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Superoxide production by cytochrome b_{559}

Mechanism of cytosol-independent activation

Vasilij Koshkin*, Edgar Pick

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

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Abstract

Purified cytochrome b_{559} relipidated with either a mixture of phosphatidylcholine and phosphatidic acid or with phosphatidylcholine only exhibits high and low superoxide (O_2^-) producing ability, respectively, in the absence of cytosolic activators [Koshkin, V and Pick, E (1993) FEBS Lett 327, 57–62] This system was used as a model for the study of the mechanism of NADPH oxidase activation It is shown that, depending on the composition of the phospholipid environment, cytochrome b_{599} binds FAD with high or low affinity, this being accompanied by changes in flavin absorbance and fluorescence High affinity binding of FAD to cytochrome b_{559} relipidated with phosphatidylcholine combined with phosphatidic acid is associated with an enhanced NADPH-driven O_2^- producing capacity A kinetic study of O_2^- production by cytochrome b_{559} reflavinated under stoichiometric FAD binding conditions revealed an FAD/heme ratio of 1 2 A further kinetic study of O_2^- production by high- and low-activity relipidated and reflavinated cytochrome b_{559}, at varying substrate concentrations, and the determination of steady-state difference spectra of such preparations, reduced by NADPH, indicated that O_2^- production is activated by facilitation of electron transfer from NADPH to FAD rather than by an enhancement of NADPH binding

Key words Superoxide, Cytochrome b_{559}, NADPH oxidase, Phospholipid, FAD

1. Introduction

Cytochrome b_{559} is an essential component of the electron transferring enzyme complex, known as NADPH oxidase, which catalyzes the NADPH-supported production of superoxide (O_2^-) by phagocytic cells [1–3] It is the only cytochrome capable of one-electron reduction of oxygen In addition to cytochrome b_{559}, the NADPH oxidase complex consists of three cytosolic components (p47-*phox*, p67-*phox* and the small G protein *rac*1 p21 or *rac*2 p21) The induction of O_2^- production is thought to be the consequence of the assembly of the isolated components into an organized complex Such assembly is elicited in the intact cell, following specific membrane receptor-ligand interactions, by a yet poorly understood transductional mechanism(s) [1]. In a cell-free system, consisting of the isolated NADPH oxidase components derived from resting cells, O_2^- production can be elicited by certain anionic amphiphiles such as arachidonate [4] or sodium dodecyl sulfate (SDS) [5] The commonly accepted view is that cytochrome b_{559} functions as the terminal O_2 reducing component ([1–3], but see [6] for a dissident view) and the existence of an NADPH-binding flavoprotein transferring electrons from NADPH to cytochrome b_{559} was proposed [7] The failure to identify this flavoprotein among either membrane or cytosolic components led to the idea that cytochrome b_{559} is a flavocytochrome also possessing the NADPH-binding site and, therefore, bearing the complete electron transport apparatus of the NADPH oxidase. The experimental evidence on which this proposal rests is less than exhaustive and includes· amino acid sequence alignment of the large subunit of cytochrome b_{599} with other flavoproteins, affinity labelling with NADPH and binding of FAD [8–11]. Recently, we provided direct experimental evidence for this proposal by demonstrating that purified and relipidated cytochrome b_{559} is capable of NADPH-driven, FAD-dependent O_2^- production in the absence of the cytosolic components of NADPH oxidase [12] The catalytic potency of cytochrome b_{559} was found to vary over a wide range depending on the nature of phospholipids utilized for relipidation The most effective activity supporting lipid was a 1 1 (w/w) mixture of phosphatidylcholine with phosphatidic acid and it was felt that this system represents a useful model for the

* Corresponding author Fax (972) (3) 642-9119

Abbreviations O_2^-, superoxide, SDS, sodium dodecyl sulfate, EGTA, [ethylenebis(oxyethylenenitrilo)]tetracetic acid, PMSF, phenylmethylsulfonylfluoride, SOD, superoxide dismutase, LiDS, lithium dodecyl sulfate

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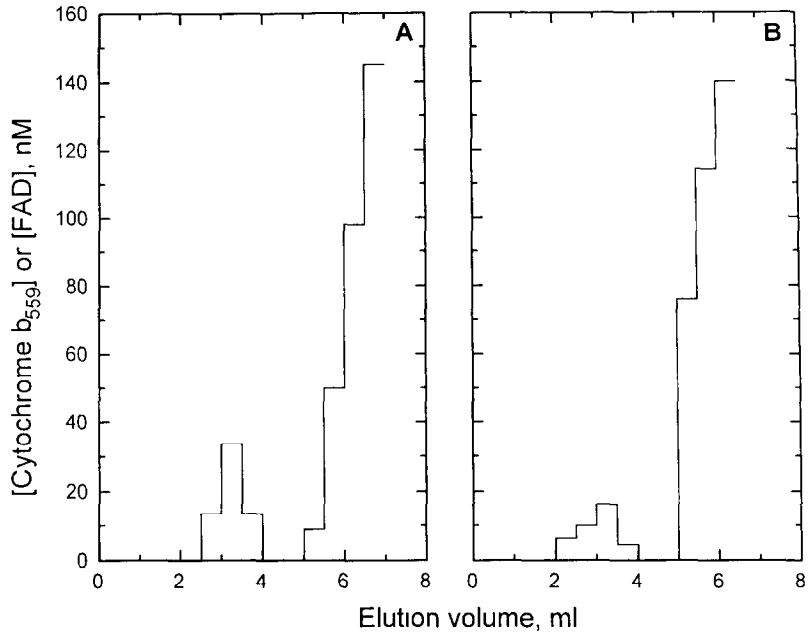

Fig 1 Gel filtration of reflavinated cytochrome b_{599} Purified cytochrome b_{559} (1 17 μM) was supplemented with phospholipids (A, 290 μg/ml phosphatidylcholine and 290 μg/ml phosphatidic acid, or B, 500 μg/ml phosphatidylcholine), 30 μM LiDS and 2 7 μM FAD in a final volume of 151 μl, diluted with 550 μl of buffer B, lacking cytochrome *c* and containing 2 μM FAD, and kept on ice for 20 min These preparations were subjected to gel filtration on PD-10 columns (Pharmacia) equilibrated with buffer B, lacking cytochrome *c*, and the eluate was analyzed for cytochrome b_{559} concentration (dashed line) and FAD concentration (solid line)

study of cytochrome b_{559} enzymology In the past, O_2^- production by purified cytochrome b_{559} could be demonstrated only in conjunction with an exogenous reductase, such as NADPH cytochrome P-450 reductase [13,14]. Relipidated cytochrome b_{559} also offers a valuable system permitting to distinguish between two alternative mechanisms of NADPH oxidase activation· facilitation of NADPH binding and the enhancement of electron transport [1,3] In the present study, we demonstrate that cytochrome b_{599} provided with a lipid environment leading to high or low O_2^- generating states, binds FAD with high or low affinity, respectively. In reflavinated high-activity cytochrome b_{559}, an FAD/heme ratio of 1·2 was measured. Finally, we offer evidence in support of cytochrome b_{559} 'activation' being the result of enhanced electron transport from NADPH to FAD

2. Materials and methods

2 1 Purification of cytochrome b_{559}

Cytochrome b_{559} was purified as described by us in the past [12,15] The principal purification steps included solubilization of guinea pig peritoneal macrophage membranes, prewashed with 1 M NaCl, with 40 mM octyl glucoside in buffer A (0 05 M Na-phosphate, pH 7 4, 1 mM $MgCl_2$, 1 mM EGTA, 2 mM NaN_3, 1 mM dithioerythritol, 1 mM PMSF, 1 μg/ml leupeptin, 20% glycerol), batch absorption with DEAE-Sepharose, affinity chromatography on heparin-agarose, and gel filtration on Superose 12 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} cm^{-1} [16] For experiments involving O_2^- generation and FAD

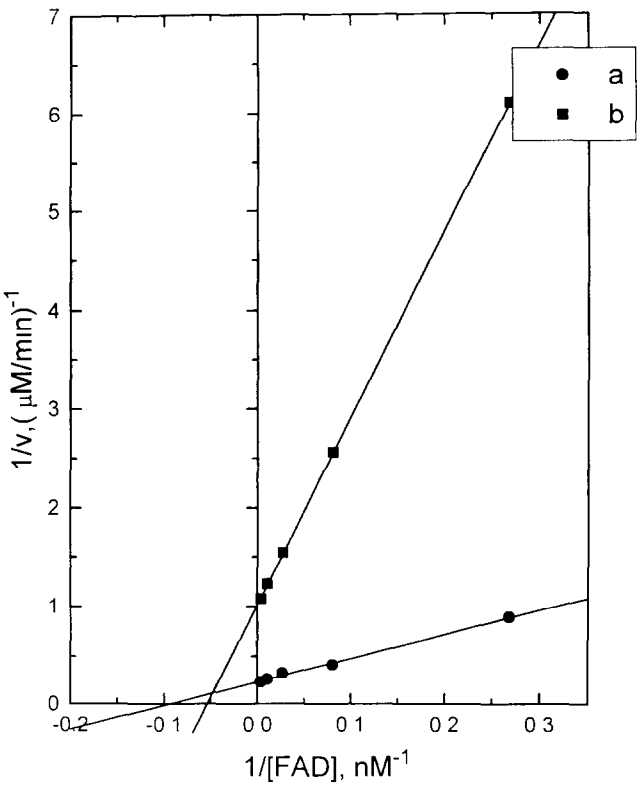

Fig 2 Dependence of the O_2^--producing activity of cytochrome b_{559} on the concentration of added FAD Purified cytochrome (0 75 μM) was supplemented with phospholipids (a, 250 μg/ml phosphatidylcholine and 250 μg/ml phosphatidic acid, b, 500 μg/ml phosphatidylcholine) and 5 μl aliquots were added to 1 ml assay mixture containing variable concentrations of FAD After 3 min incubation, the reaction was started by addition of 400 μM NADPH

binding, purified cytochrome b_{559} with a specific content of 8–11 nmol heme/mg protein was used, partially purified cytochrome b_{559}, with a specific content of 1 5–3 nmol heme/mg protein, was used for spectroscopy

2 2 Reconstitution of cytochrome b_{559} *with phospholipids and reflavination with FAD*

These procedures were performed essentially as described before [12,17] L-α-Phosphatidylcholine II-S or IV-S, from soybean, and L-α-phosphatidic acid, sodium salt, from egg yolk lecithin (all from Sigma) were dissolved in buffer A at a concentration of 4 mg/ml Cytochrome b_{559} preparations were supplemented with the desired amounts of phospholipid and allowed to stand on ice for 10–15 min This was followed by 6- to 8-fold dilution in (for O_2^- production assays) or by dialysis for 16–20 h against 200 volumes of octyl glucoside-free buffer A (for spectroscopy) [17] When intended for the performance of steady state reduction experiments, dialysis was against buffer A lacking NaN_3, in order to avoid interference with the activity of catalase added to the assay buffer Reflavination with FAD (described in detail in the legends to figures) was performed in the presence of 30 μM lithium dodecyl sulfate (LiDS), that was shown by us earlier to enhance O_2^- production by cytochrome b_{559} [12]

2 3 O_2^- production

This was assayed by the rate of superoxide dismutase (SOD) inhibitable cytochrome *c* reduction as described before [5,12] The assay buffer (buffer B) consisted of 65 mM K, Na-phosphate buffer, pH 7 0, 1 mM $MgCl_2$, 1 mM EGTA, 2 mM NaN_3, 0 1 mM ferricytochrome *c* and 30 μM LiDS Measurements were performed with a Uvikon 860 spectrophotometer (Kontron) using a 'time drive' program and rate values were obtained using the 'derivative overlay' program

2 4 Other assays

Steady-state difference spectra of NADPH-reduced vs oxidized reflavinated cytochrome b_{559} were obtained in the presence of air using a Uvikon 860 spectrophotometer at a scanning rate of 500 nm/min, the content of the assay cuvette being stirred between repeated scans The extent of reduction of the redox centers of cytochrome b_{559} was determined from absorbance differences at 559–540 nm, for heme [18], and 450–540 nm, for FAD [19] and calculated in relation to the Na dithionite-reduced state Noncovalently bound flavin was assayed by the fluorometric method of Faeder and Siegel [20] This and other fluorescence measurements were performed with a FP-770 spectrofluorometer (Jasco) Protein concentration were determined by the method of Bradford [21], with bovine gamma globulin as the standard

3. Results and discussion

We have recently shown that purified cytochrome b_{559} exhibits varying levels of electron transport (O_2^- production) activities, depending on the nature of the phospholipids serving for relipidation [12]. In order to elucidate the biochemical basis responsible for the high- and low-activity states, two types of cytochrome b_{559} preparations were investigated High-activity cytochrome b_{559} was obtained by relipidation with a mixture of phosphatidylcholine and phosphatidic acid (details of the relipidation conditions are recorded in the legends of Figs 1–5) and expressed a turnover rate of 15–30 mol O_2^-/mol cytochrome b_{559}/s. Low-activity cytochrome b_{559} was obtained by relipidation with phosphatidylcholine only and expressed a turnover rate of 4–7 mol O_2^-/mol cytochrome b_{559}/s.

3 1 Binding of FAD to relipidated cytochrome b_{559}

Binding of FAD to cytochrome b_{559}, relipidated to

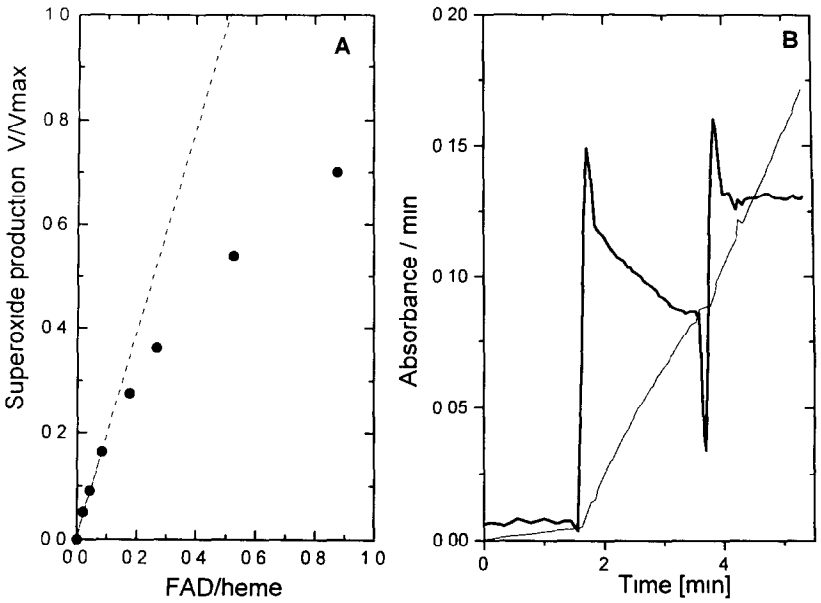

Fig 3 Determination of the FAD/heme stoichiometry in activated cytochrome b_{599} (A) Dependence of O_2^- production activity by cytochrome b_{559} on the 'added FAD'/heme ratio Purified cytochrome b_{559} (0 75 μM) was relipidated with 250 μg/ml phosphatidylcholine and 250 μg/ml phosphatidic acid, 10 μl aliquots of this preparation were supplemented with variable amounts of FAD (1 μl) and diluted in 50 μl of buffer B, containing 30 μM LiDS After 5 min incubation, the reaction was started by the addition of these samples to an NADPH-containing assay mixture Initial rates were calculated using a 'derivative overlay' program (B) O_2^- production by cytochrome b_{559} containing a half-equimolar amount of FAD Partially purified relipidated cytochrome b_{559} (1 05 μM heme, 100 μg/ml phosphatidylcholine, and 100 μg/ml phosphatidic acid, dialyzed as described in section 2) was supplemented with 30 μM LiDS and 0 52 μM FAD After 3 min of incubation a 10 μl aliquot was added to 1 ml complete assay mixture (indicated by arrow marked 1) and at the time indicated by arrow marked 2, a saturating amount of FAD (1 μM) was added Thin tracing, original record of absorbance at 550 nm, thick tracing, its first derivative

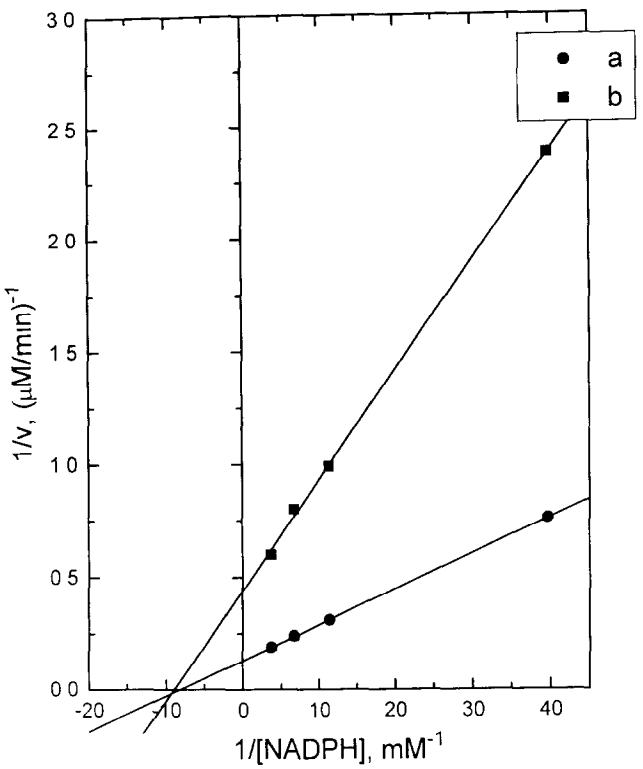

Fig 4 Dependence of the O_2^- producing activity of cytochrome b_{559} on the concentration of NADPH Purified cytochrome b_{559} was combined with phospholipids (a, 100 μg/ml phosphatidylcholine and 100 μg/ml phosphatidic acid, b, 200 μg/ml phosphatidylcholine) and 15 μl aliquots were added to 1 ml assay mixture containing 1 μM FAD The reaction was started by the addition of variable amounts of NADPH

achieve either the high- or the low-activity state, was demonstrated by separation of FAD-reconstituted cytochrome b_{559} from free FAD by gel filtration (Fig 1) High-activity reflavinated cytochrome b_{559} exhibited an O_2^- producing capacity amounting to 70% of the activity measured under conditions of an excess of free FAD in the assay medium Low-activity reflavinated cytochrome b_{559} produced only 20% of the amount of O_2^- measured in the presence of an excess of free FAD in the assay medium Further indications of FAD binding were derived from the analysis of the absorbance and fluorescence spectra of reflavinated cytochrome b_{559}, demonstrating a shift of the 450 nm absorbance maximum to 460 nm, and a decrease in fluorescence intensity by 50% (results not shown) These changes suggest the exposure of flavin to an apolar environment [22] and its interaction with aromatic amino acid residues [23] The affinity of the cytochrome b_{559}–FAD interaction, estimated by saturation kinetics measurements [24,25] and expressed as the concentration of FAD supporting half-maximal O_2^- production, was found to be 8 0 ± 2 4 nM (number of cytochrome preparations, $n = 4$) and 23 5 ± 5 6 nM ($n = 3$), for high- and low-activity cytochrome b_{599} preparations, respectively (Fig 2) This demonstrates that high-activity cytochrome b_{559} preparations have a higher affinity for FAD and suggests that tighter binding of FAD to cytochrome b_{559} leads to an enhanced catalytic capacity

The FAD/heme stoichiometry was determined by studying O_2^- production by relipidated high-activity cytochrome b_{559} as a function of the 'added FAD'/heme ratio (Fig 3A) From the initial linear part of the curve, representing the region of stoichiometric FAD binding ([FAD] ≪ [heme]) [26], a FAD/heme ratio of 1 2 was calculated Concentrated preparations of high activity relipidated cytochrome b_{559} (above 1 μM; therefore, ≫ K_{diss} of FAD) provided with a half-equimolar amount of FAD exhibited a nearly maximal initial rate of O_2 production (Fig 3B) The finding of an FAD/heme ratio of 1 2 in cytochrome b_{559} is in agreement with the ratio reported by Segal et al [9] for total neutrophil membranes, and provides functional proof for earlier proposals of a multi-heme nature of cytochrome b_{559} [27,28] Our finding is also relevant to the proposal of parallel reduction of two one-electron centers by flavin as a $n = 2$ to $n = 1$ step-down mechanism [29,30], taking into account the finding that the flavosemiquinone in phagocyte membranes is unstable [31,32]

3 2 *Electron transfer by reflavinated cytochrome* b_{559}

In order to distinguish between an increase in substrate binding and enhanced electron transport as being responsible for the high-activity state of cytochrome b_{559}, we performed a kinetic investigation of O_2^- production by high- and low-activity cytochrome b_{559} preparations Typical results are shown in Fig 4 and demonstrate a K_m for NADPH of 124 ± 23 and 146 ± 57 μM for high and low activity cytochrome b_{559} preparations, respectively, whereas the V_{max} of these preparations was 32 7 ± 8 4 and 7 6 ± 2 0 mol O_2^-/mol cytochrome b_{559}/s (data derived from 3 experiments) These results suggest that activation of cytochrome b_{559} is the result of an enhancement in its electron transport capability rather than facilitation of substrate binding to it.

The state of electron transferring centers during O_2^- production by cytochrome b_{559} was estimated from the steady-state difference spectra of NADPH-reduced cytochrome b_{559} under aerobic conditions (Fig 5) Addition of NADPH to high-activity cytochrome b_{559} led to the reduction of 10% of heme and 40% of FAD, that decreased gradually in the course of the reaction No measurable reduction of either heme or FAD was detected upon addition of NADPH to low-activity cytochrome b_{559} (Fig 5) These results indicate that the 'switch' for cytochrome b_{559} activation is located between NADPH binding and FAD reduction A similar situation is known to exist in the complete NADPH oxidase system derived from stimulated intact phagocytes [32], confirming that identical processes are taking place in the native enzyme complex and in purified relipidated and reflavinated cytochrome b_{559}

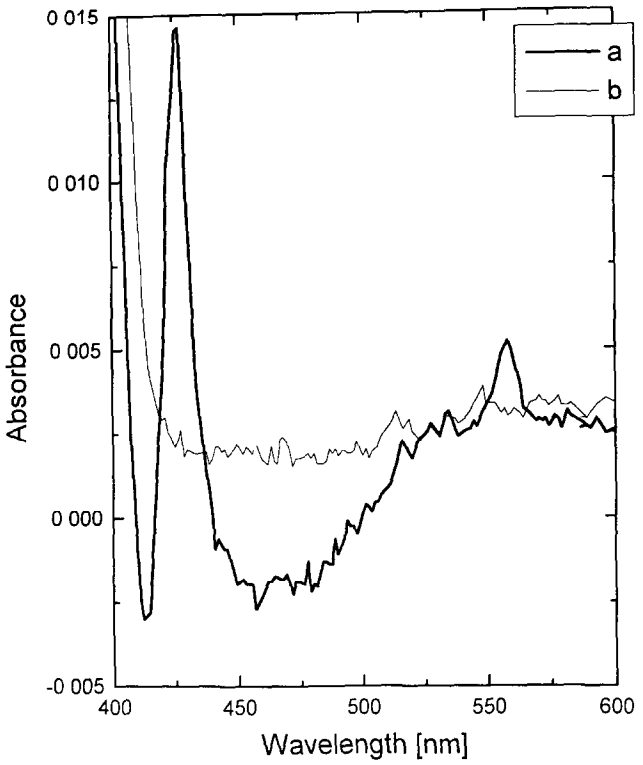

Fig 5 Difference spectra of NADPH-reduced reflavinated cytochrome b_{559} Partially purified cytochrome b_{559} (1 6 μM) was combined with phospholipids (spectrum a, 100 μg/ml phosphatidylcholine and 100 μg/ml phosphatidic acid, spectrum b, 200 μg/ml phosphatidylcholine) and dialyzed as described in section 2 To the final cytochrome b_{559} preparation (1 32 μM) were added 30 μM LiDS, 0 66 μM FAD, 10 U/ml SOD, 130 U/ml catalase After the oxidized spectrum was recorded, the reaction was started by addition of 0 58 mM NADPH and the spectra were scanned at 1 min intervals Typical initial spectra after NADPH addition (*minus* oxidized spectra) are presented

We conclude that cytochrome b_{559} is a flavocytochrome with a 1.2 FAD/heme stoichiometry. Its NADPH-dependent O_2 reducing capacity is determined by the state of the FAD group; high affinity binding of FAD, associated with a specific phospholipid environment, results in facilitation of electron transport from NADPH to FAD and, consequently, in enhanced O_2^- production at a constant affinity for the substrate It appears likely that a similar mechanism underlies activation of the complete NADPH oxidase system in the intact cell.

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