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Membrane-Bound Respiratory Chain of *Spirillum itersonii*

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The membrane-bound respiratory system of the gram-negative bacterium *Spirillum itersonii* was investigated. It contains cytochromes *b* (558), *c* (550), and *o* (558) and β-dihydro-nicotinamide adenine dinucleotide (NADH) and succinate oxidase activities under all growth conditions. It is also capable of producing D-lactate and α-glycerophosphate dehydrogenases when grown with lactate or glycerol as sole carbon source. Membrane-bound malate dehydrogenase was not detectable under any conditions, although there is high activity of soluble nicotinamide adenine dinucleotide: malate dehydrogenase. When grown with oxygen as the sole terminal electron acceptor, approximately 60% of the total *b*-type cytochrome is present as cytochrome *o*, whereas only 40% is present as cytochrome *o* in cells grown with nitrate in the presence of oxygen. Both NADH and succinate oxidase are inhibited by azide, cyanide, antimycin A, and 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide at low concentrations. The ability of these inhibitors to completely inhibit oxidase activity at low concentrations and their effects upon the aerobic steady-state reduction levels of *b*- and *c*-type cytochromes as well as the aerobic steady-state reduction levels obtained with NADH, succinate, and ascorbate-dichlorophenolindophenol suggest the presence of an unbranched respiratory chain in *S. itersonii* with the order ubiquinone → *b* → *c* → *c* → oxygen.

Within the past decade, the electron transport chains of a variety of bacteria have been studied and characterized (see 12, 20). Some of the outstanding features of bacterial respiratory chains are their extreme diversity, multiplicity, and arrangements of cytochromes and quinones (20). Additionally, many bacteria are capable of altering their cytochrome content in response to changes in growth conditions (3, 17, 18, 20). Some bacteria possess a large array of primary dehydrogenases, and most bacteria studied contain multiple terminal oxidases, and the ability to utilize compounds other than oxygen as terminal electron acceptor is not uncommon among those bacteria studied to date (see 12, 20 for review).

The gram-negative bacterium *Spirillum itersonii* has been used by this laboratory to study the regulation of heme and cytochrome biosynthesis (2-5, 8-10). Previous work has shown that *S. itersonii* possesses *b*- and *c*-type cytochromes and that it is capable of altering its cytochrome content in response to culture growth conditions. When grown with low aeration and adequate amount of iron, *S. itersonii* forms large amounts of both *b*- and *c*-type cytochromes and part of the *c*-type cytochrome is present as soluble cytochrome c_{550} (2, 3). In addition, when grown with nitrate it forms a nitrite-induced, soluble nitrate reductase (9).

Although it was known that *S. itersonii* contains *b*- and *c*-type cytochromes and a ubiquinone (3, 4), the relationship of these components to each other was unknown. In the present work, a study of the membrane-bound respiratory system was made. The data suggest that *S. itersonii* has an unbranched respiratory chain with a single terminal oxidase (cytochrome *o*).

MATERIALS AND METHODS

Maintenance and growth of *S. itersonii*. The strain of *S. itersonii* and its maintenance have been described previously (3). Cultures were grown at 30°C in 2.5-liter Fernbach flasks containing 1.5 liters of GGS medium (3) + 0.1% (wt/vol) yeast extract and were shaken on a reciprocal shaker at 60 cycles/min. For growth with nitrate, cultures were grown as described except that 2 g of KNO_3 per liter was added to the medium. In the experiments presented in Table 2, cells were grown as described above except that the media contained 10 mM substrate (succinate, malate, glycerol, or lactate) in place of the glycine, glutamate, and succinate mixture present in GGS medium, and no yeast extract was added.

Preparations of respiratory particles. Late log-phase cells were harvested, and membrane particles were isolated as previously described (5) except that

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all preparations were suspended in a buffer containing 50 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 7.8), 1 mM histidine, and 0.6 M sucrose. This buffer was used to stabilize β-dihydro-nicotinamide adenine dinucleotide (NADH) oxidase activity which was labile in Tris or phosphate buffers alone. Protein concentration was determined by the method of Lowry et al. (13).

Measurement of oxidase activity. Oxidase activities were measured with a Rank oxygen electrode at 25°C. The reaction vessel contained approximately 1 mg of protein, 50 μmol of potassium phosphate buffer (pH 7.5), and either 10 μmol of succinate or 1 μmol of NADH in a total volume of 2 ml. Some inhibitors were added in 10 μl of ethanol, and this amount of ethanol alone had no effect on the oxidase activities. Inhibitors were added to the chamber after the normal oxidase rate had first been established.

Determination of cytochromes. Cytochrome concentrations were calculated from reduced *minus* oxidized difference spectra recorded at room temperature with a Cary 14R spectrophotometer. The extinction coefficients used were: cytochrome *c*, $\epsilon = 19.2 \times 10^3$ (550 nm), cytochrome *b*, $\epsilon = 20.7 \times 10^3$ (560 nm) (19). Due to the large amount of cytochrome *c* present in most preparations, the methods and correction factors of Sinclair and White (19) were used to minimize errors attributable to spectral overlap. Individual hemes were extracted and quantified as their pyridine hemochromogens (16).

Cytochrome *o* was determined from CO-reduced *minus* reduced differences spectra using the extinction coefficient of 80×10^3 (422 − 450 nm) (18). For CO spectra, NADH was used as the reductant in anaerobic cuvettes. Low-temperature (77 K) spectra were measured with a spectrophotometer (Aminco DW-2).

Aerobic steady-state determinations. The reduction kinetics of individual cytochromes were determined with an Aminco DW-2 spectrophotometer. All measurements were performed at 25°C. The reaction mixture contained 100 μmol of potassium phosphate buffer (pH 7.5) and 1 to 4 mg of protein in a final volume of 1 ml. Reaction was initiated by addition of 3 μmol of substrate. Wavelength pairs used were: *c*-type cytochrome, 550 − 540 nm; *b*-type cytochrome, 560 − 570 nm. A 100% reduction was obtained by addition of $Na_2S_2O_4$. Measurement of ubiquinone reduction was performed by the method of Pumphrey and Redfearn (15) as described by Daniel (6).

Dehydrogenase assays. Succinate, α-glycerophosphate, D-lactate, and malate dehydrogenases were all measured by using a PMS-DCIP (phenazine methyl-sulfate-dichlorophenolindophenol) assay. Each assay contained 50 μmol of potassium phosphate buffer (pH 7.5), 0.1 μmol of DCIP, 1 μmol of PMS, 2 μmol of KCN, 10 μmol of substrate, and enzyme extract in a total volume of 1.0 ml. After establishing the endogenous rate, the substrate was added and the reaction was followed at room temperature, spectrophotometrically, at 600 nm.

Chemicals. Antimycin A, 2-*n*-heptyl-4-hydroxyquinoline (HQNO), 2,6-DCIP, PMS, and NADH were obtained from Sigma Chemical Co., St. Louis, Mo. CO in lecture-size bottles was from Matheson Gas Corp. All other chemicals were of reagent grade.

RESULTS

Cytochrome content of respiratory particles. Isolated respiratory particles of *S. itersonii* obtained from cells grown aerobically in the presence or absence of nitrate possess NADH and succinate oxidase activities (Table 1). Membrane-bound D-lactate and α-glycerolphosphate dehydrogenases were inducible. D-Lactate dehydrogenase was induced when cells were grown with lactate or glycerol as sole carbon source; α-glycerolphosphate dehydrogenase was found when glycerol, lactate, or malate, but not succinate, were carbon sources (Table 2). No membrane-bound malate dehydrogenase was detectable, although soluble nicotinamide adenine dinucleotide (NAD) malate dehydrogenase was present at high levels under all growth conditions.

Room temperature reduced *minus* oxidized difference spectra indicated the presence of *c*- and *b*-type cytochromes with an α peak at 550 nm and a shoulder at 560 nm. At liquid nitrogen temperature (77 K), two clearly separate α-

TABLE 1. *Cytochrome content and oxidase activities of S. itersonii respiratory particles*

Growth condition[a]	Cytochromes[b]			Oxidase activity[c]	
	c	*b*-type	*o*	NADH	Succinate
With nitrate	1.83	1.10	0.32	0.129	0.036
Without nitrate	1.34	0.80	0.46	0.134	0.042

[a] Cultures were grown under identical conditions except that nitrate (2 g/liter) was added to one.

[b] Cytochrome concentrations are expressed as nanomoles per milligram of protein. The *b*-type cytochrome includes both cytochromes *b* and *o*.

[c] Oxidase activities are expressed as micromoles of O_2 consumed per minute per milligram of protein.

TABLE 2. *Effect of growth conditions on dehydrogenases of S. itersonii*

Culture carbon source	Dehydrogenase[a]			
	α-Glycerophosphate	D-Lactate	Malate	Succinate
Glycerol	7.4	4.4	0	41.0
Lactate	0.9	13.4	0	44.0
Malate	1.7	0	0	28.6
Succinate	0	0	0	57.6

[a] Dehydrogenase activities are expressed as nanomoles of DCIP reduced per minute per milligram of membrane protein.

peaks were visible at 550 nm and 558 nm. No shoulder or minor peaks were discernable (Fig. 1). Succinate and NADH caused almost complete (95%) reduction of both *b*- and *c*-type cytochromes, although the *b*-type cytochrome reacted slowly and required several minutes to achieve its maximum reduction. The CO reduced *minus* reduced difference spectrum (recorded at room temperature) has a single peak at 422 nm and minor peaks at 535 nm and 565 nm (Fig. 2). Addition of 0.1% (vol/vol) Triton X-100 results in a shift of the 422 nm peak to 418 nm. Similar shifts have been noted before in *Rhodopseudomonas spheroides* and *Paracoccus denitrificans* (14). The CO reactive pigment, hereafter referred to as cytochrome *o*, combines slowly with CO taking 5 to 10 min to fully react. Therefore, for quantitation of cytochrome *o*, scans were repeated until no further spectral changes occurred.

Effect of growth conditions on cytochrome *o*. The cytochrome *o* content of *S. itersonii* was significantly influenced by culture growth conditions. Cytochrome *o* comprises approximately 60% of the total *b*-type cytochrome in cells from cultures grown aerobically but without nitrate. However, only 40% of the *b*-type cytochrome was present as cytochrome *o* in cultures grown with nitrate added.

Steady-state reduction levels. The steady-state reductions of ubiquinone and *b*- and *c*-type cytochromes are shown in Table 3. The quinone was not rigorously identified, but the spectrum of the extracted quinone indicates that only ubiquinone and not menaquinone is present. The relatively high aerobic reduction level of ubiquinone suggests that is present in the respiratory chain before cytochromes *b* and *c*.

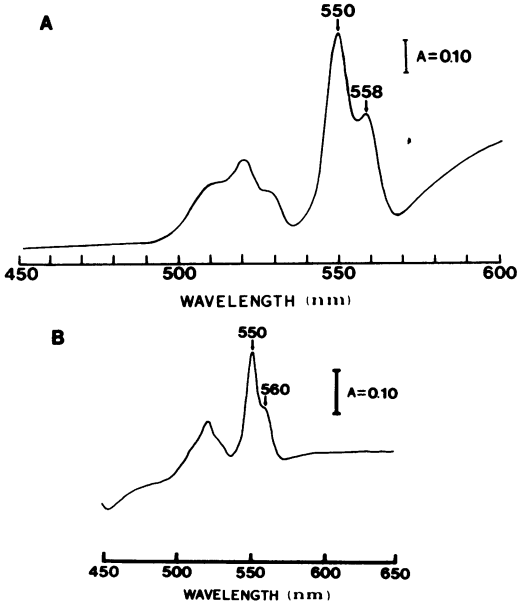

FIG. 1. *Dithionite reduced minus oxidized difference spectra of S. itersonii respiratory particles. (A) 77 K, 1-mm pathlength, 20 mg of protein/ml; (B) room temperature, 1-cm pathlength, 8 mg of protein/ml.*

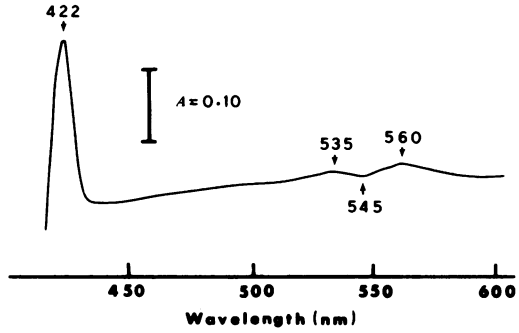

FIG. 2. *CO reduced minus reduced difference spectrum of S. itersonii respiratory particles (room temperature). Particle preparations were reduced with NADH in anaerobic cuvettes (1-cm pathlength) before the addition and equilibration with CO gas, 9.5 mg of protein/ml.*

The aerobic reduction of *c*-type cytochrome is similar to that found with mitochondrial type cytochrome *c*. The aerobic reduction of *b*-type is lower than would be expected if only a single cytochrome *b* were present occurring before cytochrome *c* in the electron transport chain. However, this lower aerobic reduction is explained by the presence of at least two *b*-type cytochromes: one occurring before and the other after cytochrome *c* (i.e., cytochromes *b* and *o*) (See Fig. 4). This hypothesis is supported by the biphasic nature of the trace in Fig. 3 as well as by data from CO-spectra that indicate approximately 50% of the total *b*-type cytochrome is present as cytochrome *o*.

Ascorbate-DCIP, which donates electrons at the level of cytochrome *c*, caused partial reduction of *b*-type cytochrome (Fig. 3). (Ascorbate alone did not reduce any cytochrome component.) The reduction by ascorbate-DCIP could not be attributable to spectral overlap by cytochrome *c* since the reduction kinetics are very different from those observed at the wavelength used to measure the reactions of cytochrome *c*. However, the reaction with its low aerobic reduction is characteristic of a terminal oxidase and is probably attributable to cytochrome *o*. The higher steady-state aerobic reduction of cytochrome *c* by ascorbate-DCIP in nitrate-grown cells, as compared to aerobically grown cells, reflects the increased cytochrome

c:cytochrome o ratio present in these preparations.

The data from both CO difference spectra (Table 1) and steady-state reduction studies with ascorbate-DCIP (Table 3) confirm the presence and the concentration of cytochrome o in the respiratory particles. These data are consistent in demonstrating that cytochrome o content is variable and that it is higher in aerobically grown cells than in cells grown with nitrate.

Effect of inhibitors of electron transport. Table 4 shows the effect of inhibitors of electron transport on the steady-state aerobic reduction levels of b- and c-type cytochromes. Similar data were obtained from cells grown with or without nitrate. Antimycin A and HQNO, which inhibit by blocking electron transfer between cytochromes b and c, both cause oxidation of cytochrome c and further reduction of the b-type cytochrome (Fig. 4). The relatively low level of reduction of the b-type cytochrome in the presence of HQNO or antimycin A can be attributed to the large contribution of cytochrome o which is not reduced under these conditions. Neither HQNO nor antimycin A had any effect on ascorbate-DCIP reduction of cytochrome o. Azide and cyanide, both of which block the terminal oxidase, cause extensive reduction of both c- and b-type cytochromes.

Both NADH and succinate oxidase activities are inhibited at low concentrations of inhibitors (Table 5). The concentrations required for inhibition are lower than those reported in other bacterial systems (6, 7, 11, 18).

DISCUSSION

The present work demonstrates that the membrane-bound respiratory system of *S. itersonii* possesses cytochromes b (558), c (550), and o (558). It also contains NADH and succinate dehydrogenases and has the ability to form inducible dehydrogenases for D-lactate and α-glycerolphosphate, but not for malate. While it is known that *S. itersonii* forms soluble cytochrome c (2) and soluble nitrate reductase (9), no effort was made to study their possible interactions with the membrane-bound respiratory chain. No a-type cytochromes were detected by either CO reduced *minus* reduced (room temperature) or reduced *minus* oxidized (77 K) spectra.

The CO-reduced *minus* reduced difference spectra, steady-state reduction kinetics, reduction by ascorbate-DCIP, and the effect of antimycin A and HQNO on the aerobic steady-state reduction demonstrate that a portion of the b-type cytochrome is present as cytochrome o. Cytochrome o of *S. itersonii* requires extended periods of gassing with CO to react completely. This is not an uncommon feature since it has been reported before by Broberg and Smith in *Bacillus megaterium* KM (1). Data from CO difference spectra and steady-state reduction studies show that approximately 60% (ca. 0.5 nmol/mg of protein) of the b-type cytochrome is present as cytochrome o in aerobically grown cells while only 40% (ca. 0.3 nmol/mg of protein) is cytochrome o in cells grown in the presence of nitrate. This is dissimilar to *H. parainfluenzae* where growth with nitrate results in increased content of cytochrome o as well as cytochromes a, b, and c, although cytochrome a_2 levels decrease (18), and in *Paracoccus denitrificans* were cytochromes c and b+o increase while cytochrome a_3 decreases (18). Of note, however, is the fact that, in both of these bacteria, one of their terminal oxidases decreases in

TABLE 3. *Substrate reduction of S. itersonii respiratory particles as determined by dual-wavelength spectrophotometry*

Growth condition[a]	Substrate	Cytochrome c[b]		b-Type cytochrome[b]		Ubiquinone[c]	
		Aerobic steady state (%)	Anaerobic state (%)	Aerobic steady state (%)	Anaerobic state (%)	Aerobic steady state (%)	Anaerobic state (%)
With nitrate	NADH	32 ± 3 (18)	95–100	20 ± 3 (32)	85–95	74	85–90
	Ascorbate-DCIP	60 ± 4 (6)	95–100	0 (9)	42 ± 2 (9)		
Without nitrate	NADH	26 ± 3 (16)	95–100	19 ± 2 (31)	85–95	65	85–90
	Ascorbate-DCIP	38 ± 2 (6)	95–100	0 (17)	58 ± 6 (17)		

[a] Growth condition refers to the nature of the terminal electron acceptor available to cells in culture. Experimental details are described in Materials and Methods.

[b] The numbers given are mean values with standard deviations and number of determinations.

[c] Ubiquinone was determined by the method of Pumphrey and Redfearn (15) with the numbers given representing the average of three experiments.

content when cells are grown with nitrate. In *S. itersonii* there is only a single detectable terminal oxidase and its content decreases when cultures are grown in the presence of nitrate.

Both NADH and succinate oxidase activities

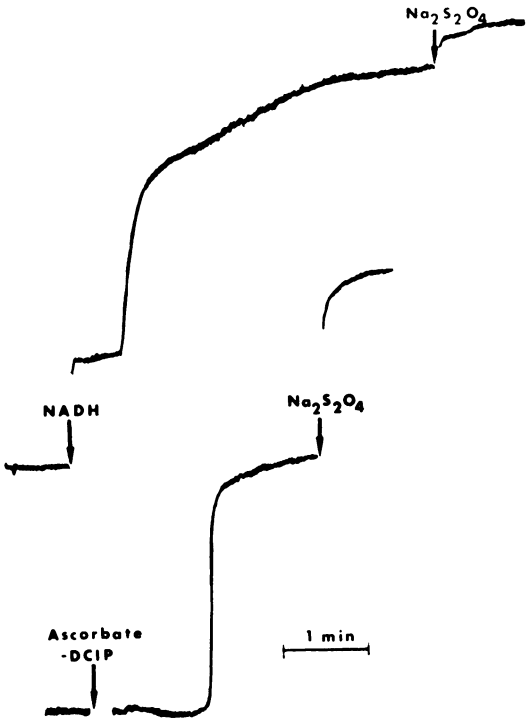

FIG. 3. *Reduction of b-type cytochromes in respiratory particles of S. itersonii as determined by dual wavelength spectrophotometry. An upward deflection of the trace indicates reduction. Upper trace, 2.2 mg of protein/ml; lower trace, 4.0 mg of protein/ml.*

TABLE 4. *Effect of inhibitors of electron transport on the aerobic steady-state reduction levels of cytochromes in respiratory particles of S. itersonii*[a]

Inhibitor	Concn	Reduction (%)	
		Cytochrome *c*	*b*-Type cytochrome
None		32	20
HQNO	5 μM	20	36
Antimycin A	5 μM	20	36
Azide	1 mM	68	65
KCN	100 μM	84	78

[a] Particle preparations were made from cells grown with nitrate. Experiments were performed as described for Table 3, and NADH was the reductant used in these experiments. After attainment of the aerobic reduced-state, inhibitors were added rapidly and mixed thoroughly. In some cases inhibitors were added in ethanol solutions.

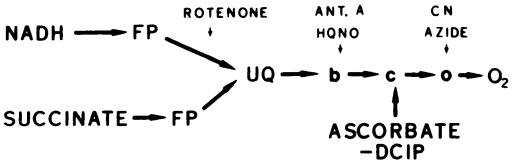

FIG. 4. *Proposed membrane-bound respiratory chain of S. itersonii. UQ, ubiquinone; FP, flavoprotein.*

TABLE 5. *Effects of inhibitors of electron transport on NADH and succinate oxidase activities in respiratory particles of S. itersonii*[a]

Inhibitor	Concn (nmol/mg of protein)	Inhibition of oxidase activity (%)	
		NADH	Succinate
Rotenone	1.5	98	0
HQNO	1.5	97	95
HQNO	0.15	50	50
Antimycin A	1.5	100	100
Antimycin A	0.15	85	84
Azide	10	40	40
Azide	100	90	88
KCN	5	32	30
KCN	50	100	100

[a] Assays were carried out as described in Materials and Methods. The numbers are averages from six experiments. Identical results are obtained with cells grown in the presence or absence of nitrate.

are equally inhibited at low concentrations of HQNO, antimycin A, KCN, and azide. The concentrations required for inhibition are lower than those reported for other bacterial systems. The respiratory chain of *S. itersonii* is even more sensitive to these inhibitors than those of *P. denitrificans* (18) and *Mycobacterium flavum* 301 (7), which are two of the most sensitive bacterial systems reported. The sensitivity of the *S. itersonii* respiratory chain to these inhibitors may be attributable to the presence of only a single terminal oxidase, cytochrome *o*, whereas these other bacteria possess multiple terminal oxidases including *a*-type cytochromes.

The aerobic steady-state reduction levels, the effects of inhibitors on the aerobic steady states of cytochromes, and the sensitivity of both NADH and succinate oxidase activities to inhibitors support the concept of an unbranched membrane-bound electron transport chain from NADH and succinate to oxygen in *S. itersonii* (Fig. 4).

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