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Generation of superoxide by purified and relipidated cytochrome b_{559} in the absence of cytosolic activators

Vasilij Koshkin and Edgar Pick

Laboratory of Immunopharmacology, Department of Human Microbiology, Sackler School of Medicine, Tel-Aviv University, Tel-Aviv 69978, Israel

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Purified cytochrome b_{559} from guinea pig macrophages was relipidated with several phospholipid mixtures. Relipidated cytochrome b_{559} was found capable of NADPH-dependent superoxide (O_2^-) production in the absence of the cytosolic components of the NADPH oxidase complex. The rate of O_2^- generation by cytochrome b_{559} varied with the type of phospholipid utilized for relipidation, was absolutely dependent on exogenous FAD, and was enhanced by a critical concentration of anionic amphiphile. It is demonstrated that exogenous FAD acts by binding to cytochrome b_{559}. These results provide firm experimental evidence for the proposal that cytochrome b_{559} comprises the complete electron transporting apparatus of the O_2^- forming NADPH oxidase and that the cytosolic components function merely as activators.

Superoxide; Cytochrome b_{559}; NADPH oxidase; Phospholipid; Phosphatidic acid; FAD

1. INTRODUCTION

Phagocytes possess an enzyme complex catalyzing the one electron reduction of molecular oxygen to superoxide (O_2^-) in conjunction with oxidation of NADPH [1]. The O_2^- forming NADPH oxidase is dormant in resting cells and is activated by various stimulants interacting with membrane receptors. Analysis of the constituents of NADPH oxidase was facilitated by the development of a cell-free system in which O_2^- production is elicited in mixtures of plasma membranes and cytosol, derived from resting cells, by anionic amphiphiles, such as arachidonate [2] or SDS [3]. Four proteins were found to be required for O_2^- production in the cell-free system: cytochrome b_{559}, which was shown to be the only component contributed by the membrane [4]; two cytosolic proteins, p47-*phox* and p67-*phox* [5,6], and the small G protein *rac*1 p21 [7] or *rac*2 p21 [8], also localized in the cytosol. It is commonly accepted that cytochrome b_{559} is the terminal NADPH oxidase component, with its heme prosthetic group acting as the electron carrier to oxygen [10]. A dissident view, questioning the redox function of cytochrome b_{559} heme has also been expressed [11]. Theoretical considerations and experimental findings also indicated that an FAD-containing flavoprotein is a component of the oxidase and, most likely, bears the NADPH binding site [12–14]. The original suggestion that the NADPH-binding flavoprotein was represented by one of the cytosolic factors [15] was contradicted by experimental evidence [16]. On the other hand, the proposal that a membrane-bound flavoprotein is an obligatory component of the NADPH oxidase [17–21] is difficult to reconcile with the finding that the only membrane protein required for O_2^- production in the cell-free system is cytochrome b_{559} [4] and with the complete reconstitution of the NADPH oxidase complex from purified cytochrome b_{559} and purified or recombinant cytosolic components [22,23].

Recently, the idea was put forward that cytochrome b_{559} is a flavoprotein, the large subunit of which contains both the FAD and the NADPH-binding sites [22,24,25]. This hypothesis is based principally on the observation that alignment of the amino acid sequence of the large subunit of cytochrome b_{559} with flavoproteins of the ferredoxin-$NADP^+$ reductase family reveals similarities in the NADPH and FAD-binding domains. The experimental evidence in support of this proposal is still rather limited and rests on the following findings. (1) Testing cytochrome b_{559} for its ability to support cell-free O_2^- production demonstrated that dependence on exogenous FAD increased in parallel with the degree of cytochrome b_{559} purity [25]. (2) Deflavinated cytochrome could be partially reflavinated, when FAD was added during NADPH oxidase assembly [22]. (3) The large subunit of cytochrome b_{559} could be photoaffinity labelled with NADP [24]. In the present study we demonstrate that purified cytochrome b_{559} incorporated in a specific lipid environment is capable of NADPH and

Correspondence address: E. Pick, Department of Human Microbiology, Sackler School of Medicine, Tel-Aviv University, Tel-Aviv 69978, Israel. Fax: (972) (3) 642-9119.

Abbreviations: O_2^-, superoxide; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; SOD, superoxide dismutase; LDS, lithium dodecyl sulfate.

FAD-dependent O_2^- production in the absence of the three cytosolic activators.

2. MATERIALS AND METHODS

2.1 *Lipids and other chemicals*

The following lipids were obtained from Sigma (product numbers appear in brackets): L-α-phosphatidylcholine of the following types: II-S, from soybean, 14% (P 5638); IV-S, from soybean, 35% (P 3644); X-E, from egg yolk, 60% (P 5394), and L-α-phosphatidic acid, sodium salt, from egg yolk lecithin, 98% (P 9511). Common laboratory chemicals were purchased from Sigma or Merck. Reagents for SDS-PAGE were from Bio-Rad. Chromatography materials were obtained from Pharmacia LKB Biotechnology.

2.2. *Purification of cytochrome b_{559}*

Guinea pig peritoneal macrophages were obtained and the membrane fraction prepared as described before [4]. Cytochrome b_{559} was purified essentially as described by us in the past [4], with some modifications. Briefly, the membrane pellet was extracted with 1 M NaCl and solubilized, at 2–3 × 108 cell equivalents/ml, in buffer A (0.05 M sodium phosphate, pH 7.4, 1 mM $MgCl_2$, 1 mM EGTA, 2 mm NaN_3, 1 mM dithioerythritol, 1 mM PMSF, 20% glycerol) containing 40 mM octyl glucoside. The soluble membrane fraction was batch-absorbed with DEAE-Sepharose CL 6B and the material not bound by the anion exchanger was supplemented with 0.05 M NaCl and diluted to 30 mM octyl glucoside before being applied to a column of heparin-agarose. The column was washed with buffer A, containing 30 mM octyl glucoside and supplemented with 0.1 M NaCl, and cytochrome b_{559} eluted by a gradient of NaCl (0.1 to 0.7 M). As a final step in the purification sequence, we used gel filtration on a Superose 12 column (HR 10/30) in buffer A containing 30 mM octyl glucoside and 150 mM NaCl, as described before [26]. The concentration of cytochrome b_{559} was determined from the difference spectrum of sodium dithionite-reduced *minus* oxidized samples, using the extinction coefficient (427–411 nm) = 200 $mM^{-1} \cdot cm^{-1}$ [13]. The spectra were recorded and stored in the memory of a Uvikon 860 spectrophotometer (Kontron).

2.3. *Reconstitution of cytochrome b_{599} with phospholipids*

Phospholipids were dissolved in buffer A, containing 40 mM octyl glucoside at a concentration of 4 mg/ml. Occasionally, solubilization of lipids was assisted by sonication. Cytochrome b_{559} preparations, derived from gel filtration on Superose 12 were supplemented with the desired amounts of phospholipid and kept in ice for a few minutes. We have shown that, for use in cell-free O_2^- production, cytochrome b_{559}–lipid–detergent mixtures have to be diluted in or dialyzed against detergent-free buffer, to reduce the concentration of octyl glucoside to 8 mM or less [4,26]. In the experiments described in this report, the cytochrome b_{559}–phospholipid mixtures were diluted 6 to 8-fold, in O_2^- assay buffer (see below), lacking cytochrome *c*, and allowed to stand in ice for at least 30 min.

2.4. *O_2^- production*

This was assayed by the rate of SOD-inhibitable cytochrome *c* reduction, as described before [3]. The assay buffer consisted of 65 mM K,Na-phosphate buffer, pH 7.0, 1 mM $MgCl_2$, 1 mM EGTA, 2 mM NaN_3 and 0.1 mM ferricytochrome *c*. Additional ingredients, when added, are indicated in the text and legends to figures. Measurements were performed with a Uvikon 860 spectrophotometer (Kontron) using a 'time drive' program.

2.5. *Other assays*

Measurements of NADPH-dependent reduction of cytochrome b_{559} were performed under anaerobic conditions that were achieved by bubbling of nitrogen and an internal oxygen scavenging system (100 mM glucose and 2.66 units/ml glucose oxidase). The establishment of true anaerobic conditions was demonstrated by the virtual absence of NADPH oxidation in the presence of all the components of the cell-free O_2^- generating system [27]. SDS-PAGE was performed on a 11.5% acrylamide separating gel, according to Laemmli [28], followed by silver staining under basic conditions [29]. Protein concentrations were determined by the method of Bradford [30], bovine gamma globulin being used as standard.

3. RESULTS AND DISCUSSION

The purified preparations of cytochrome b_{559} utilized in the experiments described in this report had a heme content in the range of 7–12 nmol/mg protein. SDS-PAGE analysis, utilizing silver staining, of typical cytochrome b_{559} preparations demonstrated that these were free of contaminating proteins (Fig. 1).

Purified cytochrome b_{559} was relipidated with a variety of phospholipids, essentially as described before [4,26] but exogenous FAD (1 μM) was present during all stages of the relipidation procedure. As apparent from Fig. 2 (panel A), relipidated cytochrome generated O_2^- upon additon of NADPH, in the absence of cytosol. The rate of O_2^- production was determined by the nature of the phospholipid utilized for relipidation. Thus, maximal O_2^- production was obtained when cytochrome b_{559} was relipidated with a mixture of a crude preparation of soybean phospholipids (14% phosphatidylcholine) and 98% pure phosphatidic acid. Significant amounts were also produced by cytochrome b_{559} relipi-

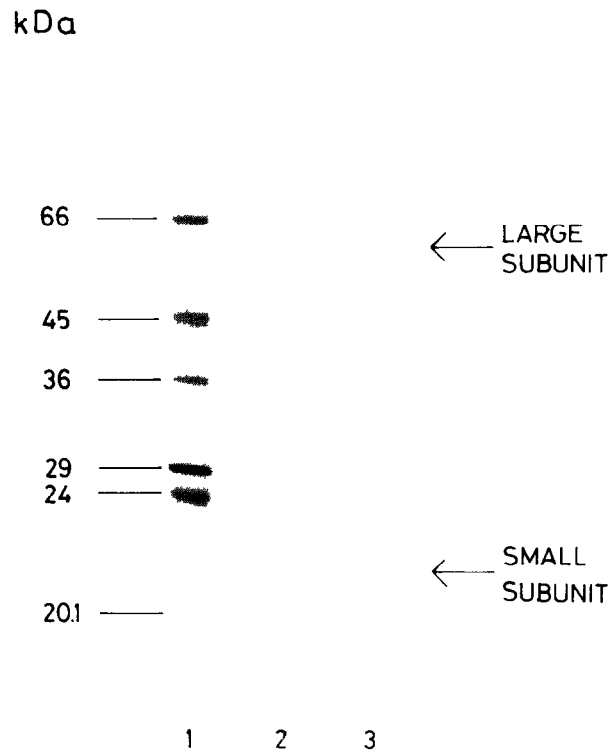

Fig. 1. SDS-PAGE of purified cytochrome b_{559} preparations. Lane 1 contains molecular weight standards. Lanes 2 and 3 contain distinct batches of purified cytochrome b_{559}. 8 pmol of each batch were applied per lane. Arrows indicate the position of the two subunits of the cytochrome b_{559} heterodimer.

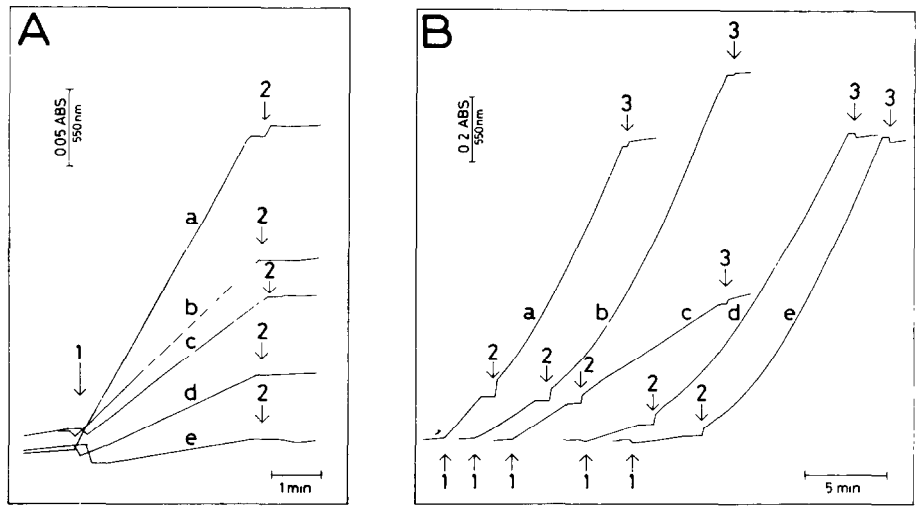

Fig. 2. O_2^- production by cytochrome b_{559} in the absence of cytosolic activators. Purified cytochrome b_{559} (8.1 nmol/mg protein) was concentrated by ultrafiltration to 808 pmol/ml and reconstituted, as described in section 2, with the following phospholipids: phosphatidylcholine, type II-S and phosphatidic and (100 μg/ml of each, tracings a); phosphatidylcholine, type II-S (200 μg/ml, tracings b); phosphatidic acid (200 μg/ml, tracings c); phosphatidylcholine, type IV-S (200 μg/ml, tracings d); and phosphatidylcholine, type X-E (200 μg/ml, tracings e). The cytochrome–lipid mixtures were supplemented with 1 μM FAD, diluted 7.4-fold in assay buffer lacking cytochrome *c* and supplemented with 1 μM FAD and 30 μM LDS, and allowed to stand for 30 min in ice. (A) At the time indicated by arrow marked 1, 2.2 pmol (in 20 μl) of the various relipidated and diluted cytochrome b_{559} preparations were added to cuvettes containing 1.1 ml complete assay buffer containing 1 μM FAD, 30 μM LDS and 400 μM NADPH. At times indicated by arrows marked 2, 100 units/ml SOD were added. (B) Identical preparations of relipidated cytochrome b_{559} were used. Arrows marked 1 indicate the addition of the cytochrome b_{559} preparations. At times indicated by arrows marked 2, 25 μl of macrophage cytosol (3.7×10^6 cell equivalants) were added and the concentration of LDS was raised to 100 μM. At times indicated by arrows marked 3, 100 units/ml SOD were added.

dated with each of the above lipids, by itself. Lower amounts of O_2^- were generated by cytochrome b_{559} relipidated with a soybean phospholipid preparation containing 35% phosphatidylcholine or an egg yolk phospholipid preparation containing 60% phosphatidylcholine. The identification of the reaction product as O_2^- is proven by the complete inhibition of cytochrome *c* reduction by SOD (Fig. 2, panel A, arrow 2). O_2^- production was absolutely dependent on NADPH. LDS (optimal concentration = 30 μM) enhanced O_2^- production 1.5–2-fold but cytochrome b_{599} was also capable of O_2^- generation in the absence of an anionic amphiphile. O_2^- generation by crude and purified cytochrome b_{559} preparations, expressed as turnover rates (mol O_2^-/mol cytochrome b_{559}/s), is summarized in Table I. The value of close to 25 mol O_2^-/mol cytochrome b_{599}/s, detected with purified cytochrome b_{559} relipidated with the optimal phospholipid combination,

Table I

O_2^- production by cytochrome b_{559} in the absence of cytosolic activators

Phospholipid preparation	O_2^- production (mol O_2^-/mol cytochrome b_{559}/s)			
	Crude cytochrome b_{559} (0.5–2 nmol/mg protein)		Purified cytochrome b_{559} (7–12 nmol/mg protein)	
	−LDS	+LDS	−LDS	+LDS
Phosphatidylcholine, type II-S + Phosphatidic acid	11.5 ± 4.9 (*n* = 2)	16.2 ± 1.2 (*n* = 3)	11.8 ± 2.8 (*n* = 2)	23.8 ± 4.8 (*n* = 4)
Phosphatidylcholine, type II-S	4.3 ± 1.8 (*n* = 3)	9.9 ± 2.4 (*n* = 3)		12.2
Phosphatidic acid	7.3	9.1		12.5
Phosphatidylcholine, type IV-S	1.5 ± 0.5 (*n* = 2)	5.1 ± 1.0 (*n* = 6)		6.0
Phosphatidylcholine, type X-E		2.4		2.5

Cytochrome b_{559} preparations were reconstituted with phospholipids, as described in section 2 (200 μg/ml, when a single lipid was used and 100 μg/ml for each lipid, when two lipids were used in combination). The relipidated cytochrome b_{559} preparations were assayed for O_2^- production in the presence of 1 μM FAD and 400 μM NADPH but in the absence of cytosol. When added, the concentration of LDS was 30 μM. 'n' in parentheses indicates the number of cytochrome b_{559} preparations tested. Results represent means ± S.D.

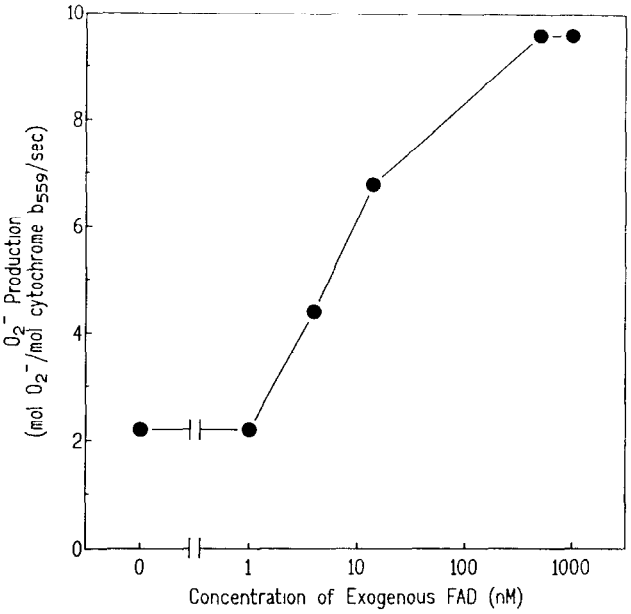

Fig 3. Dependence of O_2^- production on exogenous FAD. Purified cytochrome b_{559} was reconstituted with a mixture of phosphatidylcholine, type II-S and phosphatidic acid, in the absence of exogenous FAD, and O_2^- production was assayed, essentially as described in the legend of Fig. 2, in the presence of various concentrations of FAD added to the assay medium

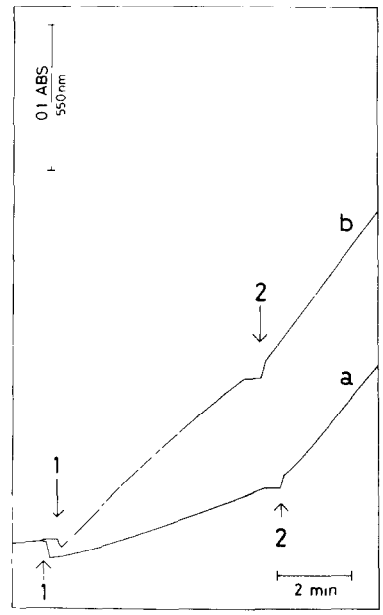

Fig 4. Preincubation of cytochrome b_{559} with FAD enhances its ability to generate O_2^-. Cytochrome b_{559} was reconstituted with a mixture of phosphatidylcholine, type II-S and phosphatidic acid in the absence of FAD and O_2^- production assayed essentially as described in the legend of Fig. 2. (Tracing a) To the cuvette containing 1.1 ml assay buffer supplemented with 30 μM LDS and 400 μM NADPH, FAD was added to a final concentration of 1 nM followed by the addition of 3 pmol cytochrome b_{559} (arrow 1). At the time indicated by arrow 2, further FAD was added, to bring its final concentration of 0.49 μM. (Tracing b) 3 pmol of cytochrome b_{559} was mixed with 55 nM FAD and preincubated for 1 min at room temperature. The mixture was added to the cuvette containing 1.1 ml assay buffer supplemented with 30 μM LDS and 400 μM NADPH at the time indicated by arrow 1. The final concentration of FAD (derived from the cytochrome b_{559}–FAD preincubation mixture) was 1 nM. At the time indicated by arrow 2, FAD was added, to bring its final concentration to 0.49 μM.

corresponds to 15–20% of the rates measured in amphiphile-activated cell-free systems composed of purified guinea pig cytochrome b_{559} [4] or porcine cytochrome b_{559} [25] and cytosolic activators. The rates measured are close (70–80%) to those recently reported for fatty acid stimulated O_2^- production by porcine cytochrome b_{559} combined with a membrane-derived nitroblue tetrazolium reductase and cytosol [31] and about 30-fold higher than those reported for cytochrome b_{559} combined with hepatic NADPH-cytochrome P450 reductase, in the absence of cytosol and amphiphile [32]. Cytosol-independent O_2^- production was evident with both crude and highly purified cytochrome b_{559} preparations (Table I). The apparently lower turnover values found with crude cytochrome b_{559} preparations represent a calculation artefact due to the presence of hemoproteins other than cytochrome b_{559} in partially purified preparations and was observed before by us [4] and Sumimoto et al. [25]. Addition of cytosol to preparation of relipidated cytochrome b_{559} engaged in NADPH-dependent O_2^- production resulted in an enhancement in the rate of O_2^- generation (Fig. 2, panel B). However, the enhancing effect of cytosol required the addition of LDS and exhibited a higher LDS concentration optimum (100 μM, as opposed to 30 μM found with cytochrome b_{559} alone). Cytosol-enhanced O_2^- production was not related to the nature of the phospholipid used to reconstitute cytochrome b_{559}, the only exception being the inability of cytosol to enhance O_2^- production by cytochrome relipidated with phosphatidic acid (Fig. 2, panel B, tracing c). Relipidated cytochrome b_{559} exhibited a substrate specificity similar to that measured in the presence of cytosolic activators [2,3], with a K_m of 75 μM, for NADPH and 1.86 mM, for NADH.

O_2^- generation by relipidated cytochrome b_{559} was strictly dependent on exogenous FAD. As seen in Fig. 3, half-maximal O_2^- production was achieved in the presence of 10 nM FAD. FMN was incapable of supporting O_2^- production. The role of exogenous FAD in cell-free O_2^- production is the subject of controversy [22,24,25,31]. To clarify whether FAD functions as a cofactor of cytochrome b_{559} or as a free electron carrier, experiments as that illustrated in Fig. 4 were performed. Tracing a represents O_2^- production by relipidated cytochrome b_{559} added directly to the assay buffer supplemented with 1 nM FAD (a concentration insufficient for supporting O_2 production; see Fig.3). Tracing b represents O_2^- production by relipidated cytochrome b_{559} preincubated with 55 nM FAD for 1 min before addition to the assay (this resulted in a final concentra-

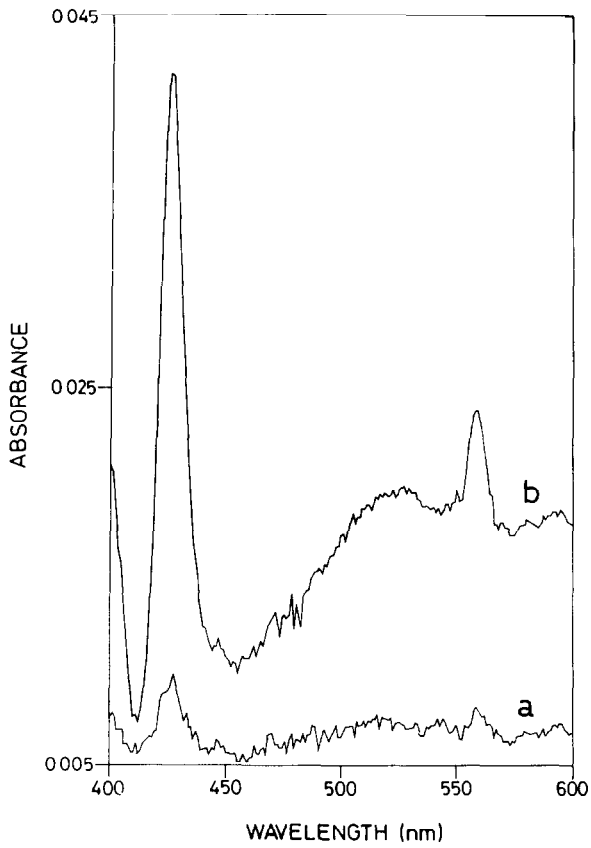

Fig. 5. Reduction of purified and relipidated cytochrome b_{559} by NADPH under anaerobic conditions Purified cytochrome b_{559} (130 pmol) relipidated with a mixture of phosphatidylcholine, type II-S and phosphatidic acid was added to a cuvette containing 0 7 ml of assay buffer supplemented with 1 μM FAD, 30 μM LDS and 140 units/ml of SOD. Anaerobic conditions were established, as described in section 2, and the absorbance spectrum of the oxidized cytochrome b_{559} was recorded (400–600 nm). This was followed by the addition of 400 μM NADPH and the absorbance spectrum recorded immediately after the addition of NADPH. Next, complete chemical reduction was induced by the addition of a few grains of sodium dithionite and the absorbance spectrum recorded again. Tracings represent the reduced minus oxidized difference spectra following the addition of NADPH (a) and sodium dithionite (b).

tion of 1 nM FAD in the assay buffer). It is apparent that preincubation with FAD leads to a higher rate of O_2^- production, approaching that measured following the addition of a saturating amount of FAD to the assay buffer (Fig. 4, arrow 2). This suggests the formation of a cytochrome b_{559}–FAD complex during preincubation.

Finally, we investigated the capability of purified and relipidated cytochrome b_{559} to interact with NADPH by examining whether NADPH can reduce cytochrome b_{559} under anaerobic conditions. Fig. 5 illustrates the reduced *minus* oxidized spectrum of purified relipidated cytochrome b_{559} incubated with NADPH under anaerobic conditions in the presence of FAD and LDS (tracing a). Positive peaks were detected at 427 nm and 558 nm and a trough, at 412 nm. These values are characteristic of guinea pig cytochrome b_{559} [4,33] and correspond precisely to those detected on the reduced *minus* oxidized spectrum of chemically reduced cytochrome b_{559} (tracing b).

We have demonstrated that cytochrome b_{559} incorporated in an optimal lipid environment and supplemented with FAD, shown to act by reflavinating the deflavinated apoprotein, is capable of NADPH-dependent O_2^- production. The presence of an anionic amphiphile, such as LDS, was not a prerequisite for O_2^- generation but had an enhancing effect. The mechanism by which relipidated cytochrome b_{559} becomes catalytically active in the absence of cytosolic components remains to be clarified. We found that the ability of a particular preparation of phosphatidylcholine to support cytochrome b_{559}-mediated O_2^- production increased with the proportion of contaminating (= other than phosphatidylcholine) lipids that it contained. This suggests that, in addition to a structural role in providing a membrane-mimicking environment, one or more of the lipids used in the relipidation procedure might function as activators, not unlike anionic amphiphiles in the complete (membrane+cytosol) cell-free system. Support for this suggestion is provided by the finding that the highest rates of O_2^- production were obtained with cytochrome b_{559} relipidated with a mixture of the least pure preparation of phosphatidylcholine (type II-S) and phosphatidic acid. It is of interest that Bellavite et al. [33] described the ability of phosphatidic acid from egg yolk lecithin (the type of preparation also used in our experiments) to elicit O_2^- production in solubilized porcine neutrophil membranes independently of cytosol. The phosphatidic acid-activable material copurified with cytochrome b_{559} and O_2^- production was dependent on the presence of FAD in the assay medium.

In conclusion, the present report offers direct experimental proof for the proposal [22,24,25] that the complete electron transporting machinery of the O_2^- generating NADPH oxidase is contained in the cytochrome b_{559} dimer.

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