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Energization of *Bacillus subtilis* Membrane Vesicles Increases Catalytic Activity of Succinate:Menaquinone Oxidoreductase

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Abstract—In this work, high $\Delta\mu H^+$-dependent succinate oxidase activity has been demonstrated for the first time with membrane vesicles isolated from *Bacillus subtilis*. The maximal specific rate of succinate oxidation by coupled inside-out membrane vesicles isolated from a *B. subtilis* strain overproducing succinate:menaquinone oxidoreductase approaches the specific rate observed with the intact cells. Deenergization of the membrane vesicles with ionophores or alamethicin brings about an almost complete inhibition of succinate oxidation. An apparent K_m for succinate during the energy-dependent succinate oxidase activity of the vesicles (2.2 mM) is higher by an order of magnitude than the K_m value measured for the energy-independent reduction of 2,6-dichlorophenol indophenol. The data reveal critical importance of $\Delta\mu H^+$ for maintaining active electron transfer by succinate:menaquinone oxidoreductase. The role of $\Delta\mu H^+$ might consist in providing energy for thermodynamically unfavorable menaquinone reduction by succinate by virtue of transmembrane electron transport within the enzyme down the electric field; alternatively, $\Delta\mu H^+$ could play a regulatory role by maintaining the electroneutrally operating enzyme in a catalytically active conformation.

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Key words: succinate:menaquinone oxidoreductase, $\Delta\mu H^+$, *Bacillus subtilis*, electrogenesis, menaquinone, fumarate

Succinate:menaquinone oxidoreductase from *Bacillus subtilis* belongs to the group of enzymes which catalyze $2e^-$ reduction of the membrane-bound quinone with succinate (succinate:quinone oxidoreductases, SQR) [1]. There is a related group of similar enzymes which catalyzes the reverse process (quinol:fumarate oxidoreductases, QFR) [2]. Apparently, the SQR and QFR functions of the same enzyme *in vivo* are never actuated at the same time (being characteristic, respectively, of the aerobic and anaerobic type of metabolism [3]) and usually an enzyme of only one type is expressed in a cell under given conditions [4]. Nevertheless, each member of the SQR/QFR family is able to carry out both reactions quite efficiently [5, 6]. The direction of a process is determined by a redox potential difference between a donor and an acceptor of electrons [5].

Depending on the structure of membrane subunit(s), the enzymes of the family are subdivided into types A-E [7]. According to this classification, SQR from *B. subtilis* belongs to B-type because it contains two hemes [8] (see Scheme). Hexacoordinated low-spin hemes *b*, with two imidazoles as the axial ligands of heme iron, are placed one on top the other with their planes normal both to each other and to the membrane [9]. E_{m7} values of the hemes are +65 mV and −95 mV [8, 10]. The high potential heme b_H is located near the cytoplasmic subunits of the enzyme, which harbor FAD and FeS cofactors, whereas the low-potential heme b_L is closer to the periplasmic protein surface [11]. Quinone reduction takes place near the b_L heme [12], i.e. at the outer side of the cell membrane. The B-type subfamily of the enzymes includes also QFR from *Wolinella succinogenes*, whose 3D structure has been solved with 2.2 Å resolution [13].

The presence of the second heme near the outer surface of the membrane is a typical feature of menaquinone-containing organisms (mostly anaerobes) where the enzyme acts as a fumarate reductase. The nearly obligate aerobe *B. subtilis* represents an exception to this rule because its di-heme SQR performs the "direct" reaction and feeds electrons into the respiratory chain

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCCD, N,N′-dicyclohexylcarbodiimide; DCPIP, dichlorophenol indophenol; FAD, flavin adenine dinucleotide; PMS, phenazine methosulfate; QFR, quinol:fumarate oxidoreductase; SQR, succinate:quinone oxidoreductase; TMPD, N,N,N′,N′-tetramethyl-*p*-phenylenediamine.

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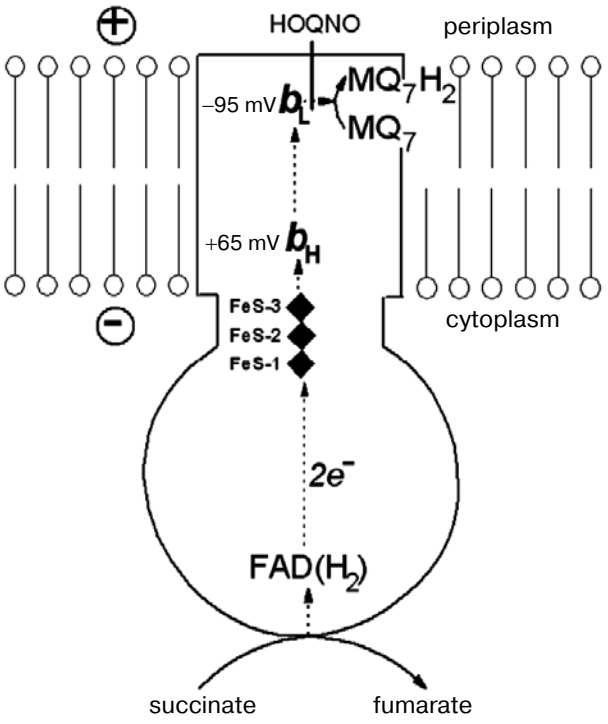

Succinate:menaquinone oxidoreductase from *B. subtilis*. The following redox cofactors are shown on the scheme: the covalently bound FAD molecule ($FADH_2$ after $2e^-$ reduction), the three FeS clusters (black diamonds), and the two hemes *b*. Electron transfer direction is shown by the dotted arrows. The curved solid arrows depict $2e^-$ reactions of succinate oxidation and MQ_7 reduction. The vertical line indicates the HOQNO inhibition site

from succinate to the quinone pool that is represented exclusively by menaquinone MQ_7 [14]. Obviously, electron transfer from the succinate/fumarate couple (E_{m7} = +24 mV [15]) to the MQ_7H_2/MQ_7 redox pair (E_{m7} = −74 mV [16]) is thermodynamically unfavorable, and it still remains unclear how this problem is solved.

There is a well established unusual circumstance: in contrast to the majority of bacteria and to mitochondria, uncouplers of oxidative phosphorylation do not stimulate respiration of *B. subtilis* cells (the so-called "respiratory control" effect [17, 18] is absent) but rather strongly inhibit oxygen consumption [19-22]. In the case of succinate-dependent respiration, this phenomenon could be explained by requirement of energy supply (in the form of $\Delta\mu H^+$) to drive the succinate:menaquinone reductase reaction in accordance with the widely held point of view that the thermodynamic barrier in the reduction of menaquinone by succinate is overcome at the expense of $\Delta\mu H^+$ produced by the other respiratory chain components [20, 23-25].

There is some evidence for such a hypothesis. In particular, it has been shown for liposome-reconstituted SQR from *B. licheniformis* (similar to the *B. subtilis* enzyme) that both the succinate:quinone reductase and quinol:fumarate reductase reactions can be coupled to the generation of $\Delta\mu H^+$ (of different sign) provided the redox potential difference between the donor and acceptor (an artificial quinone analog of menaquinone) is large enough [26]. However, no influence of $\Delta\mu H^+$ on the intraprotein electron transfer was revealed for proteoliposomes containing thermophilic SQR from *Rhodothermus marinus* [27].

A model based on the quinol:fumarate reductase reaction coupled to $\Delta\mu H^+$ generation conforms to the 3-D structure of *W. succinogenes* QFR [13, 28, 29]. The two *b* hemes are positioned across the membrane, whereas the menaquinol-binding site is located in the cavity opening into the periplasmic space. The protons released upon the oxidation of menaquinol are to be extruded outwards into the external medium, and the electrons that have to cross the membrane from the outside to the inside should generate transmembrane electric potential difference. Nevertheless, experiments with cells, subcellular vesicles, and proteoliposomes containing *W. succinogenes* QFR have demonstrated clearly that the fumarate-reductase activity of the enzyme is electroneutral [30, 31].

To reconcile the structural data with the experimental results, a so-called "E-path" hypothesis was put forward (where "E" designates a glutamate residue) [32]. According to this model, electrogenic transfer of two electrons across the membrane within *W. succinogenes* QFR is charge-compensated by a parallel transfer of two protons through the protein [33]. In the opinion of the authors, it is the E180 residue that plays a key role in this putative proton transfer pathway. Mutagenesis data confirmed the necessity of E180 for the quinol:fumarate reductase activity of the enzyme and revealed the effect of the residue replacement on the E_m value of heme b_H [34]. Moreover, replacement of Glu180 by Gln confers the ability to generate $\Delta\mu H^+$ to liposome-reconstituted *W. succinogenes* QFR during fumarate reduction, although this has been observed only in the presence of artificial electron donors much more reducing ($E_{m7} \sim -170$ mV) than MQ_7H_2 [35].

Therefore, the molecular mechanism of menaquinone reduction by the SQR/QFR family enzymes of B-type in native membrane, as well as the mechanism of the reverse reaction (menaquinol oxidation with fumarate), although of great interest, is far from clear and requires further investigation. The putative tight coupling of electron transfer with generation or consumption of $\Delta\mu H^+$ in these enzymes remains to be confirmed. It is worth noting that the well documented dependence of the succinate oxidase [20, 24, 25] and the succinate:quinone reductase [22, 24] activities of intact cells of *B. subtilis* on membrane energization can be explained alternatively by a kinetic mechanism; $\Delta\mu H^+$ could stimulate the membrane-bound SQR merely by maintaining the enzyme in its active conformation.

Until recently, the inhibition of *B. subtilis* succinate respiration induced by deenergization of the membrane

could be observed only on whole cells [20, 24, 25]. This circumstance greatly limited the experimental approaches towards investigation of the effect. Here, conditions have been found which allowed study for the first time of the energy dependence of the succinate oxidase activity with inside-out subcellular membrane vesicles.

MATERIALS AND METHODS

Chemicals. The following commercial products of high purity were used: yeast extract from Difco (USA); phenazine methosulfate (PMS) from Serva (Germany); alamethicin and acridine orange from Merck (Germany); valinomycin from Fluka (Germany); NADH from ICN (USA); *n*-dodecyl-β-D-maltoside, phenylmethylsulfonyl fluoride (PMSF), N,N′-dicyclohexylcarbodiimide (DCCD), N,N,N′,N′-tetramethyl-*p*-phenylenediamine (TMPD), menadione (vitamin K_3), DNase I, D,T-diaphorase, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), monensin, and nigericin from Sigma-Aldrich (USA). Other chemicals were mostly high-purity grade products of local Russian companies.

Purified SQR from *B. subtilis* was a kind gift of Dr. Lars Hederstedt (Lund University, Sweden).

Cell cultures. The following *B. subtilis* strains were kindly provided by Drs. Lars Hederstedt and Claes von Wachenfeldt (Lund University): 3G18 (*trpC2 ade met*, "wild type"), 3G18/pBSD1200 (the strain with enhanced content of succinate:menaquinone oxidoreductase; the content of pBSD1200 plasmid carrying the *sdhCAB* operon and the genes conferring resistance to chloramphenicol and to erythromycin is about four copies per cell [8]), LUH-17 (*trpC2 galT ΔctaCD::ble ΔqoxABCD::kan*, the mutant with caa_3 and aa_3 terminal oxidases deleted [36]). Strain LUH-17/pBSD1200 was obtained by transformation of LUH-17 cells with pBSD1200 plasmid isolated from 3G18/pBSD1200 cells. The plasmid was purified according to the Wizard Plus Minipreps DNA Purification System (Promega, USA) vacuum protocol. The transformation procedure followed the method described in [37]; the plasmid-containing clones were selected on 2% agar LB medium plate by their ability to grow in the presence of chloramphenicol (5 mg/liter).

Cells were grown in YMP medium (100 mM potassium phosphate, pH 7.0, 1 mM $MgSO_4$, 0.5% yeast extract) at 37°C with high aeration. Usually, 60 mM potassium succinate (pH 7.0) was used as an energy source; in some cases, 0.1% glucose was added to the medium to increase expression of the *bd*-type terminal oxidase. To obtain glycerol-3′-phosphate dehydrogenase containing preparations, the cells were grown in the YMP medium supplied with 0.4% glycerol. Grown cells were spun down by centrifugation (14,000*g*, 10 min), suspended in 50 mM potassium phosphate, pH 8.0, with 50 mM potassium sulfate, 1 mM $MgSO_4$, and 0.1 mM EDTA, and then spun again using the same regime; the pellet with glycerol added (20%) was frozen and stored at −30°C.

Membrane preparation. Membranes were isolated with the aid of a French press. The cells were thawed and suspended in medium (50 mM Hepes-KOH, pH 8.0, 50 mM potassium sulfate, 5 mM $MgSO_4$, and 0.5 mM EDTA) with 5 mg DNase I and 0.5 mg/ml lysozyme, and then the suspension was incubated at 37°C with careful mixing for 75 min. Spheroplasts were spun down (20,000*g*, 20 min). The pellet was resuspended in 20 ml of medium (20 mM Hepes-KOH, 50 mM sodium sulfate, 5 mM $MgSO_4$, 0.5 mM EDTA (acid), pH 7.6) supplemented by 2 mg DNase I and 1.4 mg PMSF (dissolved in ethanol). Further procedures were carried out at 2°C. Spheroplasts were disrupted by the French press. Cell debris were separated by centrifugation (12,000*g*, 20 min) and discarded. Membranes were spun down (48,000*g*, 60 min), resuspended in 0.1 M potassium phosphate, pH 6.6, and centrifuged again under the same conditions. The resulting material was suspended in medium (20 mM Hepes-KOH, pH 7.6, 50 mM sodium sulfate, 5 mM $MgSO_4$, 0.5 mM EDTA (acid)) and either used immediately or frozen in liquid nitrogen, stored at −70°C, and thawed when necessary. Protein content in the preparations was determined by the BCA reaction (Pierce, USA) using calibration against bovine serum albumin.

Spectroscopy. Most of the spectrophotometric measurements were made with a Cary-300 spectrophotometer (Varian, USA) at 20°C in a standard 1 cm optical path cell. Absolute absorption spectra were recorded at 1 nm/sec rate with 2 nm slit width (visible region) or at 0.2 nm/sec with 5 nm slit width (UV region). Anaerobic spectra were registered in a cell filled to the very top and sealed. Difference spectra were obtained by subtraction of successively recorded absolute spectra.

Enzymatic activities. *Respiration.* Oxygen consumption in the course of oxidase reactions was followed amperometrically in a closed cell with stirring, using a Clark-type electrode connected to a computer, at 20°C. Where indicated, oxidase activity was monitored in the presence of different concentrations of DCCD added from fresh ethanol stock solution (see notes in the text). When comparing the succinate oxidase activity with either the dichlorophenol indophenol (DCPIP) reductase or with the succinate dehydrogenase activities the respiration was measured in the same basic medium as the partial reaction (see below). To inhibit respiration with cyanide, fresh KCN solution was used. With menadiol as the substrate, the experimental medium was supplemented with 1 mM NADH, 100 μM vitamin K_3, and 0.15 mg/ml D,T-diaphorase; under such conditions almost all the oxidase activity observed resulted from the oxidation of menadiol by respiratory chain terminal quinol oxidases.

Succinate dehydrogenase activity of the membranes as well as of the solubilized SQR was determined by following the rate of absorption decrease of the oxidized form of

DCPIP at 600 nm (ε value under our conditions was 20.7 $mM^{-1} \cdot cm^{-1}$) in the presence of PMS. The measurements were carried out with the Cary-300 spectrophotometer in kinetic mode, with 3 nm bandwidth, at 20°C. The medium contained 20 mM Hepes/KOH, 50 mM Na_2SO_4, 5 mM $MgSO_4$, 0.5 mM EDTA, 20 mM potassium succinate, and 1 mM DCCD, pH 7.6. Control experiments showed that under our conditions, DCCD did not influence the assayed characteristics of SQR either in solubilized form or in the membrane vesicles. When the solubilized enzyme was examined, the media contained additionally 0.05% β-D-dodecyl maltoside, and SQR was added up to 0.03 μM. Membrane samples were investigated at protein concentration of ~10 μg/ml and were preincubated for 5 min in the assay medium supplemented with 1 mM KCN. The reaction was started by the subsequent additions of 0.16 mM DCPIP and 0.8 mM PMS.

DCPIP reductase activity was monitored like the succinate dehydrogenase reaction but omitting PMS.

Generation of ΔpH by membranes (internal acidification of membrane vesicles). To follow energy-dependent acidification of the inside-out membrane vesicles, a conventional method was used based on the decrease in fluorescence quantum yield of the membrane-permeable dye acridine orange [38]. The experimental medium contained 20 mM Hepes/KOH, pH 7.6, 50 mM Na_2SO_4, 5 mM $MgSO_4$, and 0.5 mM EDTA, acridine orange concentration was 2 μM, and membranes were added up to 0.1 mg protein per ml. Measurements were carried out using fluorimeters MPF-4 (Hitachi, Japan) and Fluoromax-3 (Jobin Yvon, USA), at 20°C, with stirring, in a quartz cuvette with 1 cm optical path. Excitation was at 493 nm and emission was followed at 530 nm, both beam bandwidths being set at 10 nm. Response was expressed as percent of the scale, taking the fluorescence level before addition of the respiratory substrate as 100%.

Generation of Δψ by the inside-out membrane vesicles (positive inside). Energy-dependent generation of transmembrane electric potential was monitored by following the spectral shift of the lipophilic anionic dye oxonol VI, which is accumulated inside the membrane of the vesicles [39]. Optical absorption difference (A_{628} minus A_{587}) changes were followed using an SLM-Aminco DW2000 spectrophotometer (Olis, USA) in dual wavelength kinetics mode, with 3 nm bandwidth. Oxonol VI was added to 2.5 μM concentration with 0.05-0.20 mg membrane protein per ml in a standard cuvette with 1 cm optical pathway thermostatted at 20°C, with stirring. The medium was the same as used in the ΔpH-generation experiments (see above).

RESULTS

Characteristics of the *B. subtilis* membrane preparations. The respiratory chain composition of the membranes isolated from the succinate-grown 3G18/pBSD1200 cells (approximately fourfold enhanced SQR expression as compared to the "wild type") is characterized by optical absorption difference spectra in Fig. 1. The visible range (a) presents the set of cytochromes. It is dominated by contribution of cytochromes *b* (maximum at 558 nm) and aa_3 (maximum at 601 nm). Their content is, respectively, about 3.5 and 0.5 nmol of the low-spin hemes per mg protein, which is typical of bacilli [40]. In accordance with the literature [36], cytochrome *bd* expression is minimal under the given conditions (there are no absorption bands in the 620-660 nm range). Only trace amounts of cytochrome complex bc_1 are present in *B. subtilis* and the complex harbors only one normal heme *b* [41, 42]. So, almost all the heme *b* in Fig. 1a belongs to the di-heme cytochrome *b* subunit of SQR. According to the spectrum, there is about 1.8 nmol of SQR per mg protein, which exceeds 3-4-fold the enzyme content in the succinate grown "wild type" (lacking plasmid pBSD1200).

Addition of 20 mM succinate brings about reduction of about half of the total heme *b* (spectra *1* and *2*). Most probably, this is the high-potential heme b_H which is succinate reducible, whereas reduction of the low-potential heme b_L requires addition of dithionite (spectrum *3*). Such behavior is typical of a di-heme cytochrome *b* in SQR preparations from *B. subtilis* and other bacteria [8, 43, 44]. It is noteworthy that the extent of heme *b* reduction with excess succinate in the presence of oxygen is nearly the same as under anaerobic conditions (compare spectra *1* and *2* at Fig. 1a). This result coincides with data for *B. cereus* [44] and shows that oxidation of SQR hemes by menaquinone (followed by electron transfer to oxygen) proceeds much slower than reduction of the hemes by succinate.

As one can see from comparison of spectra *2* and *3*, under anaerobic conditions succinate reduces terminal oxidases aa_3 and caa_3 (the overall maximum at 601 nm) to approximately 90%. The incomplete reduction can be caused by inability of succinate to permeate through the membrane. Accordingly, it can serve as a respiratory chain substrate only for inside-out vesicles and unsealed membrane particles. The data indicate that right-side-out vesicles can comprise about 10% of the preparation.

Absorption spectra in the near-UV range characterize the quinone pool of the membranes. Difference (reduced *minus* oxidized) spectra of membranes from the cells grown on the mixture of succinate and glycerol are shown in Fig. 1b. Glycerol in the growth medium induces membrane-associated glycerol-3′-phosphate dehydrogenase [45], which enables reduction of the respiratory chain with glycerol-3′-phosphate (spectra *1* and *3*). Glycerol-3′-phosphate (spectra *1* and *3*) as well as succinate (spectrum *2*) gives rise to spectral changes that are typical of menaquinone reduction (maximum at 248 nm, broad minimum at 260-270 nm) [14]. Anaerobically

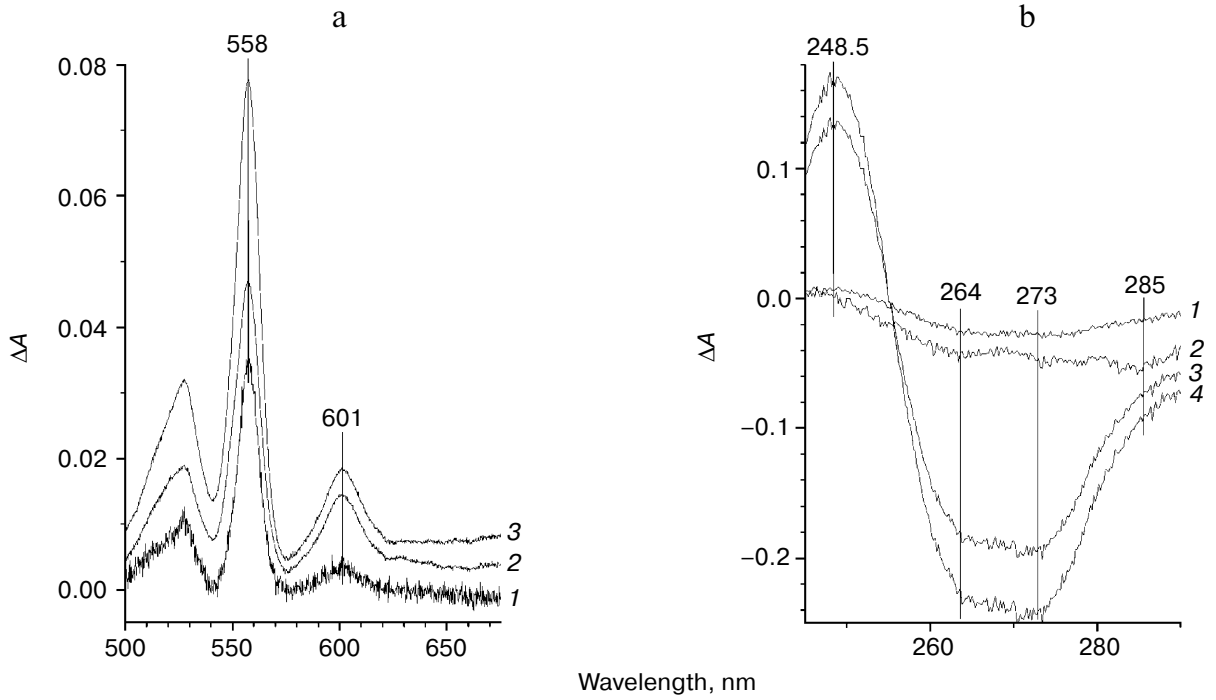

Fig. 1. Respiratory chain components of *B. subtilis* 3G18/pBSD1200 membranes. a) Cytochromes. Optical absorption difference spectra are presented for the membranes from the succinate-grown cells. Experimental medium: 20 mM Hepes/KOH, pH 7.6, 50 mM Na_2SO_4, 5 mM $MgSO_4$, 0.5 mM EDTA; membranes were added to 0.15 mg protein per ml (*1*) or to 0.8 mg protein per ml (*2*, *3*). The spectra show the absorption changes caused by the addition of: *1*) 20 mM potassium succinate, pH 7.6, aerobic conditions; *2*) 20 mM succinate, under anaerobiosis; *3*) 5 mM sodium dithionite. The signals are normalized to the protein concentration. b) Quinones. Optical absorption difference spectra are presented for the membranes from cells grown in the presence of succinate and glycerol (see "Materials and Methods"). The initially oxidized sample was reduced by 20 mM glycerol-3′-phosphate (*1*, *3*), 20 mM potassium succinate (*2*), or 5 mM sodium dithionite (*4*). Spectrum *1* is recorded aerobically, spectra *2-4* are taken under anaerobic conditions when the terminal oxidases are reduced (see text). Spectra were obtained with 50 μg protein per ml; other conditions as in panel (a).

(spectrum *3*), glycerol-3′-phosphate reduces menaquinone almost completely (compare with spectrum *4* recorded after dithionite addition). As discussed above, a small amount of menaquinone, which remains oxidized under these conditions, might belong to right-side-out vesicles in which respiratory chain is unavailable for glycerol-3′-phosphate. The total amount of menaquinone (about 10 nmol per mg protein) as estimated from spectrum *4* using an extinction coefficient of 15 $mM^{-1}\cdot cm^{-1}$ is typical for membranes from bacilli [14]. Contrary to glycerol-3′-phosphate, succinate reduces menaquinone anaerobically by only 15% (spectrum *2*), which is in agreement with the lower reducing power of this donor (E_{m7} values of the couples MQ_7H_2/MQ_7, glycerol-3′-phosphate/dihydroxyacetone phosphate, and succinate/fumarate are −74, −190 [16], and +24 mV [15], respectively). The steady-state menaquinone reduction level in the presence of oxygen and glycerol-3′-phosphate (in the course of respiration) is about 10% (spectrum *1*), whereas during respiration on succinate this value does not exceed a few percent (not shown). These data point to rapid oxidation of membrane menaquinol by terminal oxidases in both cases.

Membrane vesicles respire actively (tens-to-hundreds nmol O_2 per min per mg protein) in the presence of such substrates as NADH, glycerol-3′-phosphate, succinate, ascorbate + TMPD, and menadiol (Table 1). The sensitivity of respiration to cyanide varies significantly: thus, 1 mM KCN inhibits NADH oxidation only slightly, respiration on glycerol-3′-phosphate is inhibited about 2-fold, whereas in the other three cases an almost complete suppression of oxygen consumption is observed. The relative resistance of glycerol-3′-phosphate oxidase activity to cyanide is probably explained by the presence of a cyanide-resistant quinol oxidase *bd* [46] whose expression is induced by glycerol (see legend to Table 1). The probable reasons for resistance of NADH oxidase activity to cyanide will be discussed below.

Respiration on glycerol-3′-phosphate, succinate, or menadiol is accompanied by a well established internal acidification of inside-out membrane vesicles (see Table 1 and results below). This reports electron transfer coupled to $\Delta\mu H^+$ generation. The smaller response in the case of glycerol-3′-phosphate oxidation (as compared to succinate oxidation) might result from a significant contribution to the overall respiratory activity of *bd*-type terminal

Table 1. Respiratory activities of isolated *B. subtilis* membranes. The membranes were freshly isolated from 3G18/pBSD1200 cells grown to the beginning of the stationary phase in YMP growth medium supplemented with 60 mM potassium succinate or (for experiments with glycerol-3′-phosphate) with 0.4% glycerol

Substrate of respiration	Oxidase activity, nmol O_2/min per mg protein	Inhibition of respiration by 1 mM cyanide (remaining activity), %	ΔpH generation in the course of respiration (acridine orange fluorescence quenching), standard units
NADH, 1 mM	150	80 ± 10	⩽ 2
Glycerol-3′-phosphate, 20 mM	246	40 ± 10	15
Succinate, 6 mM	283	10 ± 5	50
Ascorbate (10 mM) + TMPD (0.1 mM)	315	⩽ 5	⩽ 5
Menadiol*, 0.1 mM	183	10 ± 5	7

Note: Measuring medium: 0.1 M Mops/KOH, pH 7.0, 0.2 mM EDTA, 5 mM $MgSO_4$.
* Reduced form of menadiol was obtained using D,T-diaphorase in the presence of NADH (see "Materials and Methods").

oxidase (see above), which is a low-efficiency $\Delta\mu H^+$ generator [46, 47]. An absence or small amplitude of the response observed with artificial donors like TMPD and menadiol might be concerned with the mechanism of their oxidation by the terminal oxidases or with their ability to permeate through the membrane, which would result in involvement in respiration of both the inside-out and right-side-out vesicles. The method of ΔpH registration by quenching the acridine orange fluorescence is not applicable for the latter fraction of the preparation, which may give rise to artifacts. The NADH-induced response could be observed only in some experiments (see curve *2* in Fig. 2a below and the respective comments).

The registration of both $\Delta\mu H$ components (ΔpH and $\Delta\psi$) using acridine orange and oxonol VI dye, respectively, is illustrated by Fig. 2. Typically, response of acridine orange to a respiratory substrate develops with $t_{1/2}$ of about 30 sec, remains stable until one of the oxidase reaction substrates is exhausted (i.e., at least for 20 min), and is eliminated completely by ΔpH-dissipating agents (panel (a)). With succinate and glycerol-3′-phosphate, acidification of the vesicles was observed with all preparations that revealed sufficient electron transfer activity with these substrates. A small but reproducible NADH-induced signal could be observed within an hour after the isolation of membranes from strain LUH-17/pBSD1200 (a mutant with cytochrome *bd* as the only terminal oxidase and with a 4-fold enhanced SQR expression) grown in the presence of glucose (curve *2*).

Examples of the oxonol VI extinction changes reporting $\Delta\psi$ generation (positive inside) coupled to succinate or glycerol-3′-phosphate respiration are shown on Fig. 2b. It has to be noted that responses of this type were seen only with some of the samples and within a short period after isolation of the membranes; also, the signal size strongly depended on experimental conditions. A low reproducibility of the results in the experiments with oxonol VI might originate in operation of multiple *B. subtilis* electrogenic antiporters [48-50] that convert $\Delta\psi$ into ΔpH, as well as by ion leaks through the membrane that diminish primarily the $\Delta\psi$ component of $\Delta\mu H^+$.

A rapid increase in proton permeability of the membranes was observed for a fresh sample incubated on ice when the vesicles had been loaded with K^+ (not shown). One could see immediate quenching of the acridine orange fluorescence when 1 μM nigericin was added with a subsequent return of the fluorescence to the initial level. The rate of the latter process depended on the extent of proton leakage. Its $t_{1/2}$ value changed from ~10 min to ~30 sec when a sample was incubated at 0°C for 35 min. Apparently, K^+ permeability of the membrane is rather low, since the amplitude of the initial response to nigericin did not change. In other experiments, 5 μM valinomycin brought about quenching of fluorescence of the dye when added to the same preparation incubated at 0°C for 45 min.

Membrane energization stimulates respiration of subcellular vesicles from *B. subtilis*. Figure 3a presents the kinetics of oxygen consumption in the course of succinate oxidation by membrane vesicles. At relatively low concentrations of Mg^{2+} (1 mM Mg^{2+} in the presence of 0.5 mM EDTA), progressing attenuation of the respiratory activity can be seen on a minute time scale. Subsequent addition of excess Mg^{2+} restores the initial rate of the process (curve *1*). The respiration kinetics remains linear in media containing about 5 mM free Mg^{2+} (curve *2*). This effect is probably concerned with the decrease in passive proton permeability of the membranes in the presence of excess magnesium ions.

Succinate oxidation accelerates also in the presence of DCCD (Fig. 3b). At 0.5-1.0 mM DCCD the respiratory rate increases by 50-60% of the initial activity.

The best results were obtained with the combination of high Mg^{2+} and DCCD treatment (cf. curve *1* at Figs. 3a and 3c). Under these conditions, oxidation of succinate does not decelerate with time and reaches ~1500 nmol O_2

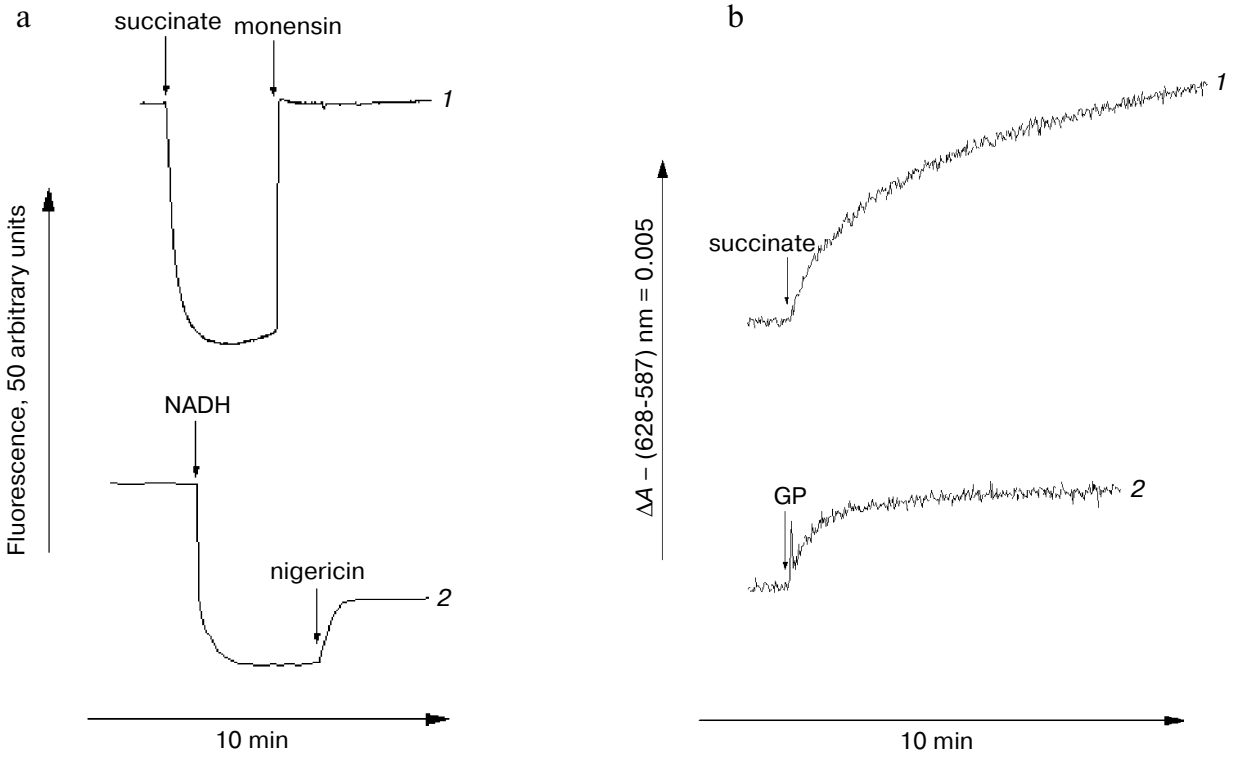

Fig. 2. Respiration-driven generation of $\Delta\mu H^+$ by the inside-out vesicles from *B. subtilis*. a) ΔpH generation. Acidification inside the membrane vesicles was determined from acridine orange fluorescence quenching. Curves: *1*) membranes (0.1 mg protein per ml) from succinate grown 3G18/pBSD1200 cells. The potassium succinate (6 mM) and monensin (5 μM) additions are indicated; *2*) freshly isolated membranes (40 μg protein per ml) from LUH17/pBSD1200 cells grown in the presence of succinate and glucose. NADH (1 mM) and nigericin (1 μM) additions are indicated. Besides the response that develops on a time scale of seconds and is associated with the vesicle acidification, the fluorescence quenching shows a rapid downward jump immediately upon addition of NADH, which is insensitive to nigericin (compare initial and final levels of fluorescence in curve *2*) and can be observed also in the absence of membranes. b) Transmembrane electric potential difference generation. Generation of Δψ was followed by oxonol VI optical absorption changes. Curves: *1*) membranes from succinate grown 3G18/pBSD1200 cells; 6 mM succinate is added; *2*) membranes from cells grown in the presence of succinate and glycerol; where indicated, 20 mM potassium glycerol-3′-phosphate (GP) is added.

per min per mg protein, which corresponds to transfer of ~60 electrons in 1 sec through an SQR molecule.

De-energization of vesicle membranes by various ionophores (curves *1-3*) or by the channel forming antibiotic alamethicin (curve *4*) results in strong inhibition of succinate respiration (Fig. 4a). The activity is suppressed completely with agents dissipating both components of $\Delta\mu H^+$. For comparison, Fig. 4d presents the effects observed upon uncoupling of *B. subtilis* whole cell respiration on endogenous substrates (see also [22]). Note that under the given conditions (cells grown on malate), the respiratory chain receives electrons mainly from NADH [51, 52].

Respiration of membranes with glycerol-3′-phosphate is also sensitive to uncouplers (Fig. 4c) though the effect is much less pronounced than in the case of succinate. Complete membrane de-energization on addition of the protonophore in combination with valinomycin brings about a two-fold inhibition of the oxidase activity. Like in the case of succinate, respiration of the vesicles with glycerol-3′-phosphate accelerates reproducibly by ~20-30% in the presence of 1 mM DCCD (not shown). The process is stimulated by Mg^{2+} as well, but it is difficult to relate the effect unambiguously to improved coupling of the membranes since magnesium ions are necessary for normal functioning of glycerol-3′-phosphate dehydrogenase itself [53, 54].

Notably, the inhibition of the NADH oxidase activity of the membranes by the uncouplers is very weak and probably unspecific (Fig. 4b, curve *1*), whereas the respiration of the intact cells on the endogenous NADH is suppressed almost completely upon membrane de-energization (Fig. 4d) [22]. This observation correlates with other peculiarities of NADH oxidation by the membrane vesicles. Thus, the process is almost insensitive to inhibition by cyanide and generates ΔpH with very low efficiency (Table 1). These facts might indicate impaired normal activity of the enzyme by which NADH reduces the menaquinone pool of the membrane. It is likely that under conditions of our experiments, the NADH dehy-

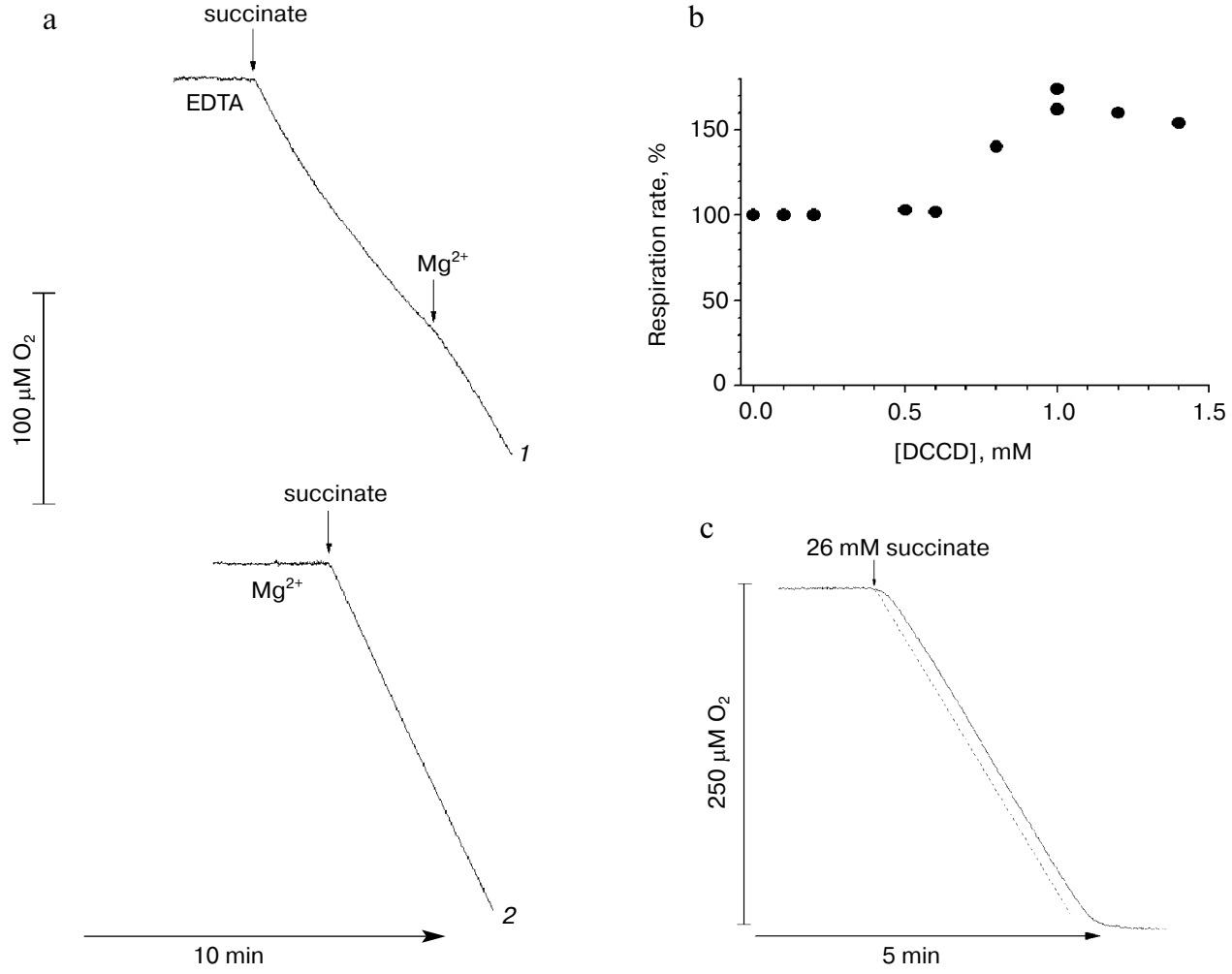

Fig. 3. Coupling agents stimulate succinate respiration of *B. subtilis* membranes. a) Effect of magnesium ions. Membranes (0.14 mg protein per ml) were isolated from strain 3G18/pBSD1200, 6 mM potassium succinate was added. Assay medium: 20 mM Hepes/KOH, pH 7.6, 50 mM Na_2SO_4. Curves: *1*) a measurement initiated in the presence of 0.5 mM EDTA and 1 mM $MgSO_4$, 5 mM $MgSO_4$ was added subsequently; *2*) the experiment was carried out in the presence of 0.5 mM EDTA and 5 mM $MgSO_4$. b) DCCD effect. The oxidase activity of the membranes was measured in medium containing 20 mM Hepes/KOH, pH 7.6, 50 mM Na_2SO_4, 5 mM $MgSO_4$, 0.5 mM EDTA, and DCCD added to the indicated final concentration; other conditions as in panel (a). Oxygen consumption rate was measured 1 min after addition of succinate. c) Succinate oxidase activity of the membranes under the conditions of maximal energization. Kinetics of oxygen consumption is shown for membranes (70 μg protein per ml) freshly isolated from 3G18/pBSD1200 strain. Assay medium: 20 mM Hepes/KOH, pH 7.6, 50 mM Na_2SO_4, 5 mM $MgSO_4$, 0.5 mM EDTA, and 1 mM DCCD. Succinate was added to the saturating concentration of 26 mM (see Fig. 5 and Table 2 below concerning an unusually high K_m for succinate in the membrane vesicles). The dotted straight line has been drawn to facilitate observation of the typical acceleration of respiration at the onset of respiration and subsequent linearity of the process under the given optimized conditions (cf. curves on Fig. 3a).

drogenase in the membrane vesicles transfers electrons, partly or entirely, not into the respiratory chain but rather directly to molecular oxygen. Alamethicin does accelerate aerobic oxidation of NADH to some extent (by a factor of 1.3-1.5), but this can probably be accounted for by the contribution of the closed right-side-out vesicles.

Dependence of the respiration rate of the coupled vesicles on concentration of added succinate is shown in Fig. 5. The data are well described by a saturating curve that corresponds to the Michaelis–Menten equation with $K_m = 2.2$ mM. Table 2 lists catalytic parameters of SQR in both solubilized and membrane-bound form for oxidation of succinate in the presence of three different electron acceptors.

Succinate:DCPIP reductase activity of the bacterial enzyme in the absence of PMS might mimic the natural activity with MQ_7 since DCPIP is likely to react with SQR at the quinone binding site [8, 55, 56]. In particular, the DCPIP reductase activity of *B. subtilis* SQR is known to be strongly suppressed by HOQNO [8] (see Scheme). However, in contrast to the intact cells where the DCPIP reduction, like respiration, is highly sensitive to $\Delta\mu H^+$

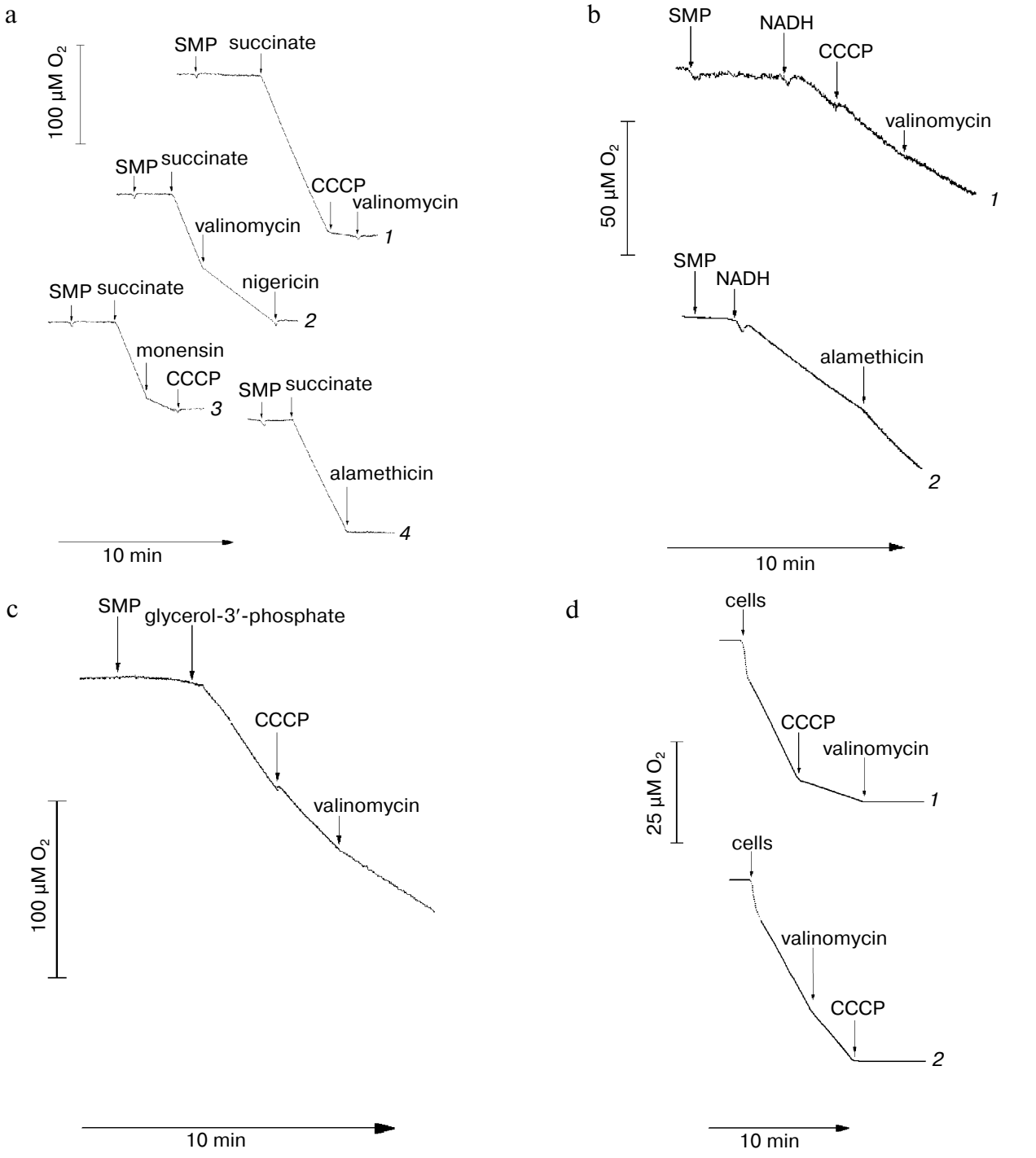

Fig. 4. Effect of membrane de-energization on *B. subtilis* respiratory chain activities with different substrates. a) Succinate oxidation by membranes. Respiration of freshly isolated subcellular membrane particles (SMP) (0.12 mg of protein per ml) from succinate grown 3G18/pBSD1200 cells in the presence of 6 mM succinate is shown. Assay medium: 20 mM Hepes/KOH, pH 7.6, 50 mM Na_2SO_4, 5 mM $MgSO_4$, 0.5 mM EDTA, and 1 mM DCCD. Additions: CCCP, 7.5 μM; valinomycin, 5 μM; nigericin, 10 μM; monensin, 5 μM; alamethicin, 100 μg per mg membrane protein. b) NADH oxidation by membranes. The membranes are added to 60 μg protein per ml; NADH, 1.5 mM. Other conditions and additions, as in panel (a), except that the medium does not contain DCCD. c) Glycerol-3′-phosphate oxidation by the membranes. Membranes (80 μg protein per ml) from 3G18/pBSD1200 cells grown in the presence of succinate and glycerol. An addition of 20 mM potassium glycerol-3′-phosphate is indicated. Other conditions and additions, as in panel (a). d) Endogenous respiration of intact *B. subtilis* cells. Cells from strain 168 A were grown with malate as a respiration substrate and added to 0.1 unit of optical density at 600 nm to medium containing 50 mM potassium phosphate, 1 mM $MgSO_4$, and 10 mM potassium malate, pH 7.0. Additions of 10 μM CCCP and 5 μM valinomycin are shown.

dissipating agents [22], the succinate:DCPIP reductase activity of the subcellular membrane particles is resistant to the uncouplers. Low specific rate of the reaction (about 12 e^- per sec per SQR molecule) is close to the residual activity of the whole cells under the conditions of membrane de-energization when DCPIP is reduced with endogenous substrates (see Table 1 in [22]).

The succinate dehydrogenase reaction (succinate → PMS → DCPIP) is also resistant to the uncouplers. The redox mediator PMS is believed to accept electrons from the FeS clusters of SQR and transfer them to DCPIP as the final acceptor [8, 56, 57]. The accelerated electron flow is followed in this case by a significant increase in both K_m (4-fold) and V_{max} (11-fold) as compared to DCPIP reduction without PMS.

Similarly, K_m and V_{max} values of the succinate oxidation increase when well-coupled membrane vesicles respire on succinate. This effect, apparently, is due to activation of the rate-limiting step of the overall process (presumably, the menaquinone reduction [44]) by $\Delta\mu H^+$ without any marked influence on the kinetic parameters of succinate binding and dissociation. The V_{max} value becomes as high as 62 e^- per sec per SQR molecule, which corresponds to ~60 O_2 molecules reduced in 1 sec by the terminal oxidases (the content of SQR in the 3G18/pBSD1200 membranes is at least 4-fold that of terminal oxidases). As compared to parameters of the specific succinate:DCPIP reductase activity (without PMS), K_m increases somewhat more (7-fold) than expected from the increase in V_{max} value (5-fold). This finding indicates that the $C_{50\%}$ value measured in the case of aerobic succinate oxidation by energized membrane vesicles might not correspond to the classical meaning of the Michaelis constant for a single enzyme molecule.

At succinate concentrations above 6 mM, the initial part of the respiration kinetics of the coupled membranes becomes nonlinear (Fig. 3c). In the first 1-2 min after onset of the respiration, the respiratory activity of the vesicles speeds up concurrently with membrane energization reaching finally the values characteristic of the high specific activity of the whole cells (compare to the linear kinetics in Fig. 3a).

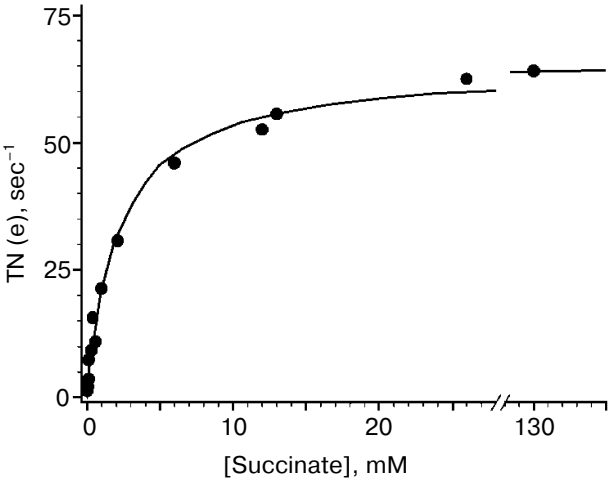

Fig. 5. Dependence of *B. subtilis* membrane respiration rate on succinate concentration. Maximal rate of the succinate oxidase reaction was measured with freshly isolated membranes from succinate grown 3G18/pBSD1200 cells. On the ordinate axis, the turnover number (TN) of SQR is plotted (i.e. the number of electrons delivered by one molecule of the enzyme from a donor to an acceptor in 1 sec). When calculating the TN value, 90% of the total membrane vesicle preparation was considered to be available for succinate. The experimental data are approximated by Michaelis–Menten dependence with K_m = 2.2 mM and V_{max} = 62 sec^{-1}.

DISCUSSION

In our previous work we have studied in detail a stimulating effect of $\Delta\mu H^+$ on the electron transfer to oxygen in the respiratory chain of the whole cells of *B. subtilis* [22] (a phenomenon first reported by Samuilov et al. [19, 21]). In this study, we have succeeded in demonstrating the energy-dependent stimulation of succinate oxidation and, to a lesser extent, of glycerol-3′-phosphate oxidation, by subcellular membranes of the same organism.

For the purpose of this work, it was desirable to obtain a preparation comprised mainly of the inside-out closed vesicles, since in this case the respiratory chain can be reduced by non-permeable substrates of the dehydro-

Table 2. Enzymatic activities of *B. subtilis* SQR in solubilized and membrane-bound forms

Preparation	Succinate→DCPIP		Succinate→PMS→DCPIP		Succinate→O_2	
	K_m, mM	V_{max}, sec^{-1}	K_m, mM	V_{max}, sec^{-1}	K_m, mM	V_{max}, sec^{-1}
Solubilized SQR	0.2	3.1	0.9	36	–	–
Membrane vesicles	0.3	12	1.2	131	2.2	62

Note: The values of K_m (substrate concentration providing for 50% activity) and V_{max} (as e^-/SQR in 1 sec) were determined using the Origin 7.0 program by fitting the data (the dependences of the SQR activity, y, on the concentration of succinate, x) by a function: $y = V_{max} \cdot x/(x + K_m)$. K_m and V_{max} values found in the succinate dehydrogenase reaction for the membranes are close to those previously reported [62] (0.9 mM and ~190 sec^{-1}, respectively).

genases. Besides, investigation of $\Delta\mu H^+$-dependent processes requires the particles to be sealed and well-coupled with low proton conductivity of their membrane.

For some reasons, previous studies of SQR in the membrane fraction of *B. subtilis* were carried out mostly with preparations obtained by osmotic shock [8, 20, 22]. This method involves treatment of the material with EDTA, which inevitably impairs the tightness of membrane coupling because of the decreased concentration of Mg^{2+} (apart from a general stabilizing effect on membrane structure [58, 59], magnesium ions are known to prevent dissociation of the outward facing F_1 subunit from the ATPase complex in the membranes [60] and thus to diminish proton leak through the F_o subunit). In the course of membrane isolation from *B. subtilis* cells, the succinate oxidase activity dropped down completely [20, 24, 25], which precluded investigation into the phenomenon of $\Delta\mu H^+$-dependent activation of SQR in the native membranes at the subcellular level.

In our study, membranes were isolated with the aid of a French press, most of the experiments were carried out with freshly prepared material, and all media contained about 10-fold excess of Mg^{2+} over EDTA. In addition, in some cases the membranes were also treated with DCCD, which is a usual practice to minimize proton leaks through F_o subunits of ATPase molecules damaged during the isolation procedure [61]. Besides, it is worth noting that in contrast to the previous work [25] carried out on "wild type" *B. subtilis* membranes obtained with the French press procedure, we have used strain 3G18/pBSD1200 with enhanced SQR expression [8].

High specific activities for oxidation of non-permeable substrates (see Table 1) point to rather small contribution of the right-side-out sealed vesicles. For example, judging from the level of cytochrome aa_3 reducibility by succinate under anaerobic conditions (cf. spectra *2* and *3* on Fig. 1a) such particles constitute about 10% of the preparation. A somewhat higher estimate (15-35% of right-side-out sealed vesicles) results from stimulation of the NADH oxidase activity with alamethicin (Fig. 4b, trace *2*; see comments in the text).

It appears to be that the preparations investigated in our work consist mostly of the inside-out membrane vesicles (65-90%), perhaps with some contamination of unsealed membrane fragments. The relative contribution of these two fractions can be estimated from the rates of respiration on succinate (Fig. 5 and Table 2). Under the coupled conditions and with the saturating substrate concentration, the succinate oxidase activity of the vesicles expressed as terminal oxidase turnover numbers (about 60 O_2 molecules reduced in 1 sec) is within the range of *B. subtilis* cell endogenous respiration rates (20-160 O_2 molecules in 1 sec; unpublished data). Since these are only the energized particles that can contribute noticeably to the respiration, the fraction of the unsealed membranes in the preparation is clearly small.

Our data show that it is membrane de-energization, which is the main cause (if not the only one) of the dramatic fall in the succinate oxidase activity, which occurs upon disruption of the *B. subtilis* cells. Indeed, the high specific respiration rate typical of the intact bacteria is completely restored with the coupled membrane, and the same pattern of inhibition of respiration by the uncouplers is observed with the cells and the membranes (cf. Figs. 4a and 4d).

It was established earlier that the inhibition of *B. subtilis* cell respiration by the uncouplers is not confined to succinate but applies to different substrates. In particular, the effect was reproduced with a mutant lacking SQR [22]. As discussed in paper [22], apart from the energy-dependent activation of SQR, there might be a more general mechanism operative that stimulates the respiratory chain activity by $\Delta\mu H^+$ at the level of menaquinone reduction. Indeed, the highest rate of glycerol-3′-phosphate oxidation by membranes was obtained in this work under the coupled conditions (see Fig. 4c and the notes in the text), though the effect of $\Delta\mu H^+$ is not so striking as compared to oxidation of succinate.

The dependence of *B. subtilis* succinate oxidase activity on energization of the membrane, first revealed with the whole cells [20, 22, 24, 25] and reproduced in this work with the subcellular membranes, might have different explanations.

It is commonly accepted that in the absence of transmembrane potential, the reaction is slow because of thermodynamic barrier that impedes the reduction of menaquinone (E_{m7} = −74 mV [16]) with succinate (E_{m7} = +24 mV [15]). Most authors assume that this barrier must be overcome at the expense of $\Delta\mu H^+$ energy. Indeed, as one can see from the Scheme, the structure of the membrane part of SQR conforms to the possibility of transmembrane electron transfer from heme b_H to heme b_L down the electric field and against the redox potential difference, with the subsequent isoenergetic reduction of membrane-bound menaquinone by the low potential heme b_L (E_m' = −95 mV [8, 10]).

It is worth noting that an alternative explanation is possible. As suggested in [12], the succinate:menaquinone-reductase reaction might yield a reduced MQ_7 tightly bound to SQR rather than free menaquinol. Such a reaction mechanism would allow overcoming the thermodynamic barrier between succinate and MQ without a consumption of $\Delta\mu H^+$ energy. The tight complex of the reduced MQ with SQR could subsequently react directly with some terminal oxidase, avoiding the step of MQ_7H_2 release into the membrane pool. In such a case, the role of membrane energization can be confined to a regulatory function maintaining SQR in the active conformation, while the electron transfer from succinate to MQ might not require dissipation of $\Delta\mu H^+$ energy.

Finally, it cannot be excluded that the enzymes of the SQR/QFR family can function both in electrogenic and

electroneutral modes. One can suppose that in the succinate oxidase reaction, which is of primary physiological importance for *B. subtilis*, it is the more energetically efficient electroneutral mechanism that is used (at least, in some cases). Our data demonstrating the non-electrogenic mechanism of the fumarate reductase activity of SQR in *B. subtilis* membrane vesicles are to be reported elsewhere.

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