Thermotolerance of the Respiratory Chain of *Bacillus coagulans*

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The thermostability of the respiratory chain of *Bacillus coagulans* grown at different temperatures was similar to the thermostolerance of the primary dehydrogenases. NADH was the major electron donor to the respiratory chain in cells grown at both 37 °C and 55 °C. The respiratory chain from cells grown at 55 °C exhibited slightly greater thermostability than that from cells grown at 37 °C. NADH-supported respiration, as well as NADH dehydrogenase activity, was much more thermostolerant than that with succinate in cells grown at both temperatures. Membrane-bound succinate dehydrogenase could be stabilized by the addition of 10% (w/v) NaCl while NADH dehydrogenase exhibited intrinsic thermostability.

**INTRODUCTION**

Facultative thermophiles such as *Bacillus coagulans* may produce thermolabile enzymes that can be stabilized to operate at high temperatures, intrinsically thermostable proteins or those which show increased thermostability at higher growth temperatures (Heinen & Lauwers, 1983). Glyceraldehyde-3-phosphate dehydrogenase in crude extracts of *B. coagulans* KU was thermolabile whether the cells were grown at moderate or high temperatures. However, it was possible to confer heat stability in excess of the high growth temperature by adjusting ionic strength with NaCl (Crabb et al., 1975). This led to the proposal that facultative thermophiles depend on extrinsic factors to stabilize their enzymes at high temperatures (Amelunxen & Murdock, 1978; Crabb et al., 1975). In contrast, ATPase activities and NADH oxidation by membrane preparations of *B. coagulans* were thermostable over the entire growth temperature range of the organism (Edwards & Jones, 1983; Jones et al., 1984; Ball et al., 1985). Four soluble tricarboxylic acid cycle enzymes from *B. coagulans* have also been studied with respect to thermostability. NaCl increased the thermostability of fumarase, aconitase and malate dehydrogenase, but not of isocitrate dehydrogenase. Heating the enzymes in the presence of their substrates increased the thermostability of isocitrate dehydrogenase, aconitase and fumarase, but not malate dehydrogenase (Jones & Spencer, 1985). *B. coagulans* therefore exhibits a diversity of mechanisms for protecting its enzymes at high growth temperatures. Increased thermostability of enzymes in the presence of their substrate has also been found in other thermophiles; for example pyruvate kinase from *Bacillus caldolyticus* was labile at growth temperature, but stability could be increased by 30% with the addition of the substrate (Lauwers et al., 1981).

In this paper we examine the thermostolerance of the respiratory chain of *B. coagulans* in the light of previous work which showed the presence of a thermostable ATPase and oxygen-dependent modulation of respiratory chain composition brought about by growth at different temperatures (Edwards & Jones, 1983; Jones et al., 1984; Ball et al., 1985; Ball & Edwards, 1986).

**METHODS**

Organism and growth. *Bacillus coagulans* (NCIB 8080) was grown aerobically in tryptone/yeast extract/glucose (TYG) broth as described by Edwards & Jones (1983) and Jones et al. (1984). Growth was monitored by following OD_{250}.

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Fractionation methods. Cultures were harvested in the mid-exponential phase of growth (OD_{600} = 0.749) by centrifugation (15000 g, 10 min) at room temperature. Pellets were washed once and resuspended in 20 mm phosphate buffer (pH 7.0). Cell suspensions were disrupted by a single passage through a French pressure cell (0-4 °C) at 8.7 × 10^4 MPa. Cell breakage was assessed using phase contrast microscopy. Unbroken cells and cell debris were removed by centrifugation (10000 g, 10 min) (pellet P1) and the resultant supernatant centrifuged (100000 g, 1 h, 18 °C). Membrane pellets (P2) were resuspended in phosphate buffer and used immediately along with the final supernatant (S1). For quantitative assessment of enzyme distribution during the fractionation procedure all pellets were resuspended to known volumes and the volume of the final supernatant was measured. A sample of broken cell suspension was retained and the activities therein were taken to represent 100%. The bulk of the remaining broken cell suspension was used for fractionation as described above.

Analytical methods. The activity of succinate dehydrogenase (EC 1.3.99.1) was measured using the method of Bernath & Singer (1962) and activity was calculated from the molar absorption coefficient at 600 nm of dichlorophenol indophenol (23 × 10^3 M^-1 cm^-1). NADH dehydrogenase (EC 1.6.99.3) activity was determined spectrophotometrically (Galanke & Hafei, 1978). The reaction was followed at 420 nm, at which the molar absorption coefficient of potassium ferricyanide is 2.7 × 10^3 M^-1 cm^-1. Assays were done at room temperature and one unit of activity was defined as 1 nmol of substrate utilized min^-1 (mg protein)^{-1}. Oxygen uptake rates were measured at 37 °C using a Rank oxygen electrode attached to a Vitatron chart recorder. Membrane samples were made up to 2.5 ml with 20 mm phosphate buffer (pH 7-0) in the chamber of the electrode. Respiration was initiated by adding 50 μl of either NADH (42 mM) or sodium succinate (1 M). The temperature of the chamber was controlled using a thermostatically controlled water heater. Temperatures of suspensions within the chamber were measured with an Edale probe thermometer (Edale Instruments, Toft, Cambridge) before addition of substrates.

RESULTS

Distribution of NADH and succinate dehydrogenases after fractionation of broken cell suspensions

Differential centrifugation of disintegrated cell suspensions from cells grown at either 37 °C or 55 °C was used to determine the relative distribution and activity of the succinate and NADH dehydrogenases (Table 1). Approximately 40% of the succinate dehydrogenase activity was located in the soluble fraction in cells grown at both temperatures, whilst a higher percentage of the enzyme was found in the membrane fraction of cells grown at 55 °C, compared to cells grown at 37 °C. Activity of the enzyme from the membrane fraction prepared from cells grown at 37 °C or 55 °C was greater than that for the soluble enzyme. In contrast, nearly all the NADH dehydrogenase activity was in the soluble fraction, less than 10% being membrane-bound in cells grown at either temperature.

Table 1. Distribution of the activities of NADH and succinate dehydrogenases after fractionation by differential centrifugation of crude extracts of B. coagulans

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein (%)</th>
<th>Specific activity [units (mg protein)^{-1}]</th>
<th>Total activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>SDH 100 (100)</td>
<td>33.6 (38-4)</td>
<td>100 (100)</td>
</tr>
<tr>
<td></td>
<td>NDH 100 (100)</td>
<td>202 (230)</td>
<td>100 (100)</td>
</tr>
<tr>
<td>Cell debris</td>
<td>P1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SDH</td>
<td>20 (22)</td>
<td>35.6 (9-03)</td>
<td>21.2 (5-3)</td>
</tr>
<tr>
<td>NDH</td>
<td>20 (17)</td>
<td>66.6 (96-3)</td>
<td>6.7 (8-6)</td>
</tr>
<tr>
<td>Membrane</td>
<td>P2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SDH</td>
<td>15 (26)</td>
<td>91.5 (68-7)</td>
<td>35.7 (63-1)</td>
</tr>
<tr>
<td>NDH</td>
<td>15 (17)</td>
<td>111 (151)</td>
<td>9.0 (2-8)</td>
</tr>
<tr>
<td>Supernatant</td>
<td>S1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SDH</td>
<td>52 (78)</td>
<td>22.9 (30-8)</td>
<td>40.0 (47-0)</td>
</tr>
<tr>
<td>NDH</td>
<td>53 (57)</td>
<td>337 (334)</td>
<td>88.6 (86-0)</td>
</tr>
<tr>
<td>Recovery</td>
<td></td>
<td>87 (126)</td>
<td>96.9 (115-4)</td>
</tr>
<tr>
<td>NDH</td>
<td>88 (92)</td>
<td>104 (3)</td>
<td>97.4 (97-4)</td>
</tr>
</tbody>
</table>
Thermotolerance in B. coagulans

The thermostability of membrane-bound and soluble NADH and succinate dehydrogenases was examined by heating fractions for 5 min at various temperatures (Fig. 1). The soluble NADH dehydrogenase from cells grown at 37 °C or 55 °C showed similar thermostability (Fig. 1a). The membrane-bound NADH dehydrogenase from cells grown at 37 °C was less thermostable (50% inhibited at approximately 68 °C) than that from cells grown at 55 °C (50% inhibited at approximately 75 °C) (Fig. 1b). Soluble succinate dehydrogenase activity showed similar thermostability from cells grown at either 37 °C or 55 °C (Fig. 1a), being 50% inhibited at approximately 60 °C. The membrane-bound activity from cells grown at 37 °C was slightly less thermostable than that from cells grown at 55 °C. These results also show that NADH dehydrogenase is more thermostable than succinate dehydrogenase. Membrane-associated activities from cells grown at 55 °C were slightly more thermostable than those from cells grown at 37 °C, implying that changes in membrane structure may occur at the higher temperature.

Thermotolerance of the two dehydrogenases

In order to investigate whether the differences in thermostability of the dehydrogenases were also reflected in the thermotolerance of the whole respiratory chain, membrane fractions were heated at various temperatures and assayed for NADH- or succinate-supported respiration (Fig. 2). Succinate-driven respiration by membranes from cells grown at both 37 °C and 55 °C showed similar thermostability, in that oxidation fell to approximately 10% of the maximum activity at 65 °C (Fig. 2). This was similar to that of the enzymic activity (Fig. 1b). The NADH-directed respiration from B. coagulans grown at 55 °C showed greater thermostability. NADH oxidation by membranes from cells grown at 37 °C fell to 10% of the original activity at a temperature of approximately 72 °C, compared to 83 °C for cells grown at 55 °C (Fig. 2). Again these results closely reflected the heat inactivation of the NADH dehydrogenase activity (Fig. 1b).

Factors promoting thermotolerance

Further studies were made on the thermostability of succinate and NADH dehydrogenases, by studying the effects of heating in the presence of either 10% (w/v) NaCl or substrate (Fig. 3). Results from fractions obtained from cells grown at 37 °C (data not shown) were similar to the results from cells grown at 55 °C. NADH dehydrogenase from soluble fractions showed greater thermostability than from membrane fractions (Fig. 3a, b). Untreated membrane-bound NADH dehydrogenase was more thermostable than membrane-bound enzyme heated in the presence of either 10% NaCl or 5 mM-NADH. Untreated enzyme activity fell to 10% of the
Fig. 2. Thermostability of substrate-driven respiration of membranes from *B. coagulans* grown at 37 °C (O, □) and 55 °C (●, ■) using succinate (O, ●) and NADH (□, ■) as substrate. Fractions were heated for 5 min at various temperatures and then immediately assayed for substrate-driven activity using an oxygen electrode. Activity is expressed as a percentage of the untreated enzyme activity (100%) assayed at 55 °C: 65.5 and 56.7 nmol O₂ min⁻¹ (mg protein)⁻¹ for NADH-driven oxidation by membranes from cells grown at 55 °C and 37 °C respectively and 2.4 nmol O₂ min⁻¹ (mg protein)⁻¹ for succinate-driven oxidation by membranes from cells grown at both temperatures.

Fig. 3. Thermal properties of NADH and succinate dehydrogenases from *B. coagulans*. Membrane-bound (a, c) and soluble (b, d) activities from cultures grown at 55 °C, were heated in the presence of 5 mM-substrate (●, O), or 10% NaCl (■, □) or without additions (▲, △). Fractions were heated at 70 °C for NADH dehydrogenase (●, ■, ▲) and 60 °C for succinate dehydrogenase (O, □, △). Activity is expressed as a percentage of the untreated enzyme activity (100%; see legend to Fig. 1) assayed at 55 °C.
original enzyme activity after approximately 17 min heating at 70 °C compared to 13 min and 11 min for membrane fractions heated in the presence of 5 mM-NADH and 10% NaCl respectively (Fig. 3a). Soluble NADH dehydrogenase activity in fractions heated in the presence of 5 mM-NADH was more thermostable than that in fractions heated in the presence of 10% NaCl or untreated (Fig. 3b). Enzyme activity in fractions heated in the presence of NADH fell to approximately 30% of the original activity after 30 min heating at 70 °C, compared to 10% for untreated enzyme and enzyme heated in the presence of 10% NaCl (Fig. 3b). Soluble succinate dehydrogenase showed similar thermostability when incubated at 60 °C irrespective of the incubation conditions (50% inhibition after approximately 16 min heating at 60 °C). Membrane-bound succinate dehydrogenase, when heated at 60 °C either in the presence of 5 mM-succinate or untreated, was quite thermostable, activity being 50% inhibited after 8 min heating (Fig. 3c). However, membrane-bound fractions heated at 60 °C in the presence of 10% NaCl were thermostable, being only slightly inhibited after 30 min incubation (Fig. 3c).

**DISCUSSION**

Growth of *B. coagulans* can occur over a temperature range of 25 °C to more than 61 °C (Sharp *et al.*, 1980). This means that cellular activities must operate over the temperature range of both mesophilic and thermophilic organisms. Growth adapts with no apparent lag to shifts up or down in temperature (Ball & Edwards, 1986), which suggests that few, if any, new proteins are synthesized as part of a thermo-adaptive response. *B. coagulans* must therefore either synthesize intrinsically thermostable proteins also capable of activity at low temperatures or stabilize proteins in some way to allow them to operate at high temperatures. In this work we have examined the thermostolerance of the respiratory chain of *B. coagulans* in the light of previous investigations which have shown that the ATPase of this bacterium is intrinsically thermostable (Edwards & Jones, 1983).

The respiratory chain of *B. coagulans* lacks cytochrome c, and the terminal oxidases include a + a3, some cytochrome o and, during growth at 55 °C, cytochrome oxidase d (Ball & Edwards, 1986). Thus physiological substrates feed in at sites 1 (NADH dehydrogenase) and 2 (flavin-linked enzymes such as succinate dehydrogenase).

Fractionation of disintegrated cell suspensions revealed that succinate dehydrogenase was predominantly associated with the membrane fraction of the cell while the bulk of the NADH dehydrogenase activity (nearly 90%) was located in the soluble fraction, and probably reflected a loose association of the enzyme with the membrane which was disrupted by the cell disintegration method employed here.

In general terms, both enzymes showed similar thermostability in both membrane and soluble fractions from cells grown at either temperature, but with the membrane-bound fractions from 37 °C grown cells being slightly less thermostable. A good correlation was found between the thermostolerance of the dehydrogenases and the stability of the full respiratory chain, indicating that all steps (e.g. oxidase activity) subsequent to the dehydrogenases were at least as thermostable. NADH-supported respiration, as well as NADH dehydrogenase activity, was much more thermostolerant than that with succinate. Growth temperature also tended to determine the thermostolerance of NADH oxidation. Soluble NADH dehydrogenase activity was more thermostolerant in the presence of NADH. Protection of enzyme denaturation by substrates has also been reported for enzymes of the tricarboxylic acid cycle in *B. coagulans* (Jones & Spencer, 1985). For succinate dehydrogenase, it was found that the membrane-bound activity could be stabilized to a high degree in the presence of 10% NaCl. A high intracellular or localized membrane ionic charge could therefore protect inactivation of the enzyme by heat. Although such high levels of NaCl will obviously not be present in non-halophiles, Damadian (1973) showed that the total intracellular ionic charge of *Escherichia coli* was 1-3 molal, so that the use of 10% NaCl in these experiments is a valid test.

The data suggest that *B. coagulans* could use a number of mechanisms to protect key metabolic enzymes from thermal denaturation. These include the synthesis of intrinsically therophilic proteins such as NADH dehydrogenase (this work) and ATP phosphohydrolase (Edwards &
Jones, 1983), protection by a high ionic charge and protection by substrate binding. Finally, the higher thermotolerance of NADH dehydrogenase, especially in cultures grown at 55 °C suggests that this bacterium will preferentially direct reducing equivalents via this part of the respiratory chain. This is borne out by the much higher activity of NADH-supported oxidation rates compared with those of succinate at 55 °C reported by Ball & Edwards (1986).

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REFERENCES


