Purified Leukocyte Cytochrome b_{558} Incorporated into Liposomes Catalyzes a Cytosolic Factor Dependent Diaphorase Activity†

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ABSTRACT: The leukocyte iodonitrotetrazolium violet (INT) reductase activity of disrupted bovine polymorphonuclear neutrophils is closely associated with the activation of the O_2^--generating NADPH oxidase in a cell-free system. It is dependent upon NADPH, cytosolic factors, and amphiphiles (such as arachidonate), the same factors required for O_2^- generation. Both O_2^- generation and INT reductase activity are inhibited by phenylarsine oxide, an inhibitor of the activation of the NADPH oxidase [Li, J., & Guillory, R. J. (1997) *J. Biochem. Mol. Biol. Biophys.* (in press)]. In this report, the INT diaphorase activity of disrupted bovine polymorphonuclear neutrophils is shown to be resolved by DEAE-Sepharose chromatography into two fractions: an NADPH−cytochrome *c* reductase-containing fraction and a cytochrome b_{558}-associated fraction. The diaphorase activity in the NADPH−cytochrome *c* reductase-containing portion is not dependent upon the presence of an amphiphile or phospholipid and is not associated with O_2^- generation. Upon incorporation into liposomes, the cytochrome b_{558}-containing fraction demonstrates high O_2^- and INT reductase activities in the presence of cytosolic factors. Both O_2^- generation and INT reductase activities are SDS and FAD dependent and further stimulated by GTPγS. Phenylarsine oxide inhibits both O_2^- generation and INT reductase activities when added prior to activation by SDS. With the cytochrome *b*-containing liposomes, the K_m values (O_2^- formation) for NADPH and NADH are 27.2 μM and 810 μM, and for INT reductase the K_m values are 27.5 μM and 1017 μM, respectively. Under anaerobic conditions and thus in the absence of O_2^- formation, the NADPH-dependent INT reductase activity does not change, indicating that the dye reduction is not due to its direct reduction by O_2^- anion but is an intrinsic property of the superoxide-generating NADPH oxidase. Cytochrome b_{558} is the essential component of the NADPH oxidase and contains all the redox centers necessary for electron flow between NADPH and oxygen. The correlation of the activation and inhibition patterns for O_2^- generation and INT reduction by cytochrome b_{558} incorporated into artificial liposomes strongly indicates that the two activities are associated with the same membrane protein, cytochrome b_{558}.

The O_2^--generating NADPH oxidase is dormant in unstimulated neutrophils and produces superoxide anion only upon activation by a number of independent stimuli. Reactive species derived from O_2^-, such as H_2O_2 and OCl^-, play an important role in the bacterial killing function of phagocytes [for reviews, see Babior (1992) and Segal (1989)]. A membrane-associate flavocytochrome (cytochrome b_{558}) is the essential component of this phagocytic NADPH oxidase. Cytochrome b_{558} consists of two subunits ($gp91^{phox}$ and $p21^{phox}$) and contains redox centers of flavin adenine dinucleotide (FAD)[1] and heme (Rotrosen et al., 1992; Segal et al., 1992; Sumimoto et al., 1992). It is generally believed that the heme is the terminal site directly involved in the transfer of a single electron to oxygen in the generation of O_2^- (Ellis et al., 1989; Isogai et al., 1991). Upon activation, at least three cytosolic factors, $p47^{phox}$, $p67^{phox}$, and a rac protein, translocate to the plasma membrane where they associate with cytochrome b_{558} to form an assembled and active NADPH oxidase complex (Heyworth et al., 1991; El Benna et al., 1994; Uhlinger et al., 1993). The activation of O_2^- generation takes place in a disrupted cell-free system in a manner which appears in a number of ways to simulate the process occurring in the intact neutrophils (Babior, 1992). The concept that cytochrome b_{558} contains the components of an electron transport chain responsible for the reduction of oxygen is reinforced by studies showing that the cytochrome contains an NADPH binding site, a FAD redox center, and two heme centers, all of which are essential elements required for the transfer of electrons from NADPH to oxygen (Doussiere et al., 1993, 1995; Quinn et al., 1992). Recent studies by Koshkin and Pick (1993) demonstrated that cytochrome b_{558} incorporated into liposome vesicles is capable of producing O_2^- independent of cytosolic factors, in contrast to the absolute cytosolic requirement for plasma membrane activation. The cytochrome can thus be considered to be the minimal component required for the NADPH-dependent reduction of oxygen in the leukocyte plasma membranes.

Cross et al. (1994) have shown that the NADPH oxidase activity of human neutrophil plasma membranes is associated with a novel INT dye reductase activity. Here we report

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[1] Abbreviations: SDS, sodium dodecyl sulfate; INT, *p*-iodonitrotetrazolium violet; OG, *n*-octyl glucoside; PMN, polymorphonuclear neutrophil; PA, phosphatidic acid; PC, phosphatidylcholine; GTPγS, guanosine 5′-(γ-thiotriphosphate); PAO, phenylarsine oxide; PMSF, phenylmethanesulfonyl fluoride; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; FAD, flavin adenine dinucleotide.

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that the dye reductase activity can be resolved into two distinguished fractions by octyl glucoside solubilization and anion exchange chromatography of the leukocyte plasma membranes. One fraction is closely associated with a resolved cytochrome *c* reductase and the other with the cytochrome b_{558}. Both O_2^- generation and INT reduction driven by NADPH require cytochrome b_{558} incorporation into a phospholipid bilayer and its reflavination. Cytosolic factors are shown to be required for O_2^- generation as well as INT reduction only with cytochrome b_{558}. The concentration dependency of both activities on cytosol indicates a regulatory role for cytosolic components. In contrast to the cytosol dependency of the cytochrome b_{558}-associated O_2^- generation and INT reduction, the INT reduction observed with the cytochrome *c* reductase preparation is not dependent upon cytosolic components nor is it associated with O_2^- generation.

EXPERIMENTAL PROCEDURES

Materials. Cytochrome *c* (from horse heart, type III), INT, NADPH, NADH, *n*-octyl glucoside (OG), phenylmethanesulfonyl fluoride (PMSF), leupeptin, pepstatin A, GTPγS, arachidonic acid (sodium salt), superoxide dismutase, galactose, galactose oxidase, DEAE-Sepharose CL-4B, heparin−agrose, phosphatidylcholine (type II-s, 20%), phosphatidic acid, Histopaque 1.119, 1.077, and PAGE standards were obtained from Sigma (St. Louis, MO). Phenylarsine oxide was purchased from Aldrich. All other regents were of the best grade commercially available.

Isolation of Bovine Polymorphonuclear Neutrophils. Bovine polymorphonuclear neutrophils were isolated from bovine blood by a procedure which includes hypotonic lysis of erythrocytes and Histopaque gradient centrifugation as described previously by Doussiere and Vignais (1985). The neutrophils were resuspended in buffer A (10 mM potassium phosphate, 150 mM NaCl, pH 7.4) supplemented with 1 mM PMSF, 1 mM EGTA, 5 μg/mL leupeptin, and 5 μg/mL pepstatin A and subjected to sonication as described below.

Subcellular Fractionation. Neutrophils were disrupted by five 15-s sonication bursts at 40% output from a microsonication probe (Heat Systems, model 350) at 0−4 °C. Unbroken cells, nuclei, granules and other cellular debris were removed from the sonicate by centrifugation at 10000*g* for 10 min at 4 °C in a Sorvall SS 34 rotor. The postnuclear supernatant was then subjected to ultracentrifugation at 120000*g* for 1 h at 4 °C to separate plasma membranes (pellet) from cytosol (supernatant). The pellet was washed with 1 M NaCl by centrifugation, resuspended in buffer A containing 1 mM PMSF, and used as the unstimulated membrane fraction. The 120000*g* supernatant was recentrifuged at the same speed and the resultant supernatant used as the cytosol fraction. Both cytosol and membranes were stored in liquid nitrogen.

Preparation of the Solubilized Membrane. The membrane fraction (2−4 mg/mL) was thawed on ice and 1 volume of extraction buffer [80 mM OG, 2 mM NaN_3, 2 mM EGTA, 100 mM phosphate, 1 mM DTT, and 2 mM PMSF, 40% glycerol (v/v), pH 7.4] added. Solubilization was assisted by gently stirring the suspension at 0−4 °C for 1 h. The detergent-treated preparation was centrifuged at 120000*g* for 1 h and the supernatant used as the solubilized membrane. The solubilized membranes were used directly for the separation of cytochrome b_{558} from the cytochrome *c* reductase activity.

Separation of Cytochrome b_{558} and Cytochrome c Reductase by DEAE-Sepharose Chromatography. The OG-solubilized membrane was mixed for 2 h at 4 °C with 0.1 volume of DEAE-Sepharose, preequilibrated with buffer B (10 mM phosphate, 40 mM OG, 1 mM PMSF, 1 mM EGTA, 1 mM DTT, and 20% glycerol, pH 7.4). The suspension was then packed into a 1.2 × 10 cm column, and the unbound proteins were collected. The column material was washed twice with buffer B (2 bed volumes). The unbound proteins and the column washes were pooled and were found to contain all of the cytochrome b_{558}. Following additional washing of the column with a 10-fold bed volume of buffer B, the DEAE-bound proteins were eluted batchwise with 250 mM NaCl.

Incorporation of Cytochrome b_{558} and Cytochrome c Reductase into Liposomes by Dialysis. Phosphatidylcholine was suspended in buffer B (10 mg/mL) by sonication at 4 °C. The cytochrome b_{558}-containing fractions (DEAE unbound) and the cytochrome *c* reductase fractions (DEAE bound) were mixed separately with a 10-fold excess of phosphatidylcholine (mass ratio). The protein−lipid mixture was sonicated 3 times, using six 1-s pulses each time at 20% output (Heat System, Model 350), and then kept on ice for 3−4 h. Excess phospholipids and detergent were removed by dialyzing the suspension against a 200-fold excess of OG-free buffer B for 20 h at 4 °C. Proteoliposome formation was confirmed and isolated by passing the dialyzed preparation through a 1.0 × 25 cm Sephadex G-75 gel filtration column. The peak fraction of the void volume contained the proteoliposomes. An alternative method for incorporation of the partially purified protein into artificial liposomes was by a dilution technique as outlined below.

Assay of O_2^- Generation. Generation of O_2^- was evaluated in a double-beam spectrophotometer (Shimadzu, UV 160A) by following the superoxide dismutase-sensitive reduction of cytochrome *c* at 550 nm as described previously (Ge & Guillory, 1994). Briefly, plasma membranes (50 μg) were incubated with cytosol (200 μg), 10 μM GTPγS, 1 mM $MgCl_2$, and 1200 nmol of arachidonate/mg of membrane protein (final concentrations in 1 mL) for 5 min at 25 °C prior to addition of SOD to the reference cuvette and 200 μM NADPH to both cuvettes to initiate O_2^- generation. NADPH oxidation when indicated was determined by the absorption changes at 340 nm.

Solubilized membranes were assayed by first mixing with phosphatidylcholine (Sigma, type-II S, 20%) at a final concentration of 200 μg/mL together with 1 μM FAD for 2 min at 25 °C in a volume of 100 μL. The mixture was diluted 7.5-fold with buffer A (in some cases, cytosol, GTPγS, and 100 μM SDS were added following the dilution) and incubated for 8 min at 25 °C. The oxidase was then activated with 100 μM SDS followed by addition of 250 μL of reaction assay buffer (final concentrations in 1 mL: 100 μM cytochrome *c*, 1 mM $MgCl_2$, 1 mM EGTA, 2 mM NaN_3, 10 mM potassium phosphate, pH 7.4). The mixture was divided equally into two cuvettes, and 150 units (10 μL) of superoxide dismutase was added to the reference cuvette. Generation of superoxide was initiated by addition of 200 μM NADPH to both experimental and reference cuvettes.

For the assay of proteoliposomes formed by dialysis (e.g., cytochrome b_{558}−liposome), unless otherwise indicated, 20

Table 1: Cofactor Dependency of the INT Reductase of the Bovine Neutrophil Plasma Membrane[a]

omitted from assay mixture	INT reduction [nmol min^{-1} (mg of membrane protein)$^{-1}$]
none	422.5
cytosol	5.5
membrane	1.8
arachidonate	16.3
GTPγS/Mg^{2+}	50.0
NADPH	10.4

[a] The NADPH-dependent INT diaphorase activity in the plasma membrane−cytosol-reconstituted cell-free system was evaluated as described under Experimental Procedures. Plasma membrane (25 μg), cytosol (100 μg), GTPγS (1 μM), Mg^{2+} (1 mM), and arachidonate (30 nmol) were incubated for 5min at 25 °C in a volume of 100 μL. The reaction mixture was then transferred to buffer A supplemented with 40 μM INT and 150 units of SOD in a total volume of 500 μL. INT reduction was initiated with 200 μM NADPH. Various components were omitted from the reaction mixture to evaluate their dependency for INT reduction. Results represent the average of two experiments using different preparations.

μg of the proteoliposomes, 200 μg of cytosol, 1 μM FAD, and 100 μM SDS were incubated for 5 min in a volume of 750 μL followed by addition of 250 μL of the reaction assay mixture. The O_2^- generating activity was assayed as described above.

Assay of INT Reductase Activity. NADPH-dependent INT reduction by either plasma membranes or solubilized membranes was assayed by monitoring the absorption change at 500 nm using the procedures outlined above for the assay of O_2^- generation except that no reaction assay buffer (which contains cytochrome *c*) was added. The total volume was adjusted to 500 μL with buffer A, and buffer A was used as a blank in the reference cuvette. INT (50 μM) was added to the experimental cuvette just prior to NADPH.

When indicated, anaerobic conditions were achieved by using N_2-purged buffer and an internal oxygen-depleting system consisting of 150 mM galactose and 6.75 units of galactose oxidase. Mineral oil (200 μL) was layed on top of the reaction mixture to exclude air, and NADPH was injected through this oil layer using a microsyringe. The mixture was mixed briefly by an internal small stirring bar and then transferred to the spectrophotometer. Unless otherwise specified, all INT reductions were measured in the presence of 150 units of SOD and were evaluated using the extinction coefficient of 11 cm^{-1} mM^{-1} (Pearse, 1960).

RESULTS

INT Diaphorase Activity in the Bovine Leukocyte Cell-Free System and Its Inhibition by Phenylarsine Oxide. Both cytosolic and membrane proteins are absolute requirements for activation of the NADPH oxidase in the plasma membrane and cytosol-reconstituted cell-free system. In addition, amphiphiles such as arachidonate (AA) or sodium dodecyl sulfate (SDS) are also required to activate the NADPH oxidase by a still undefined mechanism. As shown in Table 1, an NADPH−INT diaphorase activity (reduction of INT by NADPH) is present in this crude plasma membrane system. The INT diaphorase activity is NADPH, arachidonate, membrane, and cytosol dependent, which indicates a close association with that mechanism initiating the activation of the NADPH oxidase.

We have previously demonstrated (Li & Guillory, 1997) that phenylarsine oxide (PAO) inhibits the activation of the

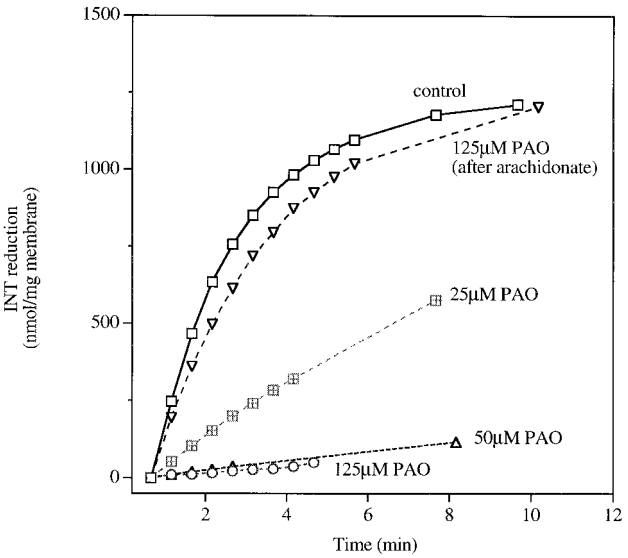

FIGURE 1: Effect of PAO on the INT diaphorase activity of the leukocyte plasma membrane. Plasma membranes (25 μg) and cytosol (100 μg) were incubated in the presence of GTPγS (1 μM), Mg^{2+} (1 mM), and 50 nmol of arachidonate for 5 min in a volume of 100 μl. Following the 5 min activation, the mixture was transferred to 400 μl of buffer A containing 50 μM INT and 150 units of SOD. The NADPH-dependent INT reduction was initiated by addition of 200 μM NADPH (control). Varying amounts of PAO were added 2 min prior to or after arachidonate activation as indicated. Each point on the curve represents the activity recorded from the data print out at that time following initiation of O_2^- generation by addition of NADPH.

NADPH oxidase in the bovine neutrophil system in confirmation of the inhibition observed by Le Cabec and Maridonneau-Parini (1995) and Kutsumi et al. (1995) with intact human leukocytes. As seen in Figure 1, the NADPH−INT diaphorase is inhibited by PAO in a dose-dependent manner with characteristics identical to the effect of PAO on O_2^- generation (Li & Guillory, 1997). The NADPH−INT diaphorase is inhibited only when PAO is added prior to the assembling/activation of the oxidase components initiated by arachidonate (Figure 1). The IC_{50}s of PAO for O_2^- generation and for NADPH−INT reduction were identical (see below and Figure 7a).

The dependency of the NADPH−INT diaphorase on amphiphiles, cytosolic factors, and NADPH (or NADH) (Table 1) and the inhibition of INT reduction by PAO in the crude membrane system (Figure 1) indicate the diaphorase activity is an intrinsic characteristic of the NADPH oxidase.

NADPH−INT Diaphorase Activity in the Detergent-Solubilized Oxidase System. Generation of O_2^- by solubilized leukocyte membranes is similar in its characteristics to that observed for the nonsolubilized plasma membranes except for the fact that there is a required relipidation and reflavination and that there is a loss of the absolute dependence on cytosolic factors for O_2^- generation (Koshkin & Pick, 1993; Li & Guillory, 1997). The cytosol-independent O_2^- generation by solubilized membranes represents only 10−20% of that seen in the plasma membrane system; the detergent-solubilized membrane, however, has the advantage of being able to be further resolved by DEAE-Sepharose chromatography, resulting in a further purification of cytochrome b_{558}. On the basis of the similarities for activation of O_2^- generation and for NADPH−

Table 2: Comparison of INT Reduction and O_2^- Formation Catalyzed by Cytochrome b_{558} and Cytochrome *c* Reductase Preparations[a]

enzymatic preparation	additional components[b]	O_2^- generation	cytochrome *c* reduction[c]	INT reduction[b]
(A) cytochrome b_{558} preparation	PC, FAD, SDS	47.56	5.4[c]	12.1
	PC, FAD, SDS, cytosol	214	14.3[c]	132
	PC, FAD, SDS, cytosol, GTPγS	815	ND[d]	429
(B) cytochrome *c* reductase preparation	none	0	44.8	27
	PC, FAD, SDS	negligible	32.9	27
	PC, FAD	0	38.1	ND
	FAD	0	31.5	28.4
	SDS	0	37.1	23.6

[a] Solubilized leukocyte plasma membrane proteins were fractionated by DEAE-Sepharose chromatography as described under Experimental Procedures. Enzymatic assays were carried out using proteoliposome-incorporated proteins formed by the dilution technique. Data represent one of at least three experiments using different preparations. Activity is presented as nmol min^{-1} (mg of membrane protein)$^{-1}$. [b] The assay mixture contained (A) the cytochrome b_{558} or (B) the cytochrome *c* reductase preparation with other components at their final concentration as indicated: phosphatidylcholine (PC, 100 μg/mL), FAD (1 μM), SDS (100 μM), GTPγS (1 μM), or 20-fold excess of cytosolic proteins. [c] In the presence of 150 units of superoxide dismutase. [d] ND, not determined.

INT diaphorase activity in the plasma membrane, we were interested in determining whether the NADPH−INT reductase activity of the plasma membrane was directly associated with cytochrome b_{558}, the known membrane component of the leukocyte NADPH oxidase, or was a reaction associated with another enzyme present in the leukocyte plasma membranes. Following DEAE-Sepharose chromatography, the unbound protein fraction which contains cytochrome b_{558} is able to produce O_2^- independent of cytosolic activators, although at about 10% of the rate of the plasma membranes (Table 2). With this preparation, only minor NADPH−INT reductase activity was seen (12.1 nmol min^{-1} mg^{-1}). However, cytosolic factors greatly stimulate both superoxide production and INT reduction by the cytochrome b_{558} preparation (Table 2).

The orientation of membrane proteins within the phospholipid bilayer is known to be crucial for their enzymatic activity as well as for their interaction with other nonmembrane proteins. Purified cytochrome b_{558} is able to generate O_2^- only when it is reflavinated and relipidated (Koshkin & Pick, 1993; Li & Guillory, 1997), and it was reasoned that the measurement of the enzymatic activities of purified cytochrome b_{558} might well be compromised depending upon its orientation into the artificial liposomal membranes formed during relipidation. In order to evaluate this possibility, we examined the incorporation of cytochrome b_{558} into proteoliposomes by a sonication/dialysis procedure rather than by direct dilution as carried out in the above-described experiments (i.e., Table 2). The cytochrome b_{558} was mixed with a 5−10-fold (mass ratio) excess of phospholipid and subjected to mild sonication followed by 3 h incubation at 0−4 °C. Proteoliposomes were then formed by dialyzing the suspension against detergent-free buffer B (a 200 excess volume for 20 h at 4 °C). The proteoliposomes (defined as cytochrome b_{558}−liposomes) were collected by passing the dialyzed preparation over a Sephadex G-75 gel filtration column (1.2 × 25 cm).

The cytochrome b_{558} incorporated into liposomes utilizing the dialysis procedure does not result in the establishment of a significant level of NADPH−INT diaphorase activity; however, in the presence of cytosol, there was a major stimulation of the NADPH−INT reductase activity as well as O_2^- generation (Figure 2). As with the plasma membranes, GTPγS stimulates these two activities to a similar extent (Table 3). As can be observed from Table 3, the enzymatic activity of the DEAE-cytochrome b_{558} preparation incorporated into liposomes by dialysis was 819 nmol min^{-1} (mg of membrane protein)$^{-1}$ when GTPγS was absent from the assay mixture and 1300 nmol min^{-1} (mg of membrane protein)$^{-1}$ when GTPγS was present. The extend of GTPγS

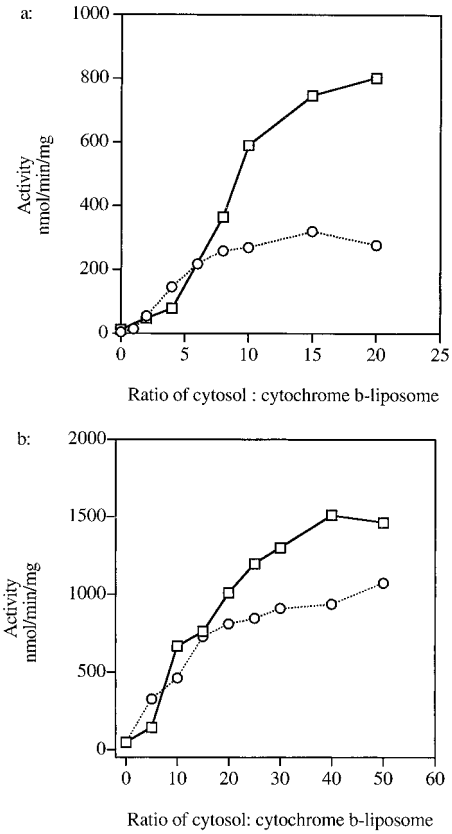

FIGURE 2: Cytosol stimulation of INT reductase activity and O_2^- generation by cytochrome b_{558}−liposomes in the presence and absence of GTPγS. Plasma membranes were solubilized with OG and treated with DEAE-Sepharose. The DEAE-nonabsorbed cytochrome b_{558} was isolated and incorporated into liposomes by dialysis as described under Experimental Procedures. For the INT reductase assay, proteoliposomes (10 μg of proteins) were incubated with 1 μM FAD, increasing amounts of cytosol, and 100 μM SDS for 5 min in a volume of 500 μL and then assayed for INT reductase as described under Experimental Procedures. For superoxide formation, 20 μg of proteoliposomes was activated with 100 μM SDS in the presence of 1 μM FAD and varying amounts of cytosol for 5 min in a volume of 750 μL followed by addition of 250 μL assay buffer (final concentrations: 100 μM cytochrome *c*, 1 mM $MgCl_2$, 1 mM EGTA, 2 mM NaN_3, 10 mM potassium phosphate, pH 7.4). Superoxide was measured as SOD-sensitive cytochrome *c* reduction as detailed under Experimental Procedures. Activities were evaluated in the absence (a) or presence (b) of 1 μM GTPγS. INT reduction (○···○); O_2^- generation (□—□).

Table 3: Effect of GTPγS on O_2^- Generation and INT Reductase Activity of the Particulate and OG-Solubilized Membrane[a]

	O_2^- generation	INT reduction
(a) Plasma Membrane and Cytosol-Reconstituted System		
+GTPγS	314	291
−GTPγS	57	36
(b) Cytochrome b_{558}−Liposome		
+GTPγS	1300	909
−GTPγS	819	609
(c) Cytochrome b_{558}, Relipidated by Dilution		
+GTPγS	832	496
−GTPγS	214	132

[a] Plasma membranes and DEAE-treated, OG-solubilized membranes from nonactivated neutrophils were evaluated for superoxide-generating NADPH oxidase activity in the presence of saturated cytosolic levels as detailed under Experimental Procedures. When added, GTPγS in the assay mixture was 1 μM. The cytochrome b_{558} preparations used were (a) plasma membranes, (b) DEAE-treated detergent-solubilized cytochrome b_{558} incorporated into phosphatidylcholine liposomes by dialysis, and (c) DEAE-treated detergent-solubilized cytochrome b_{558} combined with phosphatidylcholine followed by a 7.5-fold dilution with buffer A. The enzymatic rates are given as nmol min^{-1} (mg of membrane protein)$^{-1}$.

stimulation on O_2^- generation relative to INT reduction was the same at high and reduced levels of cytosol. In the absence of GTPγS (Figure 2a), a similar relationship held at low cytosol titers; however, at cytosol vs cytochrome *b* ratios above 5, the O_2^--generating potential continually increased while the INT reductase activity plateaued. It is most informative that the NADPH−INT reductase activity of these preparations shows a common dependence with respect to a required lipid bilayer incorporation (Table 3).

It is interesting that incorporation of cytochrome b_{558} into liposomes by the dilution or the dialysis method results in an oxidase preparation with different maximal turnovers as illustrated in Figure 3. Cytochrome b_{558} incorporated into liposomes by either dilution or dialysis was activated with cytosol, FAD, GTPγS, and SDS for a specified time after which an aliquot was assayed for O_2^- generation. In this experiment, the mass ratio of protein to phospholipid was maintained at 1:10 for the two methods. The difference in the superoxide capacity of these two preparations may indicate a structural heterogeneity of cytochrome b_{558} with respect to its topological arrangement within the two bilipid membranes.

Since exogenous FAD is required for superoxide generation by relipidated cytochrome b_{558}, we titrated FAD for its requirement in NADPH-dependent INT reduction as well as for its requirement for O_2^- generation by the cytochrome b_{558}−liposomes. The two titration profiles are superimposable (Figure 4), supporting the concept that cytochrome b_{558} catalyzes an INT diaphorase activity as well as its recognized oxidase activity. In addition, our data are confirmative in showing that cytochrome b_{558} is a flavoprotein and that both of the heme protein's activities of O_2^- generation and INT reduction require FAD. Flavin mononucleotide (FMN) would not substitute for FAD for either reaction and did not potentiate the enzymatic activity in the presence of FAD. Further correlation of the identical factor requirements for these two enzymatic activities of the cytochrome b_{558}−liposomes is demonstrated in Figure 5.

The similarity in the magnitude of the K_m (NADPH and NADH) and turnover numbers for O_2^- generation and INT reduction in two different preparations at distinctly different purity levels, i.e., the plasma membrane preparation and the DEAE-cytochrome b_{558}-proteoliposome preparation, is clearly illustrated in Table 4. The above-illustrated similarity in cofactor and activator concentration dependency and in the kinetic constants for the two activities in the two widely different preparations supports the concept that the two enzymatic activities of O_2^- generation and INT reduction are associated with the same enzyme, i.e., the flavocytochrome b_{558}.

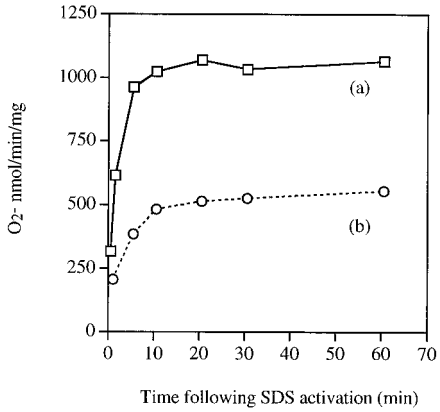

FIGURE 3: Time course of SDS activation of the cytochrome b_{558}−liposomes prepared by (a) dialysis or (b) dilution. (a) The DEAE-treated OG-solubilized cytochrome b_{558} was relipidated with a 10-fold excess of phosphatidylcholine (mass ratio) by mild sonication, dialysis against OG-free buffer B, and gel-filtration as described under Experimental Procedures. The formed cytochrome b_{558}−liposomes (120 μg) were activated with 3 mg of cytosol, 1.3 μM FAD, 1.3 μM GTPγS, and 130 μM SDS in a volume of 4.5 mL at 25 °C. At the indicated time following SDS addition, aliquots of 750 μL of the mixture were withdrawn and added to 250 μL of assay buffer (final concentrations: 100 μM cytochrome *c*, 1 mM $MgCl_2$, 1 mM EGTA, 2 mM NaN_3, 10 mM potassium phosphate, pH 7.4). Superoxide production was initiated by 200 μM NADPH and evaluated as SOD-sensitive cytochrome *c* reduction (□—□). (b) The DEAE-treated solubilized membranes (120 μg) were relipidated by incubating with phosphatidylcholine (1.2 mg) and 23.7 μM FAD in a volume of 253 μL. After 2 min, the mix was diluted with buffer A to 4033 μL and set on ice for 1 h followed by warming to 25 °C and activation with 3 mg of cytosol, 1.3 μM GTPγS, and 130 μM SDS in 4.5 mL. The final concentration of FAD was the same in cases a and b. At specified times, 750 μL aliquots were assayed for superoxide production as in case a above (○- - -○).

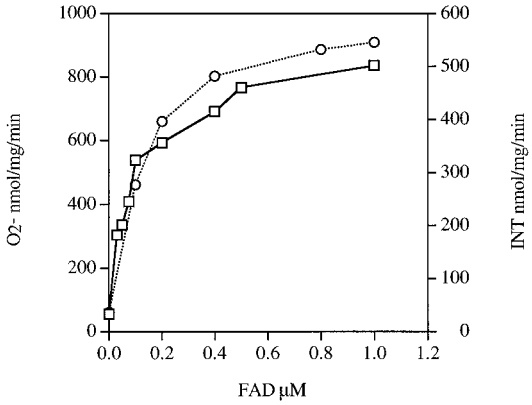

FIGURE 4: FAD dependency for INT reductase activity and O_2^- production by cytochrome b_{558}−liposomes. Cytochrome b_{558}−liposomes (10 μg of proteins) prepared by dialysis were activated with a 10-fold excess of cytosolic proteins (100 μg), varying amounts of FAD, and 100 μM SDS at 25 °C for 5 min as described under Experimental Procedures and assayed independently for INT reduction (○···○) and superoxide generation (□—□).

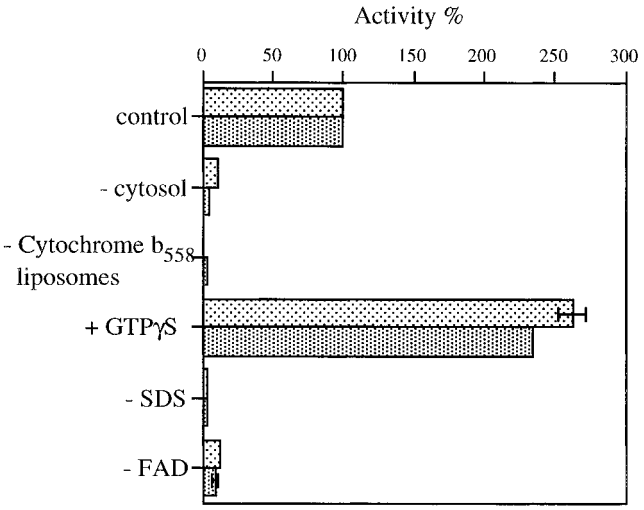

FIGURE 5: NADPH oxidase and the associated INT reductase activity of the cytochrome b_{558}−liposome preparation. Influence of cofactors. Cytochrome b_{558} from the DEAE-Sepharose preparation was incorporated into phosphatidylcholine liposomes by dialysis. The complete assay mixture contained 20 μg of cytochrome b_{558}−liposome proteins, 200 μg of cytosolic proteins, 1 μM FAD, and 100 μM SDS. After a 5 min activation period, superoxide production or INT reduction was analyzed. Components were added to or omitted from the assay mixture as indicated. The data represent the average of at least two experiments using different cytochrome b_{558} preparations. Control activities were as follows: INT reduction, 324 ± 58 nmol min^{-1} mg of liposomes)$^{-1}$ O_2^- generation, 796 ± 58 nmol min^{-1} (mg of liposomes).$^{-1}$ INT reduction (dark stippled bars); O_2^- generation (light stippled bars).

Table 4: Summary of the K_m and Turnover Numbers for O_2^- Generation, NADPH Oxidation, and INT Reductase Activity of the Plasma Membrane and Cytochrome b_{558}−Liposome Preparations[a]

preparation	activity analyzed	substrate	K_m (μM)	turnover [mol (mol of cyt b^{-1} s^{-1}]
plasma membrane	O_2^- generation	NADPH	38	61.8
	NADPH oxidation	NADPH	35.3	29.7
	INT reduction	NADPH	25.3	17.4
cytochrome b_{558}-liposome	O_2^- generation	NADPH	27.2	47.2
		NADH	810	30.7
	INT reduction	NADPH	27.5	35.3
		NADH	1017	24.2

[a] Plasma membranes and DEAE-treated, OG-solubilized cytochrome b_{558} from nonactivated neutrophils were evaluated for superoxide-generating NADPH oxidase activity as detailed under Experimental Procedures. The solubilized cytochrome b_{558} was incorporated into proteoliposomes by the dialysis method as described under Experimental Procedures.

Presence of a Distinctive Leukocyte Plasma Membrane INT Diaphorase Not Associated with Cytochrome b_{558}. In addition to the non-DEAE-Sepharose-bound cytochrome b_{558}-associated INT diaphorase, we investigated the possible existence of other diaphorases in the leukocyte plasma membrane. Our attention was directed to a particular oxidoreductase enzyme, an NADPH−cytochrome *c* reductase reported to be present in neutrophils (Nisimoto et al., 1994; Laporte et al., 1991).

Following OG solubilization, the DEAE-bound proteins were eluted with NaCl, and both O_2^- generation and NADPH−INT reduction capacities were assayed for in the effluents. Neither O_2^- generation nor cytochrome b_{558} (as judged by spectral characteristics) were found in such effluents. An NADPH−INT reductase activity was, however, detected, which was not SDS, FAD, phosphatidylcholine, or cytosol dependent and of relatively low activity [20−35 nmol min^{-1} (mg of membrane protein)$^{-1}$; Table 2]. The NADPH−INT diaphorase activity was eluted with 250 mM NaCl and coeluted with an NADPH−cytochrome *c* reductase activity (Figure 6). Its low activity, amphiphile and cytosol independence, the lack of an FAD or lipid requirement, and its association with a cytochrome *c* reductase distinguish this NADPH−INT reductase activity from that observed in the plasma membrane where both SDS (or arachidonate) and cytosol are required. In support of this is the fact that the INT reductase eluted from DEAE-Sepharose is not inhibited by PAO (Li and Guillory, unpublished data).

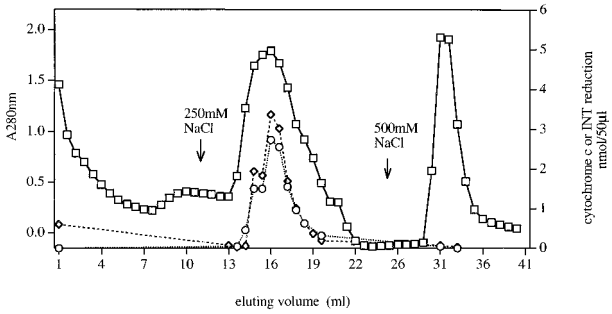

FIGURE 6: Coelution of NADPH-dependent cytochrome *c* reductase and INT diaphorase from the DEAE-Sepharose column. Octyl glucoside-solubilized plasma membranes were mixed with 0.1 volume of DEAE-Sepharose preequilibrated with buffer B for 2 h at 4 °C by gently shaking. Unbound proteins which contain cytochrome b_{558} were collected by applying the suspension to a 1.2 × 10 mL column, and the effluent was collected. After washing the column with buffer B, the bounds proteins were eluted stepwise with 250 mM and then 500 mM NaCl. Similar elution patterns were observed using a linear salt gradient. Cytochrome *c* reduction or INT reduction by effluents (50 μL) was assayed in the presence of 100 μM cytochrome *c* or 50 μM INT as detailed under Experimental Procedures. NADPH (200 μM) was added to start the reaction. INT reduction (○•••○); cytochrome *c* reduction (◇- - -◇); A_{280nm} (□−□).

It was clear that the minor INT diaphorase activity associated with the cytochrome *c* reductase is independent of O_2^- production and at a maximal represents but 5−10% of that found to be associated with cytochrome b_{558}.

NADPH−INT Reductase Activity under Anaerobic Conditions. Superoxide dismutase was found to have only a very minor effect on NADPH−INT reduction in the cell-free system, decreasing the reduction by less than 10%. It is clear that generated superoxide anion is not the reagent responsible for reduction of INT, confirming the conclusion of Cross et al. that O_2^- is not able to reduce INT (Cross et al., 1994).

Under anaerobic conditions, established by using galactose and galactose oxidase (Experimental Procedures), the rate of NADPH-dependent INT reduction was not significantly different from that observed in the presence of oxygen and is independent of the presence or absence of SOD (Figure 7a). Omission of cytochrome b_{558} abolished the NADPH-dependent INT reduction. Under anaerobic conditions, the steady-state oxidation of NADPH was reduced by 93% to 40 nmol min^{-1} mg^{-1} compared to its initial oxidation rate of 530 nmol min^{-1} mg^{-1} (Figure 7). Anaerobiosis under the conditions described (liposomal preparation in the presence of maximal cytosol and activators) has no significant influence on the rate of the NADPH-dependent INT reduction, independent as to whether the cytochrome b_{558} was incorporated into liposomes comprised of phosphati-

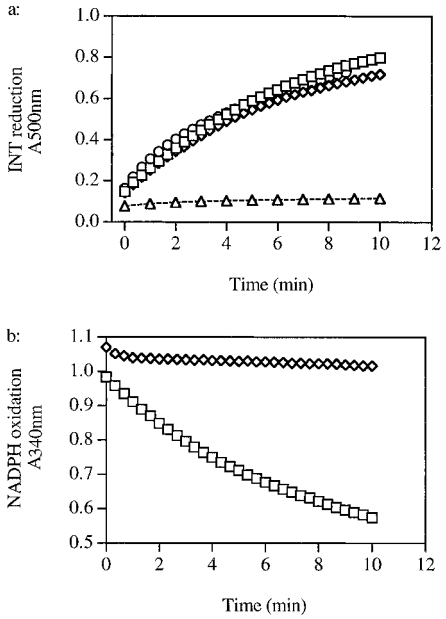

FIGURE 7: INT reductase activity under aerobic and anaerobic conditions. Proteoliposomes (20 μg) were incubated with a 10-fold excess of cytosolic proteins, 1 μM FAD, and 100 μM SDS in a volume of 1.0 mL for 5 min prior to addition of 50 μM INT. Anaerobic conditions were achieved by purging with N_2 and using an internal oxygen scavenging system (150 mM galactose and 6.75 units of galactose oxidase). The reaction mixture was overlaid with 200 μL of mineral oil through which NADPH was injected and mixed by a small magnetic stirring bar. INT reduction was 484 nmol/min/mg of proteoliposomes)$^{-1}$ aerobically and 547 nmol min^{-1} (mg of proteoliposomes)$^{-1}$ anaerobically. NADPH oxidation in the absence of INT was aerobically 523 nmol min^{-1} mg of proteoliposomes)$^{-1}$ and 27 nmol min^{-1} (mg of proteoliposomes)$^{-1}$ anaerobically. (a) Effect of anaerobiosis and SOD on INT reduction: aerobic (□—□); anaerobic (◇- - -◇); anaerobic, −SOD (○···○); anaerobic, no proteoliposomes (△- - -△). (b) NADPH oxidation under aerobic and anaerobic conditions in the absence of INT.

dylcholine or phosphatidylcholine plus phosphatidic acid (data not shown).

In addition, when enzymatic activities are only partially expressed by utilizing nonsaturating levels of cytosol or lower FAD concentrations, no significant change in the rate of INT reduction was observed under anaerobic conditions (data not shown).

Inhibitory Effect of Phenylarsine Oxide on INT Reduction, NADPH Oxidation, and O_2^- Generation. The PAO inhibition of O_2^- generation, INT reduction, and NADPH oxidation in the plasma membrane system are compared in Figure 8a. In each case, the inhibition profile follows a similar pattern with 50% of each activity being lost at a PAO concentration range of 25−30 μM. The similar inhibitory profiles indicate that the site of inhibition may be common for each of the reactions. The increased sensitivity of the purified cytochrome b_{558}−liposome preparation with 50% inhibition of both O_2^- generation and INT reduction at 15 μM PAO (Figure 8b) is consistent with the proposal that the PAO inhibition of O_2^- generation has its origin in the direct interaction of PAO with cytochrome b_{558} (Li & Guillory, 1997). The identical inhibitory profile for both O_2^- generation and INT reduction is again supportive evidence for the common origin of these two activities. PAO inhibition of the INT reduction by cytochrome b_{558}−liposome preparations was dependent upon the degree of activation of the system

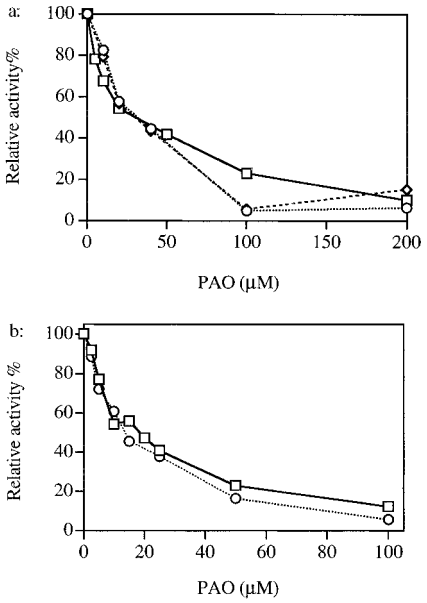

FIGURE 8: Effect of PAO on NADPH oxidation, INT reduction, and O_2^- formation in the plasma membrane and cyt b_{558}−liposome systems. Differing amounts of PAO were preincubated with plasma membranes (50 μg) or with the cytochrome b_{558}−liposome (20 μg, prepared by dialysis) for 2 min. The NADPH oxidation, INT reduction, and O_2^- formation catalyzed by the membrane or proteoliposomes were then evaluated in the presence of cytosol and SDS as detailed under Experimental Procedures. (a) Plasma membranes; (b) cytochrome b_{558}−liposomes. Control activities were for the plasma membrane: INT reduction 303 nmol min^{-1} mg^{-1}; O_2^- generation (in the absence of INT), 343 nmol min^{-1} mg^{-1}; NADPH oxidation (in the absence of INT), 598 nmol min^{-1} mg^{-1}. For the cytochrome b_{558}−liposome preparations: control INT reduction was at 636.4 nmol min^{-1} mg^{-1} and O_2^- generation at 849.3 nmol min^{-1} mg^{-1}. O_2^- generation (□—□); INT reduction (○···○); NADPH oxidation (◇- - -◇).

as was observed with plasma membranes. When added following SDS activation, PAO at 50 μM resulted in but 13% inhibition; if added prior to SDS, the inhibition was at 91%.

DISCUSSION

The NADPH-dependent INT reductase activity observed in the plasma membrane fraction of bovine neutrophils can be separated into two distinct activities by subjecting detergent-solubilized (OG) plasma membranes to fractionation by DEAE-Sepharose anion exchange chromatography. A major portion of the diaphorase activity (95%) is closely associated and fractionates with the flavocytochrome b_{558} which is a component of the leukocyte O_2^--generating system. The other portion of the diaphorase activity (5%) distributes itself in close association with an NADPH-dependent cytochrome *c* reductase. Both the NADPH-dependent cytochrome *c* reductase activity and the minor diaphorase activity are retained by DEAE-Sepharose and coeluted together using a NaCl salt gradient or batchwise elution.

Evidence that the membrane component responsible for O_2^- generation is as well the entity responsible for the major NADPH−INT diaphorase activity rests upon a number of experimental findings: (1) the INT reduction and the O_2^--generating activity of the partially purified cytochrome b_{558} incorporated into a liposome bilayer have very similar dependencies on cytosolic protein activation (Figure 2),

similar dependency for activating amphiphiles (Figure 5), and an identical requirement for FAD (Figure 4); (2) for the leukocyte cytochrome b_{558} preparation, the K_m values evaluated for FAD are 100 nM for INT reductase and 75 nM for the NADPH oxidase activity; (3) the NADPH−INT reductase and the O_2^--generating oxidase have similar K_ms for NADPH in the liposome preparation (Table 4); (4) NADH is able to function as substrate for both activities but at proportionately higher K_m values (Table 4); (5) PAO inhibits both activities with a similar inhibitory profile in both the plasma membrane and cytochrome b_{558}−liposome preparations, but only if added prior to the activating amphiphile (Figure 8 and Figure 1). The above characteristics support the concept that cytochrome b_{558} can catalyze an NADPH-dependent diaphorase activity in addition to an NADPH-dependent reduction of oxygen to superoxide.

Other evidence for the concept that cytochrome b_{558} can function as a NADPH diaphorase comes from a recent study by Cross et al. (1995) in which membrane-associated cytochrome b_{558} of a ($X91^+$) CGD patient was found to be fully functional in INT reduction but incapable of producing O_2^-. This partially functional cytochrome b appears to be modified by a single amino acid mutation ($Arg^{54} \rightarrow Ser$), indicating that Arg^{54} might be important for the electron transfer between FAD and heme or alternatively for the interaction of heme and oxygen.

The dependency of the mode of incorporation of the partially purified cytochrome b_{558} preparation into the phospholipid bilayer is instructive with respect to the correlation of such membrane orientation on the ability of the protein to catalyze specific reactions. Plasma membrane proteins solubilized by detergent have been relipidated by two defined procedures; rapidly by a dilution protocol or slowly by detergent removal by means of dialysis. Proteoliposomes prepared by dialysis exhibit a greater than 10-fold stimulation of INT reduction and O_2^- generation dependent upon cytosolic factors with a turnover for INT reduction of 35.3 mol (mol of cytochrome $b_{558})^{-1}$ s^{-1} and O_2^- formation of 47.2 mol (mol of cyt b)$^{-1}$ s^{-1}, respectively (Table 4). The difference in superoxide production by relipidated cytochrome b_{558} preparations indicates possible different orientations for cytochrome b_{558} in the lipid bilayer dependent upon the incorporation method (Figure 3).

That the NADPH−INT reductase activity associated with cytochrome b_{558} can be observed in the presence of cytosol suggests that cytosolic factors may regulate electron flow from NADPH to FAD and to INT independent of the overall pathway to oxygen. The higher cytosol requirement for O_2^- formation when compared with the requirement for INT reduction (Figure 2) may indicate a multiple cytosolic factor requirement for the partial reactions between NADPH and the oxygen electron sink. Cross and Curnutte (1995) have proposed that the cytosolic factor $p67^{phox}$ may be required for electron flow from NADPH to FAD while $p47^{phox}$ is needed for electron flow from FAD to the heme. If this is indeed the case, both cytosolic factors would be required for O_2^- generation while one ($p67^{phox}$) would be necessary for INT reduction. This possibility is consistent with our findings of a differential influence of cytosol concentration on the two activities.

In the process of plasma membrane solubilization by OG and subsequent purification of the cytochrome b_{558} by DEAE-Sepharose ion exchange chromatography, the O_2^- generation becomes absolutely dependent upon addition of exogenous FAD. This obvious dissociation of FAD from the flavocytochrome b_{558} and the consequent FAD requirement for O_2^- generation are shared by an FAD requirement for the NADPH−INT reductase. That O_2^- generation and NADPH−INT reductase have an identical requirement for FAD reinforces the suggestion of a common origin for the two activities.

The phagocytic cell-free system demonstrating superoxide generation driven by NADPH has been developed and refined for a number of specific cell types: human blood leukocytes (Mcphail et al., 1985; Curnutte, 1985), guinea pig peritoneal-derived leukocytes (Ge & Guillory, 1994; Ohtsuka et al., 1990), guinea pig macrophages (Bromberg & Pick, 1984), and bovine and pig blood leukocytes (Heyneman & Vercauteren, 1984; Ligeti et al., 1988) to mention a few. In each case, the general characteristics of the systems appear to be same. There are, however, a number of subtle differences in the mechanism of superoxide formation expressed by these systems principally with respect to system stability, overall rates of O_2^- generation, and the characteristics of arachidonate (and SDS) activation. That such represent fundamental differences in the mechanism of O_2^- generation is unlikely. With respect to the arachidonate and SDS activation, there is yet to be developed an experimental understanding of the interacting role of arachidonate (or SDS) and the cytosolic factors in O_2^- generation.

The lack of an effect on the rate of NADPH−INT reductase activity for the oxygen-depleted system observed with the purified cytochrome b_{558} incorporated into liposomes is in contrast to the competition for electrons observed by Cross et al. (1994) in the human neutrophil plasma membranes. In our hands, with a maximally activated system the rate of NADPH−INT reductase of the liposome preparations did not change more than 10% under anaerobic conditions relative to the rate observed in the presence of oxygen. Limiting the overall rates by lowing the protein: cytosol ratio or the FAD concentration did not change the relative aerobic−anaerobic INT reduction.

On the basis of the strong correlation of the inhibitory profiles and activity effects of the agents described above on INT reduction and O_2^- generation, it is clear that the two reactions are linked and are both associated with cytochrome b_{558}. A possible explanation for the inability to influence the INT reduction of the cytochrome b_{558}−liposomes by anaerobiosis can have either a structural or a kinetic basis.

In the plasma membrane preparation, one has an organized cytochrome b_{558}−membrane protein aligned in a manner closely resembling the in vivo condition. The structural alignment assures that the electron transport responsible for NADPH-driven reduction of oxygen will be carried out in a manner such that the partial reactions resulting in O_2^- generation are integrated into an essentially coupled electron transport pathway. In such a system, the arrangements of the factors required for O_2^- generation are relatively well-defined and organized. By contrast, the liposome−cytochrome *b* preparation is formed by integrating a detergent-solubilized cytochrome *b* into a liposome bilayer mainly constituted of phosphatidylcholine. The fact that the two methods used for the incorporation of purified cytochrome b_{558} into liposomes (the dilution vs dialysis method) result in preparations with different maximal rates of O_2^- generation and INT reduction is indicative of the variability in the

proper alignment of the protein into the liposomal membranes. Thus, the liposomal preparation may be a metastable loosely coupled electron transport system with possible independence of the partial reactions for NADPH to O_2^- generation. It should be mentioned that the phospholipid titers and compositions for cytochrome b_{558} relipidation have yet to be thoroughly investigated with respect to the INT reductase.

Alternatively, the rate of INT reduction and O_2^- generation in the liposomal cytochrome b_{558} preparation may be determined by different rate-limiting steps, and dependent upon which step is rate-limiting (not necessarily the same step under different conditions), one might observe either an increase, decrease, or constant INT reduction rate in the presence and absence of oxygen. The fact that the heme of cytochrome b_{558} is only moderately reduced by NADPH under anaerobic conditions and that oxygen stimulates the rate and extent of heme reduction by a mechanism yet to be defined (Gabig et al., 1982; Light et al., 1981; Foroozan et al., 1992) clearly indicates the operation of kinetic control mechanisms of which we have little understanding. Nevertheless, the constant rate of INT reduction by cytochrome b_{558} in the absence and presence of oxygen does not necessarily indicate that INT reduction is occurring by a pathway different from that utilized for oxygen reduction, a possibility which has yet to be ruled out. Current experiments are aimed at the independent activation of the partial reactions which result in INT reduction and O_2^- formation. These experiments may eventually provide a rationale for the specifics of the electron transport reactions.

In summary, bovine neutrophils possess an NADPH-dependent INT reductase activity which originates from at least two distinct membrane enzyme entities: the major portion from cytochrome b_{558} and a minor level from cytochrome *c* reductase. Some 95% of the total plasma membrane INT reductase activity is associated with cytochrome b_{558}. Both superoxide production and INT reduction catalyzed by cytochrome b_{558} are stimulated and regulated by leukocyte cytosolic factors. A similar inhibitory pattern for the vicinal sulfhydryl reagent PAO is observed for both superoxide production and INT reduction in purified cytochrome b_{558} preparations, reinforcing the concept that both activities represent aspects of the enzymatic reaction catalyzed by cytochrome b_{558}. The mechanism by which the cytosolic factors regulate and interact with cytochrome b_{558} remains to be defined.

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