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Spatial and electrogenic properties of superoxide-producing cytochrome *b*-559 incorporated into liposomes

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Abstract

Purified cytochrome *b*-559 reconstituted into liposomes, consisting of certain azolectin-based phospholipid mixtures, is capable of NADPH-supported FAD-dependent superoxide (O_2^-) production in the absence of cytosolic activators. This system, representing the simplest model of the respiratory burst NADPH oxidase, was used to study cytochrome *b*-559 enzymology and distinguish putative mechanisms of NADPH oxidase activation (Koshkin, V. and Pick, E. (1993) FEBS Lett. 327, 57–62; (1994) FEBS Lett. 338, 285–289). In the present report, representing an extension of our earlier investigations, two types of vesicle-incorporated and reflavinated cytochrome *b*-559 preparation were distinguished by their ability to catalyze vectorial electrogenic or scalar electron transport from NADPH to oxygen. This can be explained by the existence of two distinct membranal locations of cytochrome *b*-559, with NADPH-binding and O_2-reducing sites exposed on different or on the same side of the membrane. The mode of cytochrome *b*-559 insertion into the membrane depended on the reconstitution method employed. Both states of the reconstituted cytochrome *b*-559 were functionally competent judging by their susceptibility to additional activation by cytosolic NADPH oxidase components. The capability of flavocytochrome *b*-559 to function as a transmembrane electrogenic electron carrier points to its crucial role in the respiratory burst not only in its catalytical but also in its vectorial aspect. The scalar mode of its action may be related to respiratory burst pathology.

Keywords: Cytochrome *b*-559; Membrane orientation; Membrane potential; NADPH oxidase; Liposome

1. Introduction

Phagocytic cells respond to a variety of stimulants by the production of oxygen radicals, a process known as the respiratory burst. The primary oxygen radical, superoxide (O_2^-), is generated by the NADPH-driven one-electron reduction of oxygen, catalyzed by a membrane-bound NADPH oxidase complex [1–3]. This contains flavin and heme electron transport moieties and carries out transmembrane electrogenic electron transport from cytosolic NADPH to oxygen in the extracellular or vacuolar space [4–7]. For a long time this system was considered as an electron transport chain, consisting of an NADPH-binding flavoprotein and an O_2-reducing cytochrome *b*-559, which is converted from the dormant to the active state by complexing of the membranal and cytosolic components. The recent advances in the understanding of the composition and action of this complex are due to its reconstitution in vitro in a cell-free system [8,9]. This consists of purified and relipidated cytochrome *b*-559, the cytosolic components p47-*phox* and p67-*phox*, the small G protein Rac1 or Rac2 and an anionic amphiphile, serving as activator. Studies utilizing this system led to the concept that cytochrome *b*-559 represents the only catalytic component of NADPH-oxidase, bearing, in addition to the oxygen-reducing heme group, NADPH- and FAD-binding sites and performing the complete catalytic sequence of the respiratory burst upon activation by complexing with cytosolic proteins [10–12]. Resting on this concept and on the spatial properties of respiratory burst in the intact cell, it was supposed that the cytochrome *b*-559 molecule provides an electron route through the cell membrane [11,13], although experimental data on its membrane topography are very limited [14].

We recently provided direct functional proof for the catalytic competence of cytochrome *b*-559 by demonstrat-

Abbreviations: *b*-559-liposomes, cytochrome-*b*-559-containing liposomes; O_2^-, superoxide; EGTA, [ethylenebis(oxyethylenenitrilo)tetraacetic acid; LiDS, lithium dodecyl sulfate; PMSF phenylmethylsulfonyl fluoride; SOD, superoxide dismutase.

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ing its ability to function as an independent generator of O_2^- in vitro. [15,16]. Thus, we showed that cytochrome *b*-559, incorporated into phospholipid vesicles of a certain composition, is capable of NADPH-driven, FAD-dependent O_2^- production in the absence of the cytosolic components of NADPH oxidase. This system was found useful to study cytochrome *b*-559 enzymology and the mechanism of its activation. The work presented here was undertaken with the purpose to determine the spatial orientation of cytochrome *b*-559 in this system. This could provide insight into its interaction with the membrane and clear up spatial properties of the cell-free model of NADPH oxidase, which are totally unknown. For this purpose, cytochrome *b*-559 was incorporated into phospholipid vesicles by different methods and its electron transport activity with an external electron donor and an internal acceptor was studied. It is shown that cytochrome *b*-559 may insert into liposomes in two different modes; their properties are described and their possible relation to the native system is discussed.

2. Materials and methods

2.1. Purification of cytochrome b-559

Cytochrome *b*-559 was purified by the previously described method [15,17] with the slight modifications. Guinea-pig peritoneal macrophage membranes, prewashed with 1 M NaCl, were suspended in 1.5 M NaCl, 200 mM sucrose containing 15 mM phosphate buffer (pH 7.0), and separated into low-speed ($10\,000 \times g$, 30 min) and high-speed ($40\,000 \times g$, 120 min) sedimented fractions. The last fraction was used for further purification, which included solubilization with 40 mM octyl glucoside in buffer A (0.05 M sodium phosphate (pH 7.4), 1 mM $MgCl_2$, 1 mM EGTA, 0.2 mM dithioerythritol, 1 mM PMSF, 1 μg/ml leupeptin, 20% glycerol), affinity chromatography on heparin agarose and gel-filtration on Superose 12. The concentration of cytochrome *b*-559 was determined from the differential reduced-minus-oxidized spectra using the extinction coefficient (427–411 nm) = 200 mM^{-1} cm^{-1} [18]. Protein concentration was measured by the method of Bradford [19], with bovine gamma-globulin as the standard. Purified cytochrome *b*-559 (7–9 nmol heme/mg protein) was used for electron transport experiments, and the partially purified one (1.5–3 nmol heme/mg protein) for membrane potential and permeability measurements.

2.2. Reconstitution of cytochrome b-559 into liposomes

Reconsitution was accomplished by adaptation of the methods described in the literature [20–24]. Mixed phospholipids of the 'azolectin' type (L-α-phosphatidylcholine type II-S, Sigma) were dispersed under nitrogen with slight sonication in buffer A, containing 40 mM octyl glucoside, at a concentration of 7 mg/ml. Liposome formation was achieved by dialysis for 16–18 h against 200 vol. of detergent-free buffer A. Cytochrome *b*-559-containing liposomes (*b*-559-liposomes) were obtained by two methods: (a) by co-solubilization of cytochrome *b*-559 in an octyl glucoside-phospholipid mixture with slight sonication (Microson cell disruptor, 10% output power, 2×5 s) and 1–4 h incubation in ice before dialysis; and (b) by direct incorporation into preformed liposomes, i.e., mixing cytochrome *b*-559 with liposome suspension 1:10 (v/v) and brief (15–30 min) incubation in ice. Cytochrome *b*-559 binding to phospholipid vesicles was confirmed by passing through Sepharose 4B column (1×16 cm). *b*-559-liposomes with internally trapped NADPH or cytochrome c were prepared by addition of NADPH (1.45 mM) or cytochrome *c* (180 μM) to the starting phospholipid-protein mixture and, after dialysis, the loaded liposomes were separated from free solutes on a Sephadex G-75 column (1×15 cm). These liposomes showed unimodal size distribution in the region of 80–180 nm determined by quasi-elastic light scattering using Malvern system 4700 c analyzer. Cytochrome *b*-559 concentration in the cytochrome-*c*-loaded *b*-559-liposomes was estimated from the differential spectra of dithionite-reduced *minus* ascorbate-reduced samples using the extinction coefficient (559–540 nm) = 21.6 mM^{-1} cm^{-1} [25].

2.3. Electron transport activity

This was assayed by the rate of cytochrome *c* reduction [9] or NADPH oxidation [26] in buffer B, consisting of 65 mM potassium, sodium phosphate buffer (pH 7.0), 1 mM $MgCl_2$, 1 mM EGTA, with additional ingredients, when added, indicated in the text and legends to figures. Measurements were performed with a Uvikon 860 spectrophotometer (Kontron) using a 'time drive' program and rate values were obtained using 'derivative overlay' program.

2.4. Other assays

Membrane potential generation in electron-transporting *b*-559-liposomes was measured with the optical membrane potential indicator safranin [27,28] by monitoring an absorbance at 530 nm. The initial mixture for *b*-559-liposome preparation was supplemented with SOD (150 U/ml) and catalase (233 U/ml) in order to provide O_2^- scavenging on both sides of the membrane. Optical response calibration was performed by the conventional K^+-valinomycin method [27,28] using the Nernst equation $E = 60 \log [K^+]_{in}/[K^+]_{out}$ for K^+-diffusion potential calculation, where $[K^+]_{in}$ and $[K^+]_{out}$ denote the potassium concentrations inside and outside the vesicles, respectively (the cationic composition of the media employed was modified to get K^+-containing vesicle stock suspension and Na^+-containing assay medium).

Fluorometric detection of intraliposomal NADPH oxidation was performed with a FP-770 spectrofluorometer (Jasco).

3. Results and discussion

Orientation of membrane enzymes can be determined by functional assays [24] provided that the membrane permeability to related substrates is known. To define liposomes permeability to NADPH and O_2^- following two systems were used: (a) *b*-559-liposomes with internally entrapped NADPH and external NADPH oxidant glutathione + glutathione reductase; and (b) *b*-559-liposomes with internally entrapped cytochrome *c* and external O_2^- generator xanthine + xanthine oxidase. The data given in Fig. 1 illustrate the behavior of these systems in two situations: intact liposomes and after their permeabilization by freezing-thawing or lysis by detergent. One can see that NADPH inside the vesicles is inaccessible to glutathione reductase but becomes freely accessible after freezing-thawing or detergent addition (Fig. 1A). As regards O_2^- (Fig. 1B), this was found to be able to penetrate through the phospholipid membrane (curve a), in agreement with an earlier report [29], although the membrane represented a noticeable diffusion barrier for this ion (curves c,d). Reduction of internal cytochrome *c* by externally generated O_2^- was completely inhibited by externally added SOD (curve b). Both types of *b*-559-liposome possessed similar permeability properties.

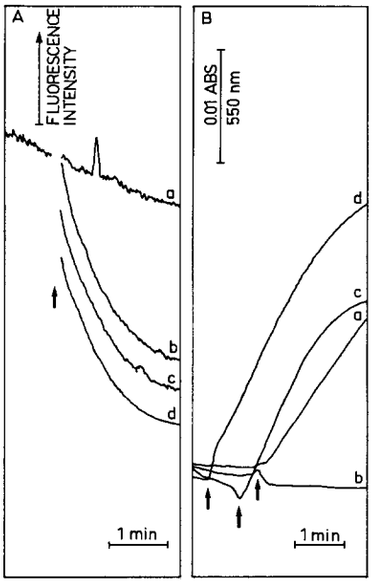

Fig. 1. Determination of the NADPH and O_2^- permeability of liposomes with incorporated cytochrome *b*-559. (A) Oxidation of intraliposomal NADPH by an external glutathione-glutathione reductase system. 1 ml of azolectin dispersion (7 mg/ml) was supplemented with 150 pmol of cytochrome *b*-559 and 1.45 mM of NADPH and treated as described under Materials and methods, 200 μl of final liposome suspension were diluted to 0.6 ml with buffer B and fluorescence (excitation 340 nm, emission 450 nm) was monitored. At the time indicated by the arrow, 1.23 mM glutathione and 0.0037 unit of glutathione reductase were added to: (a) the intact system, (b) the system treated with 30 mM octyl glucoside, (c) the system treated by freezing-thawing (−75–0° C), and (d) an equivalent amount of free NADPH. (B) Reduction of intraliposomal cytochrome *c* by external xanthine-xanthine oxidase system. 1 ml of azolectin dispersion (7 mg/ml) was supplemented with 139 pmol of cytochrome *b*-559 and 180 μM of cytochrome *c* and treated as described under Material and methods. 100 μM of final suspension were diluted with buffer B to 0.65 ml and absorbance at 550 nm was monitored. At times indicated by arrows 50 μM xanthine and 0.00036 unit xanthine oxidase were added to: (a) intact vesicles, (b) intact vesicles + 100 U/ml SOD, (c) vesicles treated by freezing-thawing (−75–0° C), and (d) vesicles treated with 30 mM octyl glucoside.

Bearing in mind these results, we investigated electron transport in cytochrome *c*-loaded *b*-559-liposomes supplemented with external NADPH. Data in Fig. 2A demonstrate that reflavinated cytochrome *b*-559, incorporated into cytochrome-*c*-loaded liposomes by cosolubilization with phospholipid, transfers electrons from external NADPH to internal cytochrome *c* with an initial rate essentially independent of external SOD (at the same time, further cytochrome *c* reduction revealed also the presence of the SOD-sensitive component). The process becomes entirely SOD-sensitive after liposome permeabilization by freezing-thawing (Fig. 2B). Evidently, SOD-resistant cytochrome *c* reduction indicates intraliposomal formation of O_2^-, i.e., it proves that in this system cytochrome *b*-559 catalyzes transmembrane electron transfer from external NADPH to internal oxygen (the SOD-sensitive component apparently reflects reduction of intravesicular cytochrome *c* due to interliposomal O_2^- transfer from other vesicles). When cytochrome *b*-559 was reconstituted into liposomes by direct incorporation, NADPH-induced cytochrome *c* reduction was completely SOD-sensitive in the intact poorly permeable vesicles (Fig. 2C), suggesting that electron transfer, in this case, was confined to the outer side of the membrane, followed by O_2^- diffusion into the liposomes.

For the investigation of the membrane potential formation in *b*-559-liposomes, SOD and catalase were included both inside and outside the vesicles, in order to quench O_2^- at the site of its formation and prevent its transmembrane diffusion. The medium was supplemented with 30 μM LiDS, which is known to accelerate electron transport in this system [15,16]. Fig. 3 illustrates a typical membrane potential measurement with the membrane-penetrating cationic dye safranin, which undergoes an absorbance shift on accumulation inside negatively charged vesicles [27,28]. The applicability of this method was confirmed by the differential spectrum (Fig. 3, inset) characteristic of accumulated safranin [27,28] and by the effect of omitting FAD (Fig. 3, curves a,b). As apparent in Fig. 3, NADPH-induced potential formation (negative inside, exceeding 45 mV from K^+-valinomycin calibration) takes place only with *b*-559-liposomes prepared by cytochrome-lipid cosol-

ubilization (curve a); *b*-559-liposome prepared by cytochrome incorporation, with only slightly lowered electron transport activity (5 against 7 e^-/heme per s), were unable to generate membrane potential (curve c). These data on the orientation of cytochrome *b*-559 in the membrane agree with those based on the effect of SOD and suggest that cytochrome *b*-559 can be incorporated into membranes in two manners, which are similar in their catalytic activity but differ in vectorial and electrogenic properties. Both variants of relipidated cytochrome *b*-559 being placed into a conventional cell-free system [9,15] could be further activated by cytosol with turnover rate rising from 5–10 to 50–60 mol O_2^-/mol cytochrome *b*-559 per s (these values should be considered as minimal because of the possibility that some fraction of cytochrome *b*-559 is inactive due to the inside-oriented NADPH-binding site). This confirms the complete functional competence of both membrane-bound forms of cytochrome *b*-559.

The data presented demonstrate the existence of two states of membrane-incorporated flavocytochrome *b*-559: (a) with a transmembranal topography of electron input and output sites, and (b) with both sites exposed on the same side of the membrane. They catalyse, respectively, vectorial electrogenic and scalar electron transfer from NADPH to oxygen. Apparently, the first state underlies the physiological transmembranal operation of the respiratory burst oxidase, whereas the second one suggests the possibility of its functioning on the one side of the membrane, which could be of interest from the standpoint of respiratory burst pathology. On the other hand, though a number of recent data prove the ability of cytochrome *b*-559 to perform NADPH-supported FAD-dependent O_2 reduction [10–12,15,16], some findings suggest that it can interact with other flavoproteins [30–32], including one located in the neutrophil membrane [33,34], and the possibility of cytochrome *b*-559 cooperating with additional electron transferring component in vivo still cannot be completely ruled out. In the light of this, it is of interest that relipidated and reflavinated cytochrome *b*-559 provides the basis not only for the catalytical, but also for the vectorial and electrogenic functions of the native NADPH oxidase. Finally, it should be mentioned that the presence of diffusion barriers in cytochrome *b*-559-containing liposomes may lead to underestimation of NADPH oxidase turnover rates measured in the cell-free system.

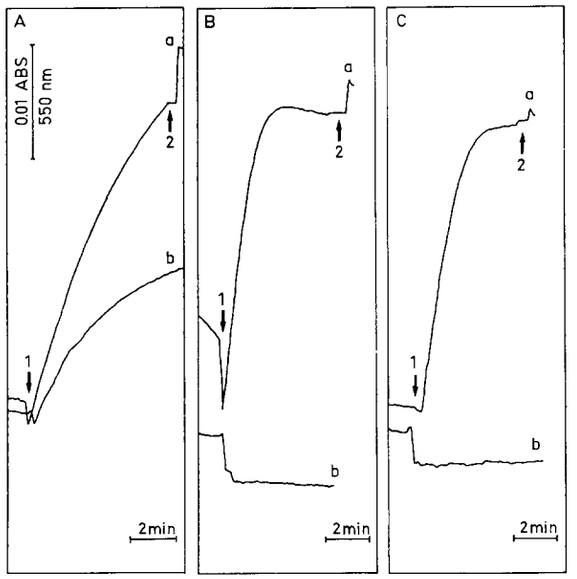

Fig. 2. Determination of spatial orientation of cytochrome *b*-559 incorporated into liposomes. (A) Electron transport in the intact cytochrome *c*-loaded *b*-559-liposomes, prepared by cytochrome *b*-559-phospholipid co-solubilization. 100 μl of *b*-559-liposomes, prepared as described in the legend to Fig. 1B, were diluted with buffer B to 0.65 ml, supplemented with 0.73 μM FAD and NADPH-driven cytochrome *c* reduction was monitored in the absence (a) or presence (b) of 100 U/ml SOD (saturating amount). Arrows marked 1 indicate the addition of 0.43 mM of NADPH, dithionite reduction is pointed by arrows marked 2. (B) Electron transport in the same liposomes, treated by freezing-thawing. (C) Electron transport in the intact cytochrome *c*-loaded *b*-559-liposomes, prepared by the direct incorporation of cytochrome *b*-559.

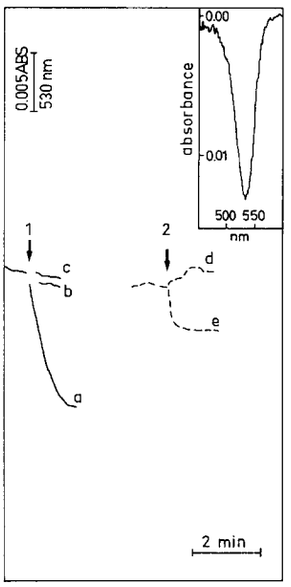

Fig. 3. Membrane potential formation in the course of the electron transport in *b*-559-liposomes. 100 μl of *b*-559-liposomes, prepared by cytochrome *b*-559 co-solubilization (curves a,b) or direct incorporation (curve c) as decribed under Materials and methods were diluted with buffer B to 0.65 ml and supplemented with 7.4 μM safranin, 30 μM LiDS and 0.73 μM FAD (curves a,c). The reaction was started by the addition of 0.22 mM NADPH indicated by arrow marked 1 and absorbance at 530 nm was monitored (dilution effects were corrected by subtracting the signal change induced by addition of an equal volume of buffer B). The dotted line represent calibrating marks obtained after addition of 0.1 μM valinomycin (arrow marked 2) to the *b*-559-liposomes prepared in K^+-containing medium and placed into K^+- (curved d) or Na^+- (curve e) containing buffer B. In the inset is shown the differential spectrum (after *minus* before NADPH addition) of the liposome suspension, curve a.

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