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Reactivity of the *Bacillus subtilis* succinate dehydrogenase complex with quinones

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The succinate dehydrogenase isolated from *Bacillus subtilis* was found to catalyze the oxidation of succinate with hydrophilic quinones. Either naphthoquinones or benzoquinones served as acceptors. The enzyme activity increased with the redox potential of the quinone. The highest turnover number was commensurate with that of the bacterial succinate respiration in vivo. The succinate dehydrogenase was similarly active in fumarate reduction with quinols. The highest activity was obtained with the most electronegative quinol. The fumarate reductase isolated from *Wolinella succinogenes* catalyzed succinate oxidation with quinones and fumarate reduction with the corresponding quinols at activities similar to those of the *B. subtilis* enzyme. Succinate oxidation by the lipophilic quinones, ubiquinone or vitamin *K*-1, was monitored as cytochrome *c* reduction using proteoliposomes containing succinate dehydrogenase together with the cytochrome bc_1 complex. The activity with ubiquinone or vitamin K-1 was commensurate with the succinate respiratory activity of bacteria or of the bacterial membrane fraction. The results suggest that menaquinone is involved in the succinate respiration of *B. subtilis*, although its redox potential is unfavorable.

Introduction

In mitochondria and Gram-negative bacteria, ubiquinone is usually the acceptor of the reducing equivalents of succinate dehydrogenase, while reduced menaquinone serves as the donor of the bacterial fumarate reductase under anaerobic conditions [1–5]. *Bacilli* and other aerobic Gram-positive bacteria contain only menaquinone [3], the redox potential of which is about 0.1 V more negative than that of the succinate/fumarate couple. Therefore, menaquinone would not be expected to serve as the electron acceptor of the succinate dehydrogenase in these bacteria [4]. Experiments with the bacterial membrane suggested that menaquinone was an obligatory component of the electron transport chain catalyzing succinate respiration in *Bacillus subtilis* [5]. The respiratory activity of the menaquinone-depleted membrane was found to be stimulated on incorporation of menaquinone. As the occurrence of artifacts could not fully be excluded in the experiments with the bacterial membranes, it was desirable to measure quinone reduction directly using the isolated succinate dehydrogenase of *B. subtilis*. In this work we measured the activities of the enzyme both in succinate oxidation and fumarate reduction using hydrophilic quinones and the corresponding hydroquinones, respectively. Furthermore, the activity of succinate oxidation with cytochrome *c* was measured with proteoliposomes containing the succinate dehydrogenase of *B. subtilis*, the cytochrome bc_1 complex from beef heart and either ubiquinone or vitamin

Abbreviations: DCPIP, 2,6-dichlorophenolindophenol; DMN, 2,3-dimethyl-1,4-naphthoquinone; $DMNH_2$, hydroquinone of DMN; HQNO, 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide; MD, 2-methyl-1,4-naphthoquinone (menadione); MDH_2, hydroquinone of MD; MK, menaquinone; MKH_2, menaquinol; NBH, 2,3-dimethoxy-5-methyl-6-*n*-nonyl-1,4-benzoquinone; NQ, 1,4-naphthoquinone; NQH_2, hydroquinol of NQ; PMS, *N*-methylphenazinium sulfate; Q_0, 2,3-dimethoxy-5-methyl-1,4-benzoquinone; Q_0H_2, hydroquinone of Q_0; Q_9, ubiquinone-9.

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K-1. Vitamin K-1 has earlier been shown to replace menaquinone as a component of bacterial electron transport [2,6,7].

For comparison these activities were also measured with the fumarate reductase isolated from *Wolinella succinogenes*. This enzyme catalyzes the oxidation of menaquinol with fumarate in vivo and is structurally very similar to the succinate dehydrogenase of *B. subtilis* [8–13]. Both enzymes are composed of two hydrophilic and a hydrophobic subunit. The larger hydrophilic subunits carry the substrate sites and covalently bound FAD, while the smaller harbour at least the [2Fe-2S] iron-sulfur centers. The enzymes contain a [3Fe-4S] and a [4Fe-4S] center in addition. The hydrophobic subunits are diheme cytochromes *b*. The cytochrome *b* of fumarate reductase has been shown to react with reduced menaquinone [13]. The amino-acid sequences of the subunits of the two enzymes are homologous [9–12].

Methods

Isolation of succinate dehydrogenase, fumarate reductase and the cytochrome bc_1 complex

The succinate dehydrogenase was isolated from a *B. subtilis* strain (3G18/pBSD1200) which overproduces the enzyme from a plasmid (Hägerhäll, C. and Hederstedt, L., unpublished data). The bacteria were grown in NSMP medium [14] at 37°C with chloramphenicol (5 mg/l) for plasmid maintenance, and harvested 1 h after entering stationary growth phase. The cells were washed once with 50 mM potassium phosphate buffer (pH 8.0) and suspended (30 g wet weight/l) in the same buffer containing 10 mM $MgSO_4$, lysozyme (0.25 g/l) DNAse and RNAse (each 6 mg/l). After incubation for 45 min at 37°C, sodium EDTA and 2 min later $MgSO_4$ were added to give 15 mM and 20 mM final concentrations. The suspension was spun for 30 min at 4°C $3000 \times g$ to remove cell debris. Membranes were isolated from the supernatant by centrifugation for 45 min at $40000 \times g$, washed once with 0.1 M potassium phosphate buffer (pH 6.6), suspended in 20 mM Mops chloride buffer (pH 7.4) (Mops buffer) and stored at −70 °C. The succinate dehydrogenase complex was solubilised from the membrane fraction using Thesit (Lubrol PX) at 6.7 g per g membrane protein in the Mops buffer at 4°C. After centrifugation at $200000 \times g$, the supernatant (250 mg protein) was applied to a DEAE-Sephacel column (35 ml) equilibrated with the Mops buffer containing Thesit (1 g/l). The column was washed with about 200 ml equilibration buffer, and an NaCl gradient (210 ml, 0–0.6 M) in this buffer was then applied. The succinate dehydrogenase eluted at about 0.3 M NaCl. Peak fractions were pooled and dialysed overnight against the Mops buffer, concentrated with poly(ethylene glycol) (M_r 20000), and stored on ice.

Fumarate reductase from *W. succinogenes* [7,8] and the cytochrome bc_1 complex from beef heart mitochondria [15] were isolated as described.

Preparation of proteoliposomes

Sonic liposomes were prepared as described by Driessen et al. [16]. Vitamin K-1 or Q_9 (80 μmol/g phospholipid) was present when indicated.

Incorporation of succinate dehydrogenase. Succinate dehydrogenase (0.9 nmol covalently bound FAD) and cytochrome bc_1 complex [15] (where indicated, 1.2 nmol) were added to 1 ml of the liposomal suspension (10 mg phospholipid) in a buffer (pH 7.4) containing 50 mM Mops and 5 mM $MgCl_2$. The mixture was shaken for 30 min with Amberlite XAD-2 (20 g/g Thesit) at room temperature. After removal of the Amberlite, the mixture was frozen in liquid N_2 and thawed at room temperature. Freeze-thawing was repeated twice.

Incorporation of fumarate reductase. Fumarate reductase (0.6 nmol), cytochrome bc_1 complex (where indicated, 1.2 nmol) and 30 mg Triton X-100 were added to 1 ml of the liposomal suspension (10 mg phospholipid) in a buffer (pH 7.6) containing 50 mM Hepes, 5 mM $MgCl_2$, 1 mM malonate and 1 mM dithiothreitol. The mixture at 4°C was passed fifteen times through an Amberlite XAD-2 column (40 g/g Triton X-100) and then freeze-thawed three times.

Enzymic activities

Cytochrome *c* reduction with succinate was recorded at 550–540 nm ($\Delta\epsilon$(reduced–oxidized) = 18.7 mM^{-1} cm^{-1}). The reaction mixture (pH 7.2, 37°C) contained 0.25 M sucrose, 50 mM potassium phosphate, 2 mM KCN, 1 mM NaN_3, 0.2 mM EDTA, 0.1% bovine serum albumin, 0.1 mM cytochrome *c* (horse heart) and 50 mM succinate. The reaction was started by the addition of proteoliposomes. Cytochrome *c* reduction with NBH was measured in the same way. However, succinate was replaced by NBH (75 μM).

The reduction of the hydrophilic quinones (DMN, MD, DMN or Q_0) with succinate, and the oxidation of the corresponding hydroquinones with fumarate was recorded photometrically at 37°C as described [5].

Other methods

Covalently bound FAD [17] and the cytochrome bc_1 complex [15] were assayed as described. Protein was determined using the Biuret method with KCN [18].

Results

Reactivity with hydrophilic quinones

Earlier various hydrophilic quinones were found to serve as acceptors of the succinate oxidation catalyzed

TABLE I

Reactivity of B. subtilis succinate dehydrogenase and of W. succinogenes fumarate reductase with hydrophilic quinones (quinols)

The activities refer to the enzymes as isolated, and are given as succinate turnovers per FAD. The FAD contents were 3.9 (succinate dehydrogenase) and 6.0 μmol/g protein (fumarate reductase). DCPIP reduction in the presence of PMS was measured as described [5].

Donor–acceptor	E_0' (mV)	Succinate dehydrogenase (s^{-1})	Fumarate reductase (s^{-1})
Succinate → DMN	−80 (19)	3.6	8.4
Succinate → MD	−1 (20)	9.2	33
Succinate → NQ	+64 (20)	36	50
Succinate → Q_0	+162 (20)	26	47
Succinate → PMS/DCPIP	–	125	125
$DMNH_2$ → fumarate	−80	58	103
MDH_2 → fumarate	−1	36	46
NQH_2 → fumarate	+64	2	≤1
Q_0H_2 → fumarate	+162	≤1	≤1

by the membrane fraction of *Bacillus subtilis* [5]. From these experiments it remained unclear whether the quinones reacted directly with the succinate dehydrogenase or whether additional components of the respiratory chain were required for activity. Therefore, the experiment was done with the succinate dehydrogenase isolated from *B. subtilis* (Table I). The preparation used contained 47% succinate dehydrogenase (molecular mass 120 kDa) as calculated from its content of covalently bound FAD (3.9 μmol/g protein). Cytochromes of the *a*- or *c*-type were not detected in low-temperature spectra of the preparation (not shown). The specific activity of DCPIP reduction with succinate of the preparation was 50-times greater than that of a homogenate of *B. subtilis* [5].

The enzyme catalyzed the reduction of the quinones listed in Table I with succinate as donor. The activities are given as turnover numbers per FAD. The activity was highest with the non-substituted naphthoquinone (NQ) and somewhat smaller with the benzoquinone Q_0. The smallest activity was observed with dimethylnaphthoquinone (DMN), although this quinone was chemically most similar to the bacterial menaquinone. The enzyme also catalyzed fumarate reduction with the corresponding hydroquinones. In this reaction $DMNH_2$ was most actively oxidized, while the activity with NQH_2 or Q_0H_2 was much smaller. Thus, the activities in both directions appeared to be determined mainly by the redox potentials rather than by the structures of the quinones. The fumarate reductase of *Wolinella succinogenes* catalyzed succinate oxidation with the quinones as well as fumarate reduction with the corresponding hydroquinones (Table I). The activities measured in both directions were similar to those observed with succinate dehydrogenase and followed the same rule which was valid with succinate dehydrogenase.

The two enzymes differed in their sensitivity to HQNO. The reduction of Q_0 with succinate, and fumarate reduction with $DMNH_2$ catalyzed by succinate dehydrogenase, were inhibited by HQNO. With 2 and 20 mol HQNO per mol FAD the inhibition was 50 and 90%, respectively. Oxidation of succinate with DCPIP in the presence of PMS was not inhibited. Fumarate reductase was insensitive to HQNO, both in succinate oxidation with quinones and in the reverse reaction. The two enzymes were insensitive to 2-thenoyltrifluoroacetone.

TABLE II

Cytochrome c reduction with succinate or NBH catalyzed by proteoliposomes

The activities are given as turnover numbers (two electrons) which were calculated from the velocities of the reactions and the FAD contents. Antimycin or myxothiazol (5 mol per mol cytochrome bc_1 complex) were applied in ethanolic solution. The same amount of ethanol had no effect on the activities. SDH, succinate dehydrogenase; FR, fumarate reductase; cyt.bc_1, cytochrome bc_1 complex.

Enzyme incorporated	Quinone	Inhibitor	$\xrightarrow[\text{cyt.}c]{\text{Succinate}}$ (s^{-1})	$\xrightarrow[\text{cyt.}c]{\text{NBH}}$ (s^{-1})
SDH	Q_9	–	0.7	–
SDH + cyt.bc_1	–	–	1.1	77
SDH + cyt.bc_1	Q_9	–	15	97
SDH + cyt.bc_1	Q_9	antimycin	0.7	–
SDH + cyt.bc_1	Q_9	myxothiazol	0.7	–
SDH + cyt.bc_1	vitamin K-1	–	2.6	73
SDH + cyt.bc_1	vitamin K-1	antimycin	0.8	–
SDH + cyt.bc_1	vitamin K-1	myxothiazol	0.5	–
FR	Q_9	–	0.6	–
FR + cyt.bc_1	–	–	0.6	39
FR + cyt.bc_1	Q_9	–	26	39
FR + cyt.bc_1	Vitamin K-1	–	5.3	39

Reactivity with lipophilic quinones

To find out whether the succinate dehydrogenase of *B. subtilis* would react with lipophilic quinones, the enzyme was incorporated into liposomes containing Q_9 or vitamin K-1, together with the cytochrome bc_1 complex from beef heart mitochondria [15]. The reduction of cytochrome *c* by the proteoliposomes was expected to occur if the quinone were to be reduced by succinate dehydrogenase, since the resulting quinol should serve as electron donor to the cytochrome bc_1 complex. The activities of cytochrome *c* reduction with succinate were compared to those of the cytochrome bc_1 complex measured with NBH as donor (Table II). Liposomes containing Q_9 catalyzed the reduction of cytochrome *c* with succinate. More than 90% of the activity was dependent on the presence of both Q_9 and the cytochrome bc_1 complex, and was blocked by antimycin or myxothiazol, which specifically inhibit the activity of the cytochrome bc_1 complex. The overall electron transport velocity was 6-times smaller than that of the cytochrome bc_1 complex, and amounted to 58% of the activity of Q_0 reduction with succinate (see Table I). Liposomes containing vitamin K-1 ($E_0' = -75$ mV [20]) instead of Q_9 ($E_0' = +112$ mV [20]) also catalyzed the reduction of cytochrome *c* with succinate. More than 70% of the activity was inhibited by antimycin or myxothiazol. The activity was 6-times smaller than that with Q_9 present. About the same ratio was calculated from the activities of succinate oxidation with Q_0 and DMN (see Table I). Thus, the lipophilic vitamin K-1 reacted with succinate dehydrogenase at the velocity expected on the basis of its redox potential. Liposomes containing the fumarate reductase of *W. succinogenes* instead of succinate dehydrogenase catalyzed the electron transport from succinate to cytochrome *c* in the presence of either Q_9 or vitamin K-1 (Table II). The activities were similar to those with succinate dehydrogenase, and the ratio of the two activities corresponded to those of Q_0 and DMN reduction by succinate measured with the isolated enzyme (see Table I).

Discussion

The results presented demonstrate that the *B. subtilis* succinate dehydrogenase can react with hydrophilic or lipophilic quinones. The reactivity is commensurate with that of the fumarate reductase of *Wolinella succinogenes* in both succinate oxidation and fumarate reduction. The succinate dehydrogenase is a member of the citrate cycle in the strictly aerobic *B. subtilis*. In contrast, *W. succinogenes* is an anaerobic bacterium. The oxidation of menaquinol by fumarate, which is catalyzed by fumarate reductase, is an essential step in the phosphorylative electron transport of *W. succinogenes* [4,7,8,13]. The quinone-reactive site of fumarate reductase is located on its cytochrome *b* [13] which is homologous to that of the succinate dehydrogenase [9,11]. Therefore, it is likely that the cytochrome *b* of succinate dehydrogenase is designed for transferring electrons to menaquinone in *B. subtilis*.

The succinate dehydrogenase of *Escherichia coli* is similar to that of *B. subtilis* with respect to the two hydrophilic subunits [10]. The enzymes differ in their hydrophobic subunits. The *E. coli* succinate dehydrogenase contains two hydrophobic subunits and one heme B group [21,22]. Neither subunit shows conclusive sequence homology to the cytochrome *b* of *B. subtilis* or of *W. succinogenes* [9,11,23]. Notably, the *E. coli* succinate dehydrogenase reacts with Q but not with menaquinone [2].

In Table III the respiratory activities with succinate of intact cells and of the membrane fraction of *B. subtilis* grown with succinate are compared to the activities of succinate oxidation catalyzed by the isolated succinate dehydrogenase and by the dehydrogenase incorporated into liposomes. The comparison is done on the basis of the apparent turnover numbers of the covalently bound FAD. Succinate dehydrogenase is the only protein of *B. subtilis* containing covalently bound flavin [24]. The velocity of succinate oxidation with Q_0 or NQ catalyzed by the isolated enzyme and that of cytochrome *c* reduction by enzyme in proteoliposomes containing Q_9 are in the same order of magnitude as that of the succinate respiration catalyzed by the intact bacteria.

The succinate oxidation with the proteoliposomes containing vitamin K-1 is considerably slower than the succinate respiration of the intact bacteria, but is close to that of the bacterial membrane fraction. Both activities are probably limited by the velocity of oxidation of the lipophilic naphthoquinols. Due to the unfavorably negative redox potentials of the two quinols, their concentrations are expected to be very small. The

TABLE III

Turnover number of the succinate dehydrogenase of B. subtilis in various reactions at 37°C

The turnover numbers are based on the contents of covalently bound FAD. The bacteria and the membrane fraction contained 0.125 and 0.25 μmol/g protein covalently bound FAD. The respiratory activities were taken from Ref. 5. The activities of Q_0 (NQ) and of cytochrome *c* reduction were taken from Table I and II.

Preparation	Reaction	Turnover number (s^{-1})
Intact bacteria	succinate → O_2	80
Membrane fraction	succinate → O_2	6
Succinate dehydrogenase	succinate → Q_0 (NQ)	26 (36)
Proteoliposomes with Q_9	succinate → cyt.*c*	15
Proteoliposomes with vitamin K-1	succinate → cyt.*c*	3

extent of menaquinone reduction in bacteria catalyzing succinate respiration is possibly increased by the interference of the electrochemical proton potential. This is suggested by the observation that more than 90% of the succinate respiratory activity of intact *B. subtilis* is lost on the addition of small amounts of a protonophore [5]. In summary, the results suggest that menaquinone is a component of the chain catalyzing succinate respiration in *B. subtilis*. The reduction of menaquinone by succinate appears to involve a mechanism resembling electron transport reversal [6].

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