Participation of Quinone and Cytochrome *b* in Tetrathionate Reductase Respiratory Chain of *Citrobacter freundii*

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Formate dehydrogenase, NADH dehydrogenase, a quinone and a *b*-type cytochrome characterized by maxima at 429 and 560 nm are shown to participate in the tetrathionate redox chain of *Citrobacter*.

Reduction of tetrathionate to thiosulphate, one of anaerobic bacterial respiratory processes, was discovered and studied first by Pollock and co-workers in the 1940s (see, e.g., Pollock & Knox, 1943; Knox & Pollock, 1944). Particulars concerning the synthesis of tetrathionate reductase (Pichinoty & Bigliardi-Rouvier, 1963; deGroot & Stouthamer, 1970) and possible energetic significance of the process (Kaprálék, 1972; Stouthamer & Bettenhausen, 1972) were published later on. The terminal tetrathionate oxidoreductase was investigated by Pichinoty & Bigliardi-Rouvier (1963) and by Stouthamer and his collaborators (see, e.g., Oltmann & Stouthamer, 1975), who used reduced Benzyl Viologen as artificial electron donor. The present knowledge of the tetrathionate respiration in bacteria was recently briefly reviewed by Thauer *et al.* (1977). Hitherto no one had undertaken a study of the whole tetrathionate redox chain previously isolated from the cell.

In the present study, washed membrane particles of *Citrobacter* bearing the active tetrathionate reductase redox chain were isolated, and the composition of this redox chain was studied.

Experimental

Cultivation

C. freundii (Sedláček, 1975) was grown at 30°C in the following medium (pH 7.5): KH_2PO_4 , 13.6 g; $(\text{NH}_4)_2\text{SO}_4$, 2.0 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g; galactose, 2.0 g; yeast extract, bacto-peptone, casamino acids (Difco, Detroit, MI, U.S.A.), 0.3 g of each; $\text{K}_2\text{S}_2\text{O}_8$ (Merck, Darmstadt, Germany), 1.0 g; all per litre. Cultivation in Erlenmeyer flasks (filled with the above medium) was used, establishing rapidly anaerobic conditions by consumption of oxygen dissolved in the medium by growing cells. Bacteria were harvested at the late-exponential phase of

growth and washed in 0.2 M-sodium/potassium phosphate buffer, pH 7.4. Chloramphenicol (100 µg/ml) was added to avoid the adaptation of cells to oxygen during the harvest.

Membrane particles

The washed bacterial cells (40–70 mg dry wt./ml) were suspended in 0.5 M-potassium phosphate buffer (pH 8.0) containing 10 mM- MgCl_2 and disrupted at 0°C by using 1 vol. of glass beads. The cell-free extract was prepared by twice centrifuging the homogenate at 13 000 g at 4°C. The washed particulate fraction was prepared by centrifuging the cell-free extract at 4°C and 160 000 g for 60 min (Spinco L2-65B centrifuge, SW 65 L Ti rotor), the washing buffer being that used for disruption. Membrane particles were stored at –14°C, if necessary.

Enzyme assays

The activity of the tetrathionate redox chain was measured at 37°C in Thunberg tubes previously flushed out and filled with N_2 . The enzyme reaction was stopped with 2.5% (w/v) trichloroacetic acid. The thiosulphate production was assayed iodometrically. The assays were done under conditions of substrate excess and the rates of reaction were linear with time over the intervals involved.

Quinone studies

Washed membrane particles were exposed to 360 nm light (Tesla lamp RVU 125 W), the distance of the light-source from membrane particles being 15 cm. The suspension of particles was cooled in water/ice during irradiation.

To restore the near-u.v.-damaged electron transfer, membrane particles were incubated with menadione (Serva, Heidelberg, Germany; 3 µmol/mg of protein) for 20 min at 20°C.

The amounts of both oxidized ubiquinone and menaquinone in a membrane preparation were determined by direct measurement of absorbance

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differences after reduction with NaBH_4 by the method of Kröger *et al.* (1971). A Cary 118 spectrophotometer was used with 1 cm-path-length cuvettes at room temperature (20°C).

Cytochrome difference spectra

The amount of a *b*-type cytochrome was calculated from absorbance differences at 427.5 and 409 nm after membrane particles had been reduced with excess of dithionite; the molar extinction coefficients for the cytochrome b_1 of *Escherichia coli* (Deeb & Hager, 1964) were used.

Protein was measured by the method of Lowry *et al.* (1951), with crystalline bovine serum albumin (BDH, Poole, Dorset, U.K.) as standard.

Results and Discussion

Dehydrogenases

To examine primary dehydrogenases participating in the tetrathionate reductase redox chain of *C. freundii*, a number of physiological electron donors were tested (Table 1). NADH, and especially formate, were found the most efficient. This may reflect an important role of formate dehydrogenase and NADH dehydrogenase in tetrathionate respiration in *Citrobacter* cells. Such a prominent role of formate among electron donors was also claimed for the nitrate respiration of *E. coli* (Enoch & Lester, 1975), which underlines similarity of both redox chains.

Succinate and lactate showed little efficiency as electron donors for the tetrathionate redox chain of *Citrobacter* (Table 1). Parallel measuring of oxidase activities with these membrane particles showed that

the efficiency of both donors was comparable with that of NADH and formate. Consequently, succinate dehydrogenase and lactate dehydrogenase, although being present in the membrane, are not effectively connected with the tetrathionate redox chain.

Quinones

Decrease of the electron transfer to tetrathionate, observed after the irradiation with 360 nm light of membrane particles (Table 2), suggests involvement of a naphthoquinone (Itagaki, 1964). The idea of participation of a quinone in the tetrathionate respiration is further supported by the ability of

Table 1. *Electron donors for the tetrathionate respiration by membrane particles*

The assay system contained: 0.25 M-sodium/potassium phosphate buffer, pH 7.4; 20 mM-electron donor (3 mM-NADH); 20 mM-tetrathionate; 0.1 mg of protein/ml of particles. The assay was carried out in Thunberg tubes, anaerobically at 37°C.

Electron donor	Rate of $\text{K}_2\text{S}_4\text{O}_6$ reduction (nmol/min per mg of protein)
Formate	528
NADH	290
Succinate	40
Lactate	17
Malate	7
Pyruvate	0
Glycerol	0
α -Oxoglutarate	0
Glucose	0
None	0

Table 2. *Sensitivity to inhibitors of tetrathionate reduction restored with menadione after irradiation by 360 nm light*
For details of the tetrathionate reductase assay, see Table 1. Inhibitors were added as a solution in dimethylformamide; the final concentration of dimethylformamide was 1% (v/v). Abbreviation used: HQNO, 2-heptyl-4-hydroxyquinoline N-oxide.

Electron donor	Membrane particles	Menadione added	Inhibitor (100 μM)	Specific activity*	Inhibition (%)
Formate	Intact	—	—	617	—
	Irradiated	—	—	80	—
		+	—	190	—
		+	Dimethylformamide	203	0
		+	Amytal	183	9
		+	Rotenone	123	40
		+	Antimycin A	37	82
		+	HQNO	8	96
NADH	Intact	—	—	247	—
	Irradiated	—	—	82	—
		+	—	122	—
		+	Dimethylformamide	122	0
		+	Amytal	122	0
		+	Rotenone	53	56
		+	Antimycin A	13	89
		+	HQNO	7	95

* nmol of tetrathionate reduced/min per mg of protein.

Fig. 1. Difference redox spectra of membrane particles

The formate-tetrathionate reductase activity was 735 nmol of tetrathionate reduced/min per mg of protein. 2 mm-path-length cuvettes were used, containing 3 mg of protein/ml; particles were reduced with several crystals of dithionite (a), 400 mM-formate (b), or 4 mM-NADH (c). Samples were measured against a blank not exogenously reduced, at 20°C. The systems with formate (FOR) and NADH were re-oxidized with 25 mM-tetrathionate (TT). A Cary 118 spectrophotometer was used.

menadione to partly restore the electron flow after damage by near-u.v. radiation (Table 2). A similar beneficial effect by menadione was observed on the NADH oxidase activity in a menaquinone-deficient strain of *Bacillus subtilis* (Bisschop & Konings, 1976).

Exogenous menadione does not seem to have

Table 3. Effects of inhibitors on tetrathionate reduction

For details of the tetrathionate reductase assay, see Table 1; the inhibitors (except cyanide and azide) were added as solutions in dimethylformamide; the final concentration of dimethylformamide was 1% (v/v). Abbreviation used: HQNO, 2-heptyl-4-hydroxyquinoline *N*-oxide.

Electron donor	Inhibitor	[Inhibitor] (mM)	Inhibition (%)
Formate	Amytal	0.1	1
	Amytal	1	26
	Rotenone	0.01	12
	Rotenone	0.1	52
	Antimycin A	0.01	58
	Antimycin A	0.1	96
	HQNO	0.01	96
	HQNO	0.1	98
	KCN	0.001	6
	KCN	0.01	23
	KCN	5	64
	NaN ₃	0.01	9
	NaN ₃	1	41
	NaN ₃	10	69
NADH	Amytal	0.1	0
	Amytal	1	21
	Rotenone	0.01	29
	Rotenone	0.1	68
	Antimycin A	0.01	45
	Antimycin A	0.1	86
	HQNO	0.01	91
	HQNO	0.1	91
	KCN	0.01	0
	KCN	0.1	0
	KCN	1	0
	NaN ₃	0.01	2
	NaN ₃	0.1	6
	NaN ₃	10	11

produced any artificial by-pass, for a similar sensitivity to respiratory inhibitors was found with the menadione-restored and the intact chain (Tables 2 and 3; Bisschop & Konings, 1976). Also, the inability of menadione to stimulate the native NADH-tetrathionate reductase favours this view.

The ubiquinone and menaquinone contents in membrane particles reducing tetrathionate amounted to about 3 μmol of ubiquinone-10 and less than 0.5 μmol of menaquinone-7/g of protein. It is impossible at present to make conclusions about which quinone is involved in tetrathionate respiration. The effect of 360 nm light may point to menaquinone, but its content in the membrane seems to be very low.

Cytochrome

The dithionite-reduced difference spectrum revealed a *b*-type cytochrome with maxima at 429 and 560 nm and minima at 408 and 455 nm bound to membrane particles reducing tetrathionate (Fig. 1). The cytochrome could be reduced by physiological

electron donors and then partly re-oxidized by tetrathionate after oxygen had been consumed by membrane particles. This redox behaviour suggests that it has a role in the electron transfer to tetrathionate. A similar *b*-type cytochrome was reported to participate in the nitrate and the fumarate respiration (Kröger *et al.*, 1971; Enoch & Lester, 1975). A rough estimation made from dithionite-reduced spectra of membrane particles gave the value of about 1 nmol of cytochrome *b*₁/mg of protein.

Inhibitors

The electron flow to tetrathionate is inhibited by rotenone, antimycin A and 2-heptyl-4-hydroxyquinoline *N*-oxide (all from Sigma, St. Louis, MO, U.S.A.), Amytal (Serva) exhibits low efficiency (Table 3). The activity of formate-tetrathionate reductase, but not of NADH-tetrathionate reductase, is lowered by cyanide and azide (Table 3), possibly owing to their effect on the formate dehydrogenase (Enoch & Lester, 1975).

Conclusion

Consequently, the tetrathionate redox chain of *C. freundii* appears to involve formate dehydrogenase NADH dehydrogenase, a quinone and the *b*-type cytochrome. Such a composition would qualify this chain to be a proton-translocating redox system (Haddock & Jones, 1977).

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