The Electron Transport System of an Extremely Thermophilic Bacterium

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Active, membrane-bound NADH and succinate oxidase activities with a temperature optimum of 75 °C were demonstrated in an extremely thermophilic bacterium. These were relatively stable in cell-free extracts and respiratory particles at 75 °C, but at 90 °C the half-lives of these oxidase systems were about 15 min in respiratory particles and 80 min in cell-free extracts. The stability of the NADH oxidase in respiratory particles at 90 °C was enhanced by 2 M-(NH₄)₂SO₄, 50% (v/v) glycerol and by NADH. A number of other substrates were oxidized by the electron transport system. Respiratory particles contained cytochromes *a*-613, *a*-602, *b*-559, cytochrome *o* and at least one *c*-type cytochrome, *c*-555. The soluble fraction contained at least two *c*-type cytochromes, at least one of which was CO-reactive. The sensitivity of NADH and succinate oxidases to a range of inhibitors was determined.

INTRODUCTION

Interest in micro-organisms living in extreme thermal environments has increased over the past few years (see, for example, Brock, 1967; Brock & Brock, 1971; Williams, 1975) and a number of enzymes from extreme thermophiles have been characterized (see, for example, Campbell & Pace, 1968; Singleton & Amelunxen, 1973; Zuber, 1976; Ljungdahl & Sherod, 1976). Despite interest in the membrane composition of such organisms (Ray *et al.*, 1971 *a, b*; Jackson *et al.*, 1973 *a*), little is known about their electron transport systems (Pask-Hughes & Williams, 1975; McFetters & Ulrich, 1972). In this paper we describe the electron transport system of a thermophilic bacterium isolated from the Rotorua thermal region of New Zealand. The organism was *Thermus*-like (H. Morgan, personal communication) and, pending the final results of taxonomic work, it will be referred to as *Thermus* T351. The electron transport system (this paper) and other physiological and biochemical activities (H. Morgan & D. A. Cowan, personal communication) displayed optimal activity between 70 and 80 °C and negligible activity below 40 °C. The organism can therefore be described as caldoactive (Heinen & Heinen, 1972; Williams, 1975) or extremely thermophilic.

METHODS

Organism. *Thermus* T351 was isolated from a hot pool (79 ± 4 °C), low in sulphide, at pH 7.8. The organism grew well at 75 °C at pH 7.5 to 7.8, but grew poorly below 60 °C; it was obligately aerobic. Cells were Gram-negative, non-motile, non-sporulating rods.

Maintenance and growth. Cultures were maintained on a medium consisting of Allen's salts (Jackson *et al.*, 1973 *b*) with 0.1% (w/v) yeast extract (BBL) and 0.1% (w/v) trypticase (BBL) in liquid culture at 75 °C. The medium was adjusted to pH 8.2 before autoclaving; the final pH was 7.5.

The organism was grown at 75 °C on a similar medium, but with 0.3% yeast extract and 0.3% trypticase. Batches of 500 ml were grown in 2 l Erlenmeyer flasks in an orbital incubator; bacteria were either harvested

directly or used to inoculate a 20 l fermenter. Bacteria were harvested during the late-exponential phase (10 to 12 h after inoculation) at an A_{650} of about 1.4 (about 2.5×10^7 cells ml⁻¹).

Preparation of subcellular fractions. Fractions were prepared as described by Daniel (1970), except that small particles were sedimented at 250000 *g* for 1 h.

Measurement of oxidase activities. Oxygen uptake was measured polarographically using a Rank oxygen electrode (Rank Bros, Bottisham, Cambs.). The reaction mixture (total vol. 2.5 ml) contained 0.1 M-KH₂PO₄/Na₂HPO₄ buffer, pH 7.0, a suitable amount of membrane particle protein and either 5 μ mol NADH or 50 μ mol of the other substrates. Buffers were equilibrated at the desired temperature with sparged air for 30 min. Particles were equilibrated in the electrode for 2 min, and oxidase activities were determined during the first minute after the addition of substrate.

Spectrophotometry. Difference spectra were obtained at room temperature with a Cary model 17 recording spectrophotometer. The concentration of individual cytochromes was determined from the dithionite-reduced minus oxidized difference spectra, and for cytochrome *o*, from the reduced+CO minus reduced difference spectra. The following wavelength pairs and molar absorption coefficients (l mol⁻¹ cm⁻¹) were used: *c*-type cytochrome, 553 and 540 nm, $\epsilon = 19000$ (Chance *et al.*, 1955); total *b*-type cytochrome, 560 and 575 nm, $\epsilon = 17500$ (Deeb & Hager, 1964); cytochrome *o*-CO, 417 and 429 nm, $\epsilon = 170000$ (Daniel, 1970); *a*-type cytochrome, 602 and 630 nm and 613 and 630 nm, $\epsilon = 24000$ (Van Gelder, 1966).

RESULTS AND DISCUSSION

Both NADH and succinate oxidase systems were membrane-bound (Table 1). NADH oxidase activity in respiratory particles, especially in the large particles, rose markedly on the addition of supernatant, but the succinate oxidase activity was unaffected. When large or small particles were subjected to additional washings, NADH oxidase activity decreased further. Little or no stimulation of activity was observed on adding purified *c*-type cytochromes derived from the supernatant. This suggests that the NADH dehydrogenase is only loosely attached to the cell membrane and that, as in other bacteria (e.g. Smith, 1968), the rate-limiting step in respiration is the dehydrogenase.

Washed membrane particles could oxidize other substrates, including glutamate and malate [$> 0.05 \mu$ mol O₂ min⁻¹ (mg protein)⁻¹], and lactate, citrate, fumarate, glycerol and glucose [0.005 to 0.02μ mol O₂ min⁻¹ (mg protein)⁻¹]. Except in the case of succinate and lactate, activities were enhanced by added supernatant and by NAD, suggesting that these other dehydrogenases are NAD-linked and at least partially soluble. Acetate, sucrose, mannitol and ethanol were not oxidized.

At 75 °C, the optimum pH and optimum concentration of phosphate buffer for both NADH and succinate oxidases were pH 7.0 and 0.1 M, respectively. After 2 min preincubation at temperatures between 40 and 95 °C, NADH and succinate oxidases were most active at 75 °C in cell-free extract and in large or small particles. The NADH oxidase activity in respiratory particles was particularly temperature-sensitive, the activities at 70 and 80 °C being about half that at 75 °C, and the activity at 40 °C was less than one-tenth of that at 75 °C.

At 75 °C, apart from an initial partial loss of activity, the respiratory activities of unbroken bacteria, cell-free extract and respiratory particles were relatively stable, although there was a substantial short-term increase in succinate respiration of unbroken bacteria (Fig. 1). The endogenous respiration (not shown) followed a similar pattern. At 90 °C, short-term increases were observed for unbroken bacteria and cell-free extracts, but not washed respiratory particles. At 90 °C the succinate oxidase of unbroken bacteria and the NADH oxidase of washed respiratory particles were substantially less stable than the oxidase activities of cell-free extract. A likely explanation is that the succinate transport system is thermolabile, and that cytoplasmic factors are not only able to stabilize oxidase activities in respiratory particles but are also implicated in the activation effect.

These stabilities are appreciably greater than those reported for NADH oxidase from *Bacillus stearothermophilus* protoplasts (Wisdom & Welker, 1973). Pask-Hughes & Williams (1975) reported similar thermostabilities for malate and isocitrate dehydrogenases in crude

Table 1. Rates of oxygen uptake by fractions from starved *Thermus* T351 at 75 °C

Oxygen uptake rates were determined after 2 min preincubation at 75 °C and are expressed as $\mu\text{mol O}_2$ consumed min^{-1} (mg protein^{-1}). Numbers in parentheses are rates in the presence of added supernatant (10 mg protein).

Substrate	Rates of oxygen uptake by different cell fractions			
	Intact organisms	Large particles	Small particles	Supernatant
Endogenous	0.013	< 0.001 (< 0.001)	< 0.001 (< 0.001)	< 0.001
NADH	—	0.073 (0.370)	0.170 (0.410)	< 0.001
Succinate	0.019	0.180 (0.180)	0.114 (0.114)	< 0.001

Fig. 1. Effect of preincubation at 75 °C (a) and 90 °C (b) on the oxidase activities of *Thermus* T351 suspensions and membrane particles. Assays were performed at 75 °C as described in Methods: ●, succinate oxidase activity of unbroken bacteria; ○, succinate oxidase activity of cell-free extract; □, NADH oxidase activity of cell-free extract; ■, NADH oxidase activity of large membrane particles. Rates of oxygen uptake are expressed as a percentage of the rate without preincubation.

extracts from *Thermus* strains YT1, NH and DI at 90 °C. An initial increase in activity of isocitrate dehydrogenase from YT1 was attributed to cold inactivation of the enzyme. We have no evidence that this is the case for the activations observed here (Fig. 1). Purified glyceraldehyde-3-phosphate dehydrogenase from *Thermus aquaticus* at 90 °C apparently had similar or greater thermostability (Hocking & Harris, 1973). However, the thermostability of NADH oxidase reported here is presumably that of the least thermostable component in the electron transport system.

The thermostability of the NADH oxidase activity of respiratory particles at 90 °C over a 15 min period was unaffected by phosphate buffer concentration (0.01 to 2 M), 1 M-MgSO₄ or by 10 mg casein ml⁻¹. Stability was enhanced about twofold by 50% (v/v) glycerol, 2 M-(NH₄)₂SO₄, and 10 mg NADH ml⁻¹. Rates were determined at 75 °C.

The absorption peaks of *a*-, *b*- and *c*-type cytochromes in washed respiratory particles were at 613 and 602, 559 and 555 nm, respectively (Fig. 2a). The major *a*-type cytochrome had an absorption peak at 613 nm, which is unusual; the troughs at 615 and 444 nm in the CO spectra suggest that at least one of the *a*-type cytochromes is a terminal oxidase. The trough at 561 nm and the peak at 417 nm indicate the presence of cytochrome *o*, and the trough at 550 nm suggests that there was some CO-reactive *c*-type cytochrome in the respiratory particles. The soluble fraction (Fig. 2b) contained at least two soluble *c*-type

Fig. 2. Difference spectra at room temperature of (a) the small membrane particle fraction ($5.0 \text{ mg protein ml}^{-1}$) and (b) the supernatant ($6.0 \text{ mg protein ml}^{-1}$) prepared from *Thermus* T351: —, dithionite-reduced minus oxidized difference spectra; ---, dithionite-reduced + CO minus dithionite-reduced difference spectra.

cytochromes, since the ratio of the peaks at 420 and 426 nm varied somewhat between preparations, and at least one of these was CO-reactive. *b*- and *c*-type cytochromes in *Thermus* NH have been reported by Pask-Hughes & Williams (1975), and *a*-605 and *b*- and *c*-type cytochromes in a *Thermus aquaticus* type organism by McFetters & Ulrich (1972).

Cytochrome concentrations [$\mu\text{mol cytochrome (g protein)}^{-1}$] in respiratory particles were: *a*-602, 0.03; *a*-613, 0.05; total *b*-type, 0.89; *o*, 0.21; total *c*-type, 0.64; and in the supernatant, 0.79 for cytochrome *c* and 0.02 for the CO-reactive cytochrome *c*. These concentrations are similar to those found in other aerobes (Gel'man *et al.*, 1975).

The sensitivity to respiratory inhibitors was within the range found for other bacteria (e.g. Daniel, 1970; Erickson & Parker, 1969; Taniguchi & Kamen, 1965) (Table 2) and there was no evidence that active sites are less exposed than in non-thermophiles. Terminal oxidase inhibitors and amytal affected NADH and succinate oxidases equally. Rotenone had more effect on the NADH oxidase activity, but bathophenanthroline, 2-heptyl-4-hydroxyquinoline-*N*-oxide and antimycin A were all more effective inhibitors of succinate oxidase. Bathophenanthroline was specific for succinate oxidase at the concentrations used.

Bacterial electron transport systems vary widely in their oxidase activities, components and inhibitor sensitivities. Apart from its thermostability and the presence of an *a*-type cytochrome with an unusual absorption peak, the electron transport system of this extreme thermophile does not fall outside the range of this variation and we have no evidence that

Table 2. *Effect of inhibitors on the NADH and succinate oxidase activities of respiratory particles at 75 °C*

Activities are expressed as a percentage of the uninhibited oxidase activity. Each assay contained 4 mg protein.

Inhibitor	Concn (μM)	Activity retained (%)	
		NADH oxidase	Succinate oxidase
KCN	10	87	80
	100	56	40
	1000	28	26
NaN ₃	500	86	85
CO/O ₂	(0.3*)	92	—
	(2.4*)	60	—
	(14.3*)	20	—
Amytal	500	88	77
Rotenone	200	48	89
Bathophenanthroline	50	100	76
	200	100	24
2-Heptyl-4-hydroxyquinoline-N-oxide	25	40	11
Antimycin A	25	66	47

* Concentrations of CO/O₂ are expressed as the molar ratio in solution.

the function of the bacterial membrane is in any way unusual. Furthermore, despite the largely hydrophobic environment of the electron transport system, its thermostability seems similar to that exhibited by soluble proteins from other extreme thermophiles.

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