots shown were carried with anti-β-subunit antiserum, anti-α-subunit antiserum, and two preimmune serums (Pre I-1 and Pre I-2) obtained from the rabbits that were injected later with the C-ter peptides of subunits β and α of cytochrome b_{558}, respectively (cf. Materials and Methods). (Panel B) (a) Anti-β-subunit plus anti-α-subunit antiserums as in panel A; (b) same as (a) in the presence of the C-ter peptide of the α and β subunits; (c) mixture of preimmune serums 1 and 2 (1/1). (Panel C) Anti-α-subunit plus anti-β-subunit antiserums as in panel B and preimmune serums 1 and 2.

a regulatory site and a catalytic site. (3) The dehydrogenase component of the oxidase may exist under various conformations.

Combined Experiments of Covalent Photolabeling of Bovine Neutrophil Plasma Membranes with [^{3}H]Azido-NADPH and Immunodetection of Cytochrome b_{558} by Western Blot. In the experiment illustrated in Figure 4, left-hand side, neutrophil plasma membrane proteins photolabeled with [^{3}H]-azido-NADPH were resolved by SDS–PAGE, using a 12% acrylamide slab gel. The gel was cut into strips. One of the strips was used for localization of the photolabeled proteins. The other strips were blotted on nitrocellulose sheets to test the localization of the α and β subunits of cytochrome b_{558} with specific antibodies. The profile of covalently radiolabeled proteins is illustrated in the upper part of the figure, and the localization of the immunodetected α and β subunits of cytochrome b_{558} is shown in the lower part of the figure. A major radioactive peak containing photolabeled protein(s) was found at 80–100 kDa, accompanied by several less intensively radioactive peaks scattered between 70 and 25 kDa. An excess of NADPH (0.5 mM) added prior to the azido probe was able to efficiently quench all labeled peaks except the one migrating to the front of the gel. Photoirradiation was performed under conditions which favored the selection of visible light (cf. Materials and Methods), which made the quenching effect of NADPH rather selective. The photolabeled band migrating in the front of the gel consisted probably of the free probe or of the probe bound to small-size proteins or membrane phospholipids. In the Western blotting experiment run in parallel, a major immunoreactive band corresponding to the β subunit of cytochrome b_{558}, revealed by the anti-β-C-ter (b_{558}) antiserum, was found to coincide with the major peak of photolabeled protein(s) at 80–100 kDa. Other photolabeled bands of smaller size in the 25–30-kDa region of the gel were also found to coincide with faintly immunoreactive bands detected with anti-β-C-ter (b_{558}) antibodies. Western blotting carried out with anti-α-C-ter (b_{558}) antibodies revealed a major immunoreactive protein with a mass of 22 kDa which did not possess a photolabeled counterpart. Preimmune serums (PreI-1, PreI-2) were found to probe only minute amounts of scattered proteins.

Decreasing the acrylamide concentration in the slab gel from 12% to 10% (Figure 4, middle part) or to 8% (Figure 4, right-hand side) modified the electrophoretic mobility of the proteins. However, in all cases, the major photolabeled band at 80–100 kDa coincided with that of the β subunit of cytochrome b_{558}. The specificity of the immunodetection of the α and β subunits of cytochrome b_{558} was made evident by the strong quenching effect of the α and β C-ter peptides added to the immunodetection medium and acting there by competition with the respective subunits (Figure 4, middle part, a and b). By contrast, the immunoreactive bands in the range of 25–30 kDa were not displaced by the two C-ter peptides of cytochrome b_{558}, and in addition, they were detected by a mixture of preimmune serums (Figure 4, middle part, c). Obviously, they were not specific.

To ascertain that the protein in the photolabeled band at 80–100 kDa was the β subunit of cytochrome b_{558}, the photolabeled plasma membranes were solubilized in 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS buffered with 50 mM Tris (pH 7.8). The extract was divided into two fractions, which were treated by anti-β-subunit antiserum, either in the absence or in the presence of a saturating concentration of the C-ter peptide of the β subunit; the immune complexes in the two fractions were adsorbed on protein A–agarose beads, extensively washed and eluted as detailed in the Materials and Methods. The bound radioactivity in the two fractions corresponding to the photolabeled species

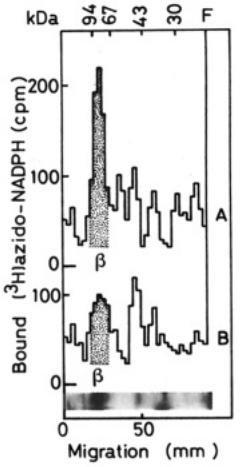

FIGURE 5: Enhancing effect of 0.05% sodium deoxycholate on photolabeling of proteins in crude plasma membrane vesicles. A plasma membrane-enriched fraction was photolabeled with [^{3}H]azido-NADPH in the presence of 0.05% sodium deoxycholate (A) or in the absence of deoxycholate (B). The proteins (600 μg) were separated by SDS–PAGE (10% acrylamide). The bar graph shows the distribution of radioactivity in the gels. The lower part of the figure illustrates the localization of the β subunit of cytochrome b_{558} determined by Western blot with an anti-β-C-ter (b_{558}) antiserum as in Figure 3.

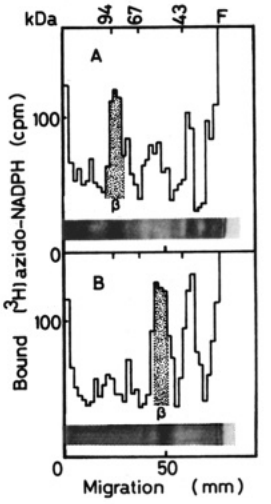

FIGURE 6: Effect of glycosidase treatment on the molecular masses of the neutrophil plasma membrane proteins photolabeled by [^{3}H]-azido-NADPH and on that of the immunodetected β subunit of cytochrome b_{558}: (A) control neutrophil membranes; (B) glycosidase-treated neutrophil membranes. A plasma membrane fraction from bovine neutrophils (600 μg of protein) was photolabeled with [^{3}H]-azido-NADPH in the presence of 0.05% sodium deoxycholate. Following denaturation, proteins were subjected to the action of glycosidase as described in the Materials and Methods, and then separated by SDS–PAGE. Two parallel 8% acrylamide gels were run. In one of them, the radioactivity of the photolabeled proteins was determined (upper part of the panels). The other one was used for immunodetection of the β subunit of cytochrome b_{558} by Western blotting (lower part of the panels).

was determined. The fraction that had been supplemented with the C-ter peptide of the β subunit contained 10 times less covalently bound [^{3}H]azido-NADPH than the nontreated fraction. This result strengthens the conclusion that the photolabeled protein of 80–100 kDa is the β subunit of cytochrome b_{558}.

A final control, namely, a prephotolysis experiment, was carried out to rule out unspecific irreversible binding of reactive long-lived byproducts accumulating during photolysis of [^{3}H]-azido-NADPH. The probe was therefore subjected to photoirradiation in standard medium without membranes for 1 min at 4 °C, and then neutrophil plasma membranes were added and allowed to incubate for 5 min at 4 °C. The membranes were washed in PBS, and the mixture was subjected to SDS–PAGE (10% acrylamide). The radioactivity profile of the resolved proteins (not shown) was flat, with an average amount of bound radioactivity of 30–40 cpm by gel slice, compared to values of 200–300 cpm in the region of 80–100 kDa corresponding to the localization of the β subunit of cytochrome b_{558} (cf. Figure 4). This rules out any binding artifact due to reactive photolysed byproducts.

Orientation of the NADPH Site of the Oxidase Complex in Crude Plasma Membrane Vesicles and in Phagosomes. All preceding photolabeling experiments were carried out with crude plasma membrane vesicles permeabilized with 0.05% sodium deoxycholate, as it was found that this treatment markedly enhanced access of NADPH to the oxidase complex (Figure 1B, lower trace). Consistent with this premise, comparison of photolabeling of crude plasma membrane vesicles in the absence or presence of 0.05% sodium deoxycholate shows that sodium deoxycholate increased by 2–3-fold the 80–100-kDa photolabeled band coinciding with the β subunit of cytochrome b_{558} (Figure 5, B *vs* A). In the case of phagosomes, sodium deoxycholate had no effect on oxidation of the added NADPH (Figure 1B, upper trace), and in agreement with this, the extent of photolabeling of the 80–100-kDa protein band was not modified by addition of sodium deoxycholate (not shown), leading to the conclusion that, in phagosomes, the NADPH binding sites of the oxidase complex and the sites labeled by azido-NADPH are both exposed to cytosol.

Effect of Enzymatic Deglycosylation on the Migration in SDS–PAGE of the Photolabeled 80–100-kDa Protein Band and on That of the Immunodetected β Subunit of Cytochrome b_{558}. The β subunit of cytochrome b_{558} is a glycoprotein whose 25–30% of the mass corresponds to carbohydrate (Parkos et al., 1987). Removal of carbohydrate by glycosidase treatment should increase the mobility of the β subunit in SDS–PAGE without altering its immune reactivity. The plasma membrane-rich fraction from bovine neutrophils was photolabeled with [^{3}H]azido-NADPH, and processed for treatment by glycosidase as described in the Materials and Methods. As illustrated in Figure 6, upon deglycosylation, both most of the radioactivity (more than 80%) of the major photolabeled band in SDS–PAGE and the totality of the immunodetected β subunit in Western blot, which initially migrated with an apparent mass of 80–100 kDa, now moved with a mass of 55–65 kDa. This result affords evidence that the β subunit of the plasma membrane-bound cytochrome b_{558} is the major protein photolabeled by [^{3}H]azido-NADPH.

Effect of Aging of Bovine Neutrophil Plasma Membranes on the Photolabeling of Proteins by [^{3}H]Azido-NADPH. Upon aging at room temperature, plasma membranes from PMA-activated bovine neutrophils not treated with antiproteases gradually lose their capacity to generate O_2^- (Figure 7). The 80–100-kDa photolabeled band, which comigrates with the β subunit of cytochrome b_{558}, also decreased upon aging, apparently a little less rapidly than the respiratory capacity. After 2 h, the decrease amounted to 80% of the control value. At this point, it should be recalled that the oxidase complex present in the plasma membranes of activated neutrophils consists of the assembly of the redox component, namely, cytochrome b_{558}, proteins of cytosolic origin, referred to as p47 and p67 and a small G protein belonging to the rac subclass [for a review, cf. Morel et al. (1992)]. The fact that the oxidase activity is more susceptible to aging than the photolabeling of cytochrome b_{558} may be due to a higher

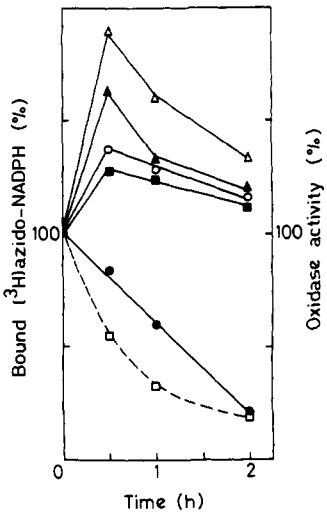

FIGURE 7: Effect of aging of plasma membranes in the absence of antiproteases on the photolabeling of proteins by [^{3}H]azido-NADPH. A plasma membrane fraction of PMA-activated neutrophils (600 μg of protein) was subjected to aging at 22 °C for 0 h (control), 0.5 h, 1 h, and 2 h, prior to photolabeling with [^{3}H]azido-NADPH, in the presence of 0.05% sodium deoxycholate (cf. Materials and Methods). Proteins were separated by SDS–PAGE (10% acrylamide), and the profile of bound radioactivity in the gel was determined. The figure shows the time course of the changes in the bound radioactivity contained in the most significative photolabeled bands with the following molecular masses: 26 kDa (△), 40 kDa (▲), 70 kDa (○), 29 kDa (■), and 80–100 kDa (●). In parallel to photolabeling, the oxidase activity (NADPH cytochrome *c* reductase activity inhibitable by SOD) was determined (□). The data are expressed in percent of the control value (no aging).

sensitivity of the cytosolic factors, and (or) the disassembly of the oxidase complex. Decrease of the photolabeled peak at 80–100 kDa was accompanied by the transient appearance of several photolabeled peaks, notably at 70, 40, 29, and 26 kDa. Proteins present in most of these peaks were not immunoreactive, except the 80–100-kDa peak and the 40-kDa peak (not shown), suggesting that they were degradation products of the β subunit of cytochrome b_{558}, devoid of the C-ter region. The fact that photolabeling of the 80–100-kDa band (probably identical to the β subunit of cytochrome b_{558}) is decreased during aging, whereas the β subunit remains immunodetectable, suggests that the native structure of cytochrome b_{558} is of importance for recognition of NADPH.

Dependence of Photolabeling of the 80–100-kDa Protein on Neutrophil Activation and on Subcellular Localization. Most of the experiments reported in this paper were carried out with plasma membranes from PMA-treated neutrophils whose NADPH oxidase was activated. Photoirradiation of nonactivated neutrophil membranes with [^{3}H]azido-NADPH led to a similar pattern of photolabeled proteins. However, unexpectedly, the extent of photolabeling of the 80–100-kDa band of the plasma membrane fraction was significantly (20–30%) higher with membranes from resting neutrophils than with those from activated neutrophils.

Photoirradiation of a granule fraction consisting of azurophil and specific granules, particularly rich in cytochrome b_{558} (Morel et al., 1985), with [^{3}H]azido-NADPH yielded a modest photolabeling of the 80–100-kDa band compared to that shown with the same amount of plasma membrane fraction (10–20%), contrasting with a strong immunoreactivity of cytochrome b_{558} subunit β to anti-β-C-ter (b_{558}) antibodies (not shown). It should also be noted that, in contrast to a cell-free system that was very efficient in O_2^- production by combination of bovine neutrophil cytosol and plasma membranes, supplemented with arachidonic acid and GTP (Ligeti et al., 1988), a cell-free system, where the plasma membrane fraction was replaced by the granule fraction, exhibited a relatively modest NADPH oxidase activity. This oxidase activity was 5–7 times lower than that expected on the basis of the amount of cytochrome b_{558} present in the granule fraction. Although cytochrome b_{558} species in neutrophil plasma membranes and granule membranes are apparently similar by spectroscopy and immunoreactivity, they may assume different conformational states depending on the protein or lipid environment in their respective membranes.

DISCUSSION

The experiments reported in the present paper were carried out to answer the question of whether the β subunit of cytochrome b_{558} possesses a binding site for NADPH and behaves as an NADPH dehydrogenase. The presence of a specific NADPH binding site on the β subunit of the plasma membrane-bound cytochrome b_{558} of bovine neutrophils is supported by a number of results based on photolabeling with [^{3}H]azido-NADPH and immunodetection of the β subunit of cytochrome b_{558}. (1) Following SDS–PAGE of photolabeled proteins from a plasma membrane fraction with gels differing in acrylamide concentration, namely, 12%, 10%, and 8%, the major photolabeled peak at 80–100 kDa was always found to coincide with the immunodetected β subunit of cytochrome b_{558} (Figure 4). (2) The immune precipitate obtained by mixing the photolabeled neutrophil membrane extract with anti-β-subunit antibodies in the presence of Sepharose-bound protein A contained a photolabeled protein material; 90% of this labeled protein material was released upon addition of the C-ter peptide of the β subunit that had been used to generate the antibodies. (3) Enzymatic release of the carbohydrate part of the β subunit of cytochrome b_{558} resulted in the disappearance of the major photolabeled peak of 80–100 kDa and the appearance of a new photolabeled peak of 55–60 kDa which coincided with the truncated β subunit immunodetected by anti-β-subunit antibodies. This latter result can be compared with that of a recent study (Ravel, 1992), conducted with human neutrophil membranes treated by [^{3}H]pyridoxal-5′-diphosphoadenosine, an inhibitor of the NADPH oxidase competing with NADPH (Ravel & Lederer, 1991). In this study, it was shown that the molecular mass of a radioactively labeled protein band of 76 kDa of nonidentified nature, but playing most likely a role in the oxidase complex, was shifted to 53 kDa by treatment of the membranes with endoglycosidase F.

The following findings favor the view that the photolabeled protein of 80–100 kDa, presumably the β subunit of cytochrome b_{558}, in bovine neutrophil plasma membrane, is endowed with an NADPH dehydrogenase function in the oxidase complex: (1) Azido-NADPH in the absence of photoirradiation competitively inhibited NADPH oxidation by plasma membranes from activated neutrophils. Competition was highly efficient since the K_i for azido-NADPH was 6–7-fold lower than the K_M for NADPH (Figure 2). (2) The K_i value for azido-NADPH coincided with the K_d value determined for a category of high-affinity binding sites. A saturation plateau for these sites was found to correspond to about 90 pmol of bound azido-NADPH/mg of plasma membrane protein (Figure 3), i.e., an amount of the same order of magnitude as that of the amount of cytochrome *b* contained in the same type of membranes (Morel et al., 1985). (3) Permeabilization of activated neutrophil plasma membrane vesicles by low concentrations of sodium deoxycholate en-

hanced both the NADPH oxidase activity and the extent of photolabeling of the β subunit of cytochrome b_{558}, most probably by facilitating the accessibility of the NADPH binding site of the dehydrogenase component of cytochrome b_{558} to NADPH and [^{3}H]azido-NADPH, respectively (Figures 1 and 5). Whereas in vesicles consisting of crude plasma membranes, the azido-NADPH and NADPH sites were predominantly exposed to the inside, in phagosomal vesicles, the azido-NADPH and NADPH sites were both essentially exposed to the outside and therefore accessible to their ligands in the absence of sodium deoxycholate.

Whereas it is evident that, in the plasma membrane of neutrophils, cytochrome b_{558} possesses an NADPH binding site probably associated with an NADPH dehydrogenase function, a small number of other proteins which are photolabeled by [^{3}H]azido-NADPH, but are not immunodetected, in particular some in the range of 55–75 kDa, are potential candidates as NADPH dehydrogenases. Along this line, recent reports on the reconstitution of NADPH oxidase activity from partially purified components of the oxidase complex in rabbit neutrophils (Laporte et al., 1991) and pig neutrophils (Miki et al., 1992) suggested that, in addition to the β subunit of cytochrome b_{558}, other proteins, including a protein of 77 kDa (Laporte et al., 1991), might act as auxiliary NADPH dehydrogenases. Such an event is not unexpected since a similar situation is encountered in the mitochondrial respiratory chain in which, in addition to the NADH dehydrogenase complex (complex I), a number of branched dehydrogenases, including succinate dehydrogenase and fatty acyl dehydrogenases, deliver electrons to the cytochrome chain.

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