Comparative Study of Energy-Transducing Properties of Cytoplasmic Membranes from Mesophilic and Thermophilic Bacillus Species

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The properties of enzymes involved in energy transduction from a mesophilic (Bacillus subtilis) and a thermophilic (B. stearothermophilus) bacterium were compared. Membrane preparations of the two organisms contained dehydrogenases for NADH, succinate, l-α-glycerophosphate, and l-lactate. Maximum NADH and cytochrome c oxidation rates were obtained at the respective growth temperatures of the two bacteria. The enzymes involved in the oxidation reactions in membranes of the thermophilic species were more thermostable than those of the mesophilic species. The apparent microviscosities of the two membrane preparations were studied at different temperatures. At the respective optimal growth temperatures, the apparent microviscosities of the membranes of the two organisms were remarkably similar. The transition from the gel to the liquid-crystalline state occurred at different temperatures in the two species. In the two species, the oxidation of physiological (NADH) and nonphysiological (N,N,N',N'-tetramethyl-p-phenylenediamine or phenazine methosulfate) electron donors led to generation of a proton motive force which varied strongly with temperature. At increasing temperatures, the efficiency of energy transduction declined because of increasing H+ permeability. At the growth temperature, the efficiency of energy transduction was lower in B. stearothermophilus than in the mesophilic species. Extremely high respiratory activities enabled B. steatorhermophilus to maintain a high proton motive force at elevated temperatures. The pH dependence of proton motive force generation appeared to be similar in the two membrane preparations. The highest proton motive force generation was observed at low external pH, mainly because of a high pH gradient. At increasing external pH, the proton motive force declined.

The effect of environmental temperature on the physical state, lipid composition, and physiological function of biological membranes has been studied thoroughly in mesophilic bacteria (18, 20, 24–26). Thermophilic microorganisms, which are able to grow at a wide range of temperatures, are well suited for a study of thermoadaptive processes in cytoplasmic membranes.

Several adaptations are required for biological membranes for optimal functioning at high temperatures. In general, the phospholipid composition of bacteria changes with the growth temperature (13). Adaptations at the protein level occur to allow enzymes from thermophilic microorganisms to operate at elevated temperatures (2). The intrinsic heat resistance of proteins of obligate thermophiles has been obtained by simple amino acid substitutions or additional salt bridging, often without gross changes in the conformation of the enzymes (1, 17, 21, 28). In thermotolerant microorganisms, the heat stability or lability of some enzymes has been suggested to be adjusted by alterations in intracellular environments (8).

Membrane-bound enzymes of thermophiles receive increased attention, mainly because of their (thermo)stability, and therefore are suitable for purification-reconstitution studies. The respiratory chains of thermophilic species, consisting of highly organized multi-enzyme systems, such as dehydrogenases, electron carriers, and terminal oxidoreductases, have been investigated in terms of their redox-carrier composition, activity, and sensitivity to classical inhibitors of electron transfer (6, 9). Also, energy-transducing properties of several cytochrome components and ATPases from thermophilic organisms have been studied (10, 16).

Aspects which have received little attention are the capacity of thermophiles to generate a proton motive force by respiration and the temperature dependence of these processes.

In the genus Bacillus, representatives of mesophilic, thermotolerant, thermophilic, and even caldoactive species are found (13). In this paper, the energy-transducing properties of a mesophilic (Bacillus subtilis) and a thermophilic (B. stearothermophilus) species and the effect of temperature on these processes were studied.

MATERIALS AND METHODS

Cell growth and preparation of membrane vesicles. B. subtilis W23 was grown at 37°C with vigorous aeration in a medium containing 0.8% (wt/vol) Tryptone (Difco Laboratories), 86 mM NaCl, 25 mM KCl, and 150 µl of micronutrient solution per liter. This micronutrient solution contained MnCl₂ (2.2%), ZnSO₄ · 7H₂O (0.05%), H₃BO₃ (0.5%), CoNO₃ · 6H₂O (0.46%), and 5 ml of a concentrated H₂SO₄ solution per liter. The medium was supplemented with CaCl₂ · 2H₂O (50 mg/liter) and FeSO₄ · 7H₂O (50 mg/liter). B. stearothermophilus ATCC 7954 was grown at 55°C with vigorous aeration in a medium containing Tryptone (10 g/liter), yeast extract (5 g/liter), and NaCl (172 mM) adjusted to pH 7.0.

Logarithmically growing cells were harvested at an A₆₆₀ of 0.8 to 1.0. Membrane vesicles of the cell preparations were prepared essentially as described by Konings et al. (11), except that the lysis temperature for the thermophilic organism was increased to 50°C to facilitate cell lysis. The

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membrane preparations were suspended and washed in various buffers and stored in a concentrated form (20 to 30 mg of protein per ml) in liquid nitrogen.

**Determination of enzyme activities.** Cytochrome c oxidase activity was measured spectrophotometrically by monitoring the decrease in the absorbance of the alpha peak of cytochrome c with an extinction coefficient (reduced minus oxidized) of ε_{550-540} = 19.5 mM⁻¹ cm⁻¹.

Oxygen consumption by membrane vesicles was measured polarographically in a 2- or 4-ml vessel with a Clark-type electrode (Yellow Springs Instrument Co.) The oxygen- or air-saturated medium contained 50 mM potassium phosphate at the appropriate pH and 5 mM MgSO₄, NADH, D,L-malate, succinate, L-lactate, or L-α-glycerophosphate at a final concentration of 10 mM or a combination of nonphysiological donors such as K⁺-ascorbate (10 mM) with N,N,N,N-tetramethyl-p-phenylenediamine (TMPD; 10 to 700 μM) or phenazine methosulfate (PMS; 5 or 200 μM) was used as an electron donor. Because the measurements were made at different temperatures, a correction was applied for a temperature-dependent variation in the oxygen solubility of the medium.

**Enzyme inactivation.** To determine the rates of inactivation of various enzyme systems, membrane vesicles were incubated for different periods at different temperatures. Directly after the incubation period, the samples were put on ice until use. Residual enzyme activities were then determined at 30°C; no detectable inactivation of the enzyme activities tested occurred during the assay time. From the inactivation patterns at different temperatures, rate constants were determined as described by Warth et al. (27) and plotted semilogarithmically versus the reciprocal of the absolute temperature. From the linear relationships obtained, the so-called inactivation temperature (Tᵢ), which is defined as the temperature at which enzyme activity is reduced by 50% in 10 min, was determined.

**Proton motive force measurement (electrical potential and pH gradient).** The transmembrane electrical potential (Δψ) was determined by two independent methods. From the distribution of tetryraphenylphosphonium ion (TPP⁺) across the cytoplasmic membrane, as measured with a TPP⁺-sensitive electrode, the membrane potential was quantified as previously described (14). A correction for concentration-dependent probe binding was applied according to the model of Lolkema et al. (14). For measurements at different temperatures, oxygen-saturated media which contained a final TPP⁺ concentration of 2 μM were used. The second method is based on the use of the membrane potential indicator 3,3'-dipropylthiacarboylcyanine iodide [diSC(3)] and measurement of membrane potential-dependent absorbance changes (A_{683} minus A_{660}). Calibration was performed by valinomycin-induced potassium diffusion potentials in a medium containing 50 mM potassium phosphate (pH 7.0), 5 mM MgSO₄, 2 μM diSC(3)], and 100 to 200 μg of membrane protein per ml. The measurements were performed at various temperatures.

The transmembrane pH gradient (interior alkaline) was determined by measuring the fluorescence changes of entrapped pyranine as described by Clement and Gould (3). Incorporation of pyranine was achieved by mixing 200 nmol of pyranine with 1 ml of membrane vesicles (10 to 20 mg of protein per ml), followed by a freeze-thaw sonication cycle. To remove external pyranine, the membrane vesicles were chromatographed over a Sephacry G-25 column (5 × 1 cm) which was preequilibrated with the buffer used during the measurements. Fluorescence changes were measured by using the excitation-emission pair 460 and 508 nm with a Perkin-Elmer spectrofluorometer. Calibration was performed by titration with acid or base in the uncoupled membrane vesicles (i.e., in the presence of 2 mM of 5-chloro-3-tert-butyl-2'-chloro-4'-nitrosalicylanilide per mg of membrane protein).

**Determination of proton fluxes through membranes.** Δψ-induced proton flux measurements in *B. steaerothermophilus* and *B. subtilis* membrane vesicles were performed at different temperatures in a well-stirred thermostat-equipped 2-ml cuvette, with phenol red (0.02 mg/ml final concentration) as the external pH indicator, by monitoring its absorbance change at 560 minus 610 nm. The rate of external pH change, as reflected by the absorbance changes of phenol red, was converted into H⁺ flux by using pulses of calibrated amounts of oxalic acid or KOH.

Membrane vesicles were washed three times in a buffer containing 0.5 mM 2-(N-morpholine)ethanesulfonic acid, 0.5 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) adjusted to the appropriate pH, and 200 mM KCl. Valinomycin-induced potassium diffusion potentials were imposed across the cytoplasmic membranes by 100-fold dilution of the membrane vesicles (14 to 15 mg of protein per ml) in the same medium, in which sodium ions were substituted for potassium ions and additional phenol red was added. The potential was initiated by addition of a small sample of valinomycin (2 μM final concentration).

**Microviscosity determinations.** The thermotropic behavior of the bacterial membrane preparations was determined from steady-state fluorescence polarization measurements of the fluorophore 1,6-diphenyl-1,3,5-hexatriene (DPH; Aldrich Chemical Co., Inc.) with a Perkin-Elmer spectrofluorometer. The experimental procedure used was described elsewhere (12). All measurements were performed in a medium containing 100 mM potassium phosphate (pH 7.0) supplemented with DPH-preincubated membranes. Measurements with *B. steaerothermophilus* membranes were performed at a DPH-protein ratio of 1.53 (nanomoles per milligrams of protein), and with *B. subtilis* membranes a ratio of 2.02 (nanomoles per milligrams of protein) was used. The apparent microviscosity (γ) was calculated after correction for a grating factor (G) of 0.78 and for the light scattering of the membranes was applied as described elsewhere (12).

**Protein determination.** Protein was determined by the method of Lowry et al. (15) with bovine serum albumin as a standard.

**Materials.** Laurylmaltoside, *Saccharomyces cerevisiae*, and horse heart cytochrome c were obtained from Sigma Chemical Co. DiSC(3)5 was purchased from Molecular Probes Inc., and phenol red was obtained from E. Merck AG.

**RESULTS**

**Membrane-bound dehydrogenases.** Enzyme-linked reduction of Fe(CN)₆³⁻ was monitored to detect the dehydrogenases in detergent-treated membranes (Table 1). *B. subtilis* membranes possess NADH, succinate, L-lactate, and L-α-glycerophosphate dehydrogenase activities. The same activities were detected immunologically with zymogram staining techniques (W. De Vrij, Ph.D. thesis, University of Groningen, The Netherlands, 1986). In *B. subtilis* membranes, no immunological response was observed for glucose or D,L-malate dehydrogenase. *B. steaerothermophilus* membranes also contained relatively high activities of NADH and succinate dehydrogenases and significant D,L-malate dehy-
TABLE 1. Substrate-linked dehydrogenase activities and oxidation rates in membrane preparations

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Fe(CN)₆³⁻ reduction (nmol/min per mg of protein)</th>
<th>Oxidation rate (nmol of O₂/min per mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B. subtilis</td>
<td>B. stearothermophilus</td>
</tr>
<tr>
<td>NADH</td>
<td>1,196</td>
<td>3,052</td>
</tr>
<tr>
<td>Succinate</td>
<td>379</td>
<td>570</td>
</tr>
<tr>
<td>Succinate-PMS</td>
<td>50</td>
<td>136</td>
</tr>
<tr>
<td>L-α-Glycerophosphate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-α-Glycerophosphate-PMS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Lactate</td>
<td>5</td>
<td>12</td>
</tr>
<tr>
<td>L-Lactate-PMS</td>
<td>0</td>
<td>223</td>
</tr>
<tr>
<td>D,L-Malate</td>
<td>0</td>
<td>92</td>
</tr>
<tr>
<td>D,L-Malate–PMS</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Oxygen consumption was determined with membrane preparations from B. subtilis and B. stearothermophilus. Measurements were performed in oxygen-saturated 50 mM potassium phosphate (pH 7.0)–5 mM MgSO₄ at 25 or 45°C, as indicated. All substrates were added at 10 mM concentrations, whereas PMS was used at 200 μM. Ferricyanide reduction was measured as described in the text, in the same medium containing 0.05% (wt/vol) lauryl maltoside.

Substrate-linked dehydrogenase activity. The activities in the latter membranes were recorded at a suboptimal temperature (45°C), and the measured activities thus do not reflect maximal turnover rates of the enzymes. Membrane preparations also had high NADH oxidase activity (Table 1). B. stearothermophilus membranes also showed a significant rate of D,L-malate oxidation, but the oxidation rates of the other substrates tested were extremely low.

Low oxidation rates of substrates for which highly active dehydrogenases are present could be caused by ineffective coupling of the dehydrogenase to the respiratory chain. This latter explanation holds for the low oxidation rate of succinate. In the presence of the nonphysiological membrane-permeable electron mediator PMS (200 μM), significant stimulation of the succinate oxidation rates were observed in the two membrane preparations. The low oxidation rates of L-lactate and D,L-malate, even in the presence of PMS, appeared to be due to the low activities of the respective dehydrogenases.

Temperature dependencies of respiratory enzymes. Membrane preparations were derived from the mesophilic and thermophilic bacilli grown at 37 and 55°C, respectively. The temperature dependencies of the enzymes involved in energy transduction are shown in Fig. 1a and b.

NADH oxidation involves a sequential series of oxidoreductase reactions, and the temperature dependency shown reflects the dependency of the overall process under substrate-saturating and steady-state conditions (Fig. 1a). The temperature optima observed for NADH oxidation corresponded rather well with the respective optimal growth temperatures of the two organisms. It should be noted that the oxidation rates of B. stearothermophilus membranes at elevated temperatures are extremely high. The relevance of this phenomenon will be discussed below.

Similar temperature dependencies were observed for cytochrome c oxidation in the two membrane preparations (Fig. 1b). The cytochrome c oxidase activities in membranes increased with temperature up to 60°C in B. stearothermophilus, whereas in B. subtilis membranes maximum oxidation rates were reached at lower temperatures (35 to 40°C). Further temperature increase resulted in lower activities. The decline in activity above 60°C in B. stearothermophilus membranes was extremely rapid.
membranes could be attributed to denaturation of the substrate cytochrome c. When cytochrome c was preincubated at temperatures above 60°C and subsequently used as a substrate for cytochrome c oxidase at lower temperatures, a similar decrease in activity was observed (data not shown).

Thermostability of respiratory enzymes. NADH oxidation \((T_i = 58°C)\) and cytochrome c oxidation \((T_i = 62°C)\) in \(B. subtilis\) membranes appeared to be rather temperature sensitive (Fig. 2a and b). In the thermophile, the two activities were much less sensitive to incubation at high temperatures. Cytochrome c oxidase was found to be extremely (thermo) stable \((T_i = 86°C)\), whereas NADH oxidation turned out to be slightly more sensitive to high temperatures \((T_i = 77°C)\) (Fig. 2c and d). The thermostability of the overall process of substrate (NADH) oxidation in \(B. stearothermophilus\) membranes is thus not limited by the terminal cytochrome c oxidase.

Microviscosity of membrane preparations. Growth at elevated temperatures also requires adjustment of the phospholipid or fatty acyl chain composition of the membranes so that the microviscosity at high temperatures allows optimal functioning of the embedded proteins. Information about the microviscosity of the two membrane preparations was obtained from DPH fluorescence polarization (Fig. 3). At all temperatures, the apparent microviscosity of \(B. stearothermophilus\) membranes was higher than that of \(B. subtilis\) membranes. Furthermore, drastic changes in microviscosity, indicative of a transition from the thermotropic gel to the liquid-crystalline phase, occurred at different temperatures in the two types of membranes. For \(B. subtilis\) membranes, the transition took place between 15 and 30°C, and for \(B. stearothermophilus\) it occurred between 25 and 40°C. Membranes from \(B. stearothermophilus\) appeared to be more rigid in the gel state than membranes from the mesophilic species, whereas microviscosities tended to converge above phase transition temperatures. It is striking that the microviscosities of the two organisms were very similar at the respective optimal growth temperatures.

Proton motive force generation in membrane vesicles. Oxidation of various electron donors by the membrane vesicles led to the generation of a transmembrane electrochemical proton gradient \(\Delta p\) (Table 2). Under the experimental conditions used, the \(\Delta p\) was composed only of the electrical potential \(\Delta \psi\). In \(B. subtilis\) membranes, a \(\Delta \psi\) of about –85 mV and in \(B. stearothermophilus\) membranes a \(\Delta \psi\) of about –110 mV were generated with NADH and reduced TMPD. Reduced PMS was also effective (data not shown). Reduced forms of PMS and TMPD donate electrons mainly at the level of the terminal oxidase. Succinate oxidation did not

![FIG. 2. Thermostability of cytochrome c and NADH oxidation in membrane vesicles.](image)

![FIG. 3. Temperature dependence of microviscosity of membranes. Microviscosity was determined by measuring the fluorescence polarization of membrane-bound DPH as described in Materials and Methods.](image)
result in the generation of a significant Δψ in either of the membrane preparations (data not shown). In _B. subtilis_ membranes, the stimulatory effect of PMS on succinate oxidation resulted in the generation of a Δψ at suboptimal temperatures (Table 2).

The Δψ generation was measured at different oxidation rates of ascorbate-TMPD in the two membrane preparations (Fig. 4). The oxidation rates were manipulated by varying the TMPD concentration. In membrane preparations of the two organisms, the Δψ increased with the oxidation rate, and the relationship between Δψ generation and respiration rate was temperature dependent. At 20°C, higher respiratory activities were necessary to maintain a certain Δψ in _B. subtilis_ membrane vesicles than in _B. stearothermophilus_ membrane vesicles. For _B. subtilis_ membrane vesicles, the highest respiratory activities shown in Fig. 4 represent the maximal oxidation rates of TMPD (_Vmax_) at the given temperatures. At the growth temperature of _B. subtilis_ (37°C), significantly higher respiratory activities were necessary to obtain a certain Δψ. The relationship between oxidation rate and Δψ generation at 37°C was very similar to the relationship observed for _B. stearothermophilus_ membranes at 45°C. Although much higher respiratory activities could be obtained in _B. stearothermophilus_ membranes at 45°C than at 20°C, the maximum Δψ at 45°C was slightly lower than that at 20°C. This strongly suggests increased ion permeabilities of these membrane preparations at higher temperatures.

To determine the proton permeability of the membrane preparations, K⁺ diffusion potentials (Δψ = -120 mV) were applied and the rates of H⁺ influx were measured at various temperatures. These H⁺ influx rates are indicative of membrane proton permeability (Fig. 5). With increasing temperatures, increasing H⁺ influx rates were observed in the two membrane preparations. _B. subtilis_ membranes showed higher H⁺ influx rates than _B. stearothermophilus_ membranes at all temperatures. At the respective optimal growth temperatures of the organisms, the membranes from _B. subtilis_ were less permeable to protons than were _B. stearothermophilus_ membranes. This was confirmed by observations of the decay rates of K⁺ diffusion potentials at various temperatures (data not shown).

TPP⁺ measurements were difficult to perform at high temperatures because of instability of the ion-selective electrode. An alternative method was therefore used to obtain information about the Δψ at high temperatures. Absorbance measurements with the Δψ indicator diSC(3)5 were performed to demonstrate Δψ generation by substrate oxidation at various temperatures in _B. stearothermophilus_ membranes. The absorbance change with NADH (2.5 mM) as the electron donor in the presence of nigericin declined with increasing temperatures (20 to 60°C), indicating a decrease in generated Δψ (Fig. 6). However, when reduced TMPD (400 μM) was used as the electron donor in the presence of nigericin, no significant decline in the absorbance change was observed at high temperatures. The differences observed with the electron donors at high temperature (60°C) can be explained by the higher oxygen consumption rates with reduced TMPD as the substrate (6,640 nmol of O₂ per min per mg of protein). At all three temperatures tested, a significant change of absorbance was observed upon the addition of nigericin, which is indicative of interconversion of the transmembrane pH gradient (ΔpH) to Δψ.

**pH dependence of Δψ generation.** At temperatures suboptimal for the two types of membranes (25°C for _B. subtilis_ membrane vesicles and 40°C for _B. stearothermophilus_ membrane vesicles), the components of the Δψ, i.e., Δψ and ΔpH, were determined at different pHs (Fig. 7a and b). The Δψ was determined with a TPP⁺-selective electrode, whereas the ΔpH was determined with entrapped pyranine. The Δψ in _B. subtilis_ membranes was generated by oxidation of reduced PMS (20 μM), whereas for _B. stearothermophilus_ membranes reduced TMPD (400 μM) was used as the electron donor. In the two membrane vesicles, the Δψ declined with increasing pH from ~150 to ~160 mV at pH

### TABLE 2. Substrate oxidation and membrane potential generation in membrane vesicles

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Oxidation rate (mmol of O₂/min per mg of protein)</th>
<th>Membrane potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>B. subtilis</em></td>
<td><em>B. stearothermophilus</em></td>
</tr>
<tr>
<td>NADH</td>
<td>452</td>
<td>1,733</td>
</tr>
<tr>
<td>Succinate-PMS</td>
<td>187</td>
<td>74</td>
</tr>
<tr>
<td>Ascorbate-TMPD</td>
<td>261</td>
<td>2,426</td>
</tr>
<tr>
<td>Ascorbate-cytochrome c</td>
<td>160</td>
<td>520</td>
</tr>
</tbody>
</table>

*Oxygen consumption and Δψ were measured in oxygen-saturated 50 mM potassium phosphate (pH 6.6) and 5 mM MgSO₄ at 25°C for _B. subtilis_ membranes and at 45°C for _B. stearothermophilus_ membranes in the presence of 100 nM nigericin. The substrates NADH, succinate, and ascorbate were added at a final concentration of 10 mM and adjusted to the appropriate pH, whereas TMPD (400 μM), cytochrome c (20 μM), or PMS (200 μM) was added as a supplement to ascorbate or succinate.*
FIG. 5. Temperature dependence of the proton influx rate in membrane preparations. Membrane vesicles (14 to 15 mg of protein per ml), washed in a buffer containing 0.05 mM 2-(N-morpholino)ethanesulfonic acid, 0.5 mM HEPES, and 200 mM KCl and adjusted with KOH to pH 7.0, were diluted 100-fold in a medium in which sodium ions were substituted for potassium ions and additional phenol red (20 μg/ml) was added. To initiate the potassium diffusion potential, valinomycin (final concentration, 2 μM) was added. Subsequently, absorbance changes were measured at 560 minus 610 nm to determine external pH changes caused by proton influx as a compensatory effect of the imposed diffusion potential. The absorbance change was converted to J of H⁺/OH⁻ (nanomoles per milligram of protein per minute) by titration with defined amounts of oxalic acid or KOH. Measurements were performed with B. subtilis (■) or B. stearothermophilus (□) membranes.

5.5 to −90 to −100 mV at pH 8.0. This decline was mainly due to a decrease in the ΔpH, which was essentially zero at pH 8.0 in B. stearothermophilus membrane vesicles. In B. stearothermophilus membranes, the Δψ reached an optimum at pH 6.5 (−120 mV) (Fig. 7b), whereas the Δψ in B. subtilis membranes appeared to be pH independent (Fig. 7a).

DISCUSSION

The temperature optima and maximum rates of substrate oxidation were drastically different in the two membrane preparations. The temperature optima of NADH and cytochrome c oxidation corresponded rather well to the optimum temperature for growth. The electron transfer chain enzymes from the thermophilic microorganism showed much higher turnover rates (see, for example, cytochrome c and NADH oxidation) at optimal temperatures than did enzymes of the mesophilic counterpart.

Growth at high temperatures requires not only adaptation of membrane-bound enzymes with respect to activity and stability but also adaptation at the level of membrane phospholipid composition. This latter adaptation is reflected in the relationship between microviscosity and temperature in each membrane preparation. Increased growth temperatures for the two microorganisms resulted in a shift of the thermotropic gel to the liquid-crystalline phase transition temperature. In B. stearothermophilus, the effect of temperature on membrane composition has been studied in great detail (23). It was demonstrated that at various levels alterations occur by variation of the growth temperature. Phosphatidylglycerol and cardiolipine (diphosphatidylglycerol) form 90% of the cellular phospholipids. An increase in phosphatidylglycerol content with a decrease in cardiolipine content occurred at increased growth temperatures. Furthermore, with increasing growth temperatures, a change in the acyl chain composition of all membrane lipids occurred, and a gradual depletion of unsaturated fatty acids, a prominent increase in the *iso-anteiiso* fatty acid ratio, and an increase in the abundance of longer homologs of saturated linear fatty acids were observed (23). The effect of growth temperature on the thermotropic behavior of the membranes in situ can be interpreted as maintenance of the apparent microviscosity (homoviscous adaptation) (23). A distinct apparent microviscosity of the membranes of the mesophilic and thermophilic species at their respective growth temperatures appears to be essential, since the apparent microviscosities of the membranes of the two bacteria at their respective growth temperatures are remarkably similar.

FIG. 6. Membrane potential measurements with the indicator DiSC(3)5 in B. stearothermophilus membranes. Absorbance changes of DiSC(3)5 at 683 minus 660 nm were monitored upon energization of B. stearothermophilus membrane vesicles (0.13 mg of protein per ml) with NADH (2.5 mM) as the electron donor. Measurements were performed in a 2-ml thermostat-equipped cuvette containing 50 mM potassium phosphate (pH 7.0), 5 mM MgSO₄ and 2 μM DiSC(3)5. After addition of the electron donor, nigericin (NIG.; 50 nM) and valinomycin (VAL.; 100 nM) were added at 20°C (a), 40°C (b), and 60°C (c) or at 60°C in the presence of ascorbate (10 mM) and TMPD (400 μM) (d).
LITERATURE CITED


Cytochromes play an important role in energy transduction. The two organisms tested contain two terminal oxidases (the o type and the aα type) (4; W. De Vrij et al., submitted for publication). B. subtilis contains an aα-type cytochrome c oxidase, whereas this enzyme in B. stearothermophilus membranes has an additional covalently attached heme c moiety. The two enzymes have been purified and reconstituted into artificial membranes, and it has been established that they function as Δp-generating components (5; De Vrij et al., submitted). The enzymes are composed of three subunits with similar molecular weights (4; De Vrij et al., submitted). Despite this similarity, the two enzymes have distinctly different properties, such as turnover rate and thermostability.

In our opinion, the most striking difference between the two membrane preparations tested is the efficiency of transducing redox energy into electrochemical energy. In the membranes from the thermophilic organism, extremely high oxidation rates are necessary to maintain a significant Δp at the growth temperature of the organism, indicating high ion permeability of the membranes. This has been confirmed by measuring H' permeability in B. stearothermophilus membranes at the growth temperature. Clearly, this organism is not capable of adjusting the membrane composition in such a way that at high temperatures an efficient way of energy transduction can be performed. Whether this is a general feature of thermophilic microorganisms is not known, but in the thermophilic organisms B. caldotenax and Thermus thermophilus HB8 much higher maintenance energies are found than in mesophilic organisms at their respective optimal growth temperatures (19). This high maintenance energy is most likely due to increased inefficiency of energy transduction.

Despite significant differences in the turnover capacity of the respiratory chain, the thermostability of enzymes, and the ion permeabilities of the membranes, the pH dependence of Δp generation in the membrane vesicles shows some similarities at suboptimal temperature conditions. In the two membrane preparations, Δp was highest at low external pH, which is mainly caused by the high ΔpH. The high Δp at low external pH cannot be explained by pH-dependent ion or H' permeabilities of the membranes, because H' permeabilities were highest at low external pH (data not shown). The decline in ΔpH with increasing external pH indicates the presence of a regulatory mechanism(s) that controls the internal pH in the physiological range. In some microorganisms, cation-H' antiporers are involved in pH homeostasis (22, 29). In a related thermophilic strain of B. stearothermophilus, PS-3, an Na'\textsuperscript{+}/H'\textsuperscript{+} exchanger functions at high pH (7), underlining the significance of these exchangers in thermophilic microorganisms also.