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MEMBRANE PHASE TRANSITIONS AND SUCCINATE OXIDASE ACTIVITY IN AN EXTREMELY THERMOPHILIC BACTERIUM

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ESR and succinate oxidase activity were used to investigate the membrane phase-transitions of an extreme thermophile, *Thermus* T351, over an 80°C temperature range in whole cells, membrane particles, and extracted lipid suspension. Three phase transitions were observed using both techniques. These occurred at about 19°C, 39°C and 66°C. The transition at 19°C is unusual in that the Arrhenius plot for succinate oxidase is concave upwards, implying an increase in activation energy (E_a) with increased temperature.

Introduction

Lipids in biomembranes undergo reversible thermotrophic phase changes as the temperature is increased, and these fluidity changes can affect the activity of membrane-bound enzymes (see, for example, Refs. 1–3). Even a minor change in fluidity can grossly change enzyme activity.

Studies on the effect of temperature on succinate oxidase activity in mitochondria and mesophilic bacteria have made a considerable contribution to this field (see, for example, Refs. 1, 4, 5). Such studies have necessarily been confined to the comparatively narrow temperature range between the freezing point of water and the denaturation temperature of the membrane proteins. However this range is large for extremely thermophilic bacteria, and earlier work [6] has shown that the stability of the succinate oxidase system is sufficient in some cases to allow studies up to 85°C. Some studies of this type have already been done [7], but the full temperature range available was not exploited.

The work presented here is designed to show the effect of lipid fluidity on membrane bound enzyme activity over a wider temperature range, and thus a wider fluidity range, than is possible using mesophilic organisms.

Materials and Methods

Maintenance and growth of cells. Laboratory cultures were maintained freeze-dried or in 20% (w/v) maltose solution at −20°C. The media consisted of Allen's salts ($(NH_4)_2SO_4$, 1300; KH_2PO_4, 280; $MgSO_4 \cdot 7\,H_2O$, 250; $CaCl_2 \cdot 2\,H_2O$, 74; $FeCl_3 \cdot 6\,H_2O$, 19; $Na_2B_4O_7 \cdot 10\,H_2O$, 4.4; $MnCl_2 \cdot 4\,H_2O$, 1.8; $ZnSO_4 \cdot 7\,H_2O$, 0.22; $CuCl_2 \cdot H_2O$, 0.05; $Na_2MoO_4 \cdot 2\,H_2O$, 0.03; in mg/l of distilled water), with 0.3% w/v yeast extract (B.B.L.) and 0.3% w/v trypticase peptone (B.B.L.). Media was adjusted to pH 8.2 with NaOH and autoclaved, after which the pH was 7.5. Cells were grown in 2-l Erlenmeyer flasks containing 500 ml of media, in a Gallenkamp orbital incubator at 75°C at 200 rev./min, and harvested during the late-exponential phase.

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Respiratory particle preparation. Cells were suspended in two volumes of precooled phosphate buffer (0.1 M KH_2PO_4/0.1 M Na_2HPO_4, pH 7.0) using a tissue grinder. After addition of DNAase (Sigma), 0.2 mg · ml^{-1}, cells were disrupted by three consecutive passages through a French Pressure cell (Aminco, Silver Springs, MD) at 60000 kPa. Unbroken cells and cell debris were removed by centrifugation at $10000 \times g$ (r_{av} 4.25 cm) for 15 min, using a precooled rotor. The top three-quarters of the supernatant was carefully removed using a Pasteur pipette and was centrifuged at $350000 \times g$ (r_{av} 6.42 cm) for 60 min to yield respiratory particles. These were washed in 0.1 M phosphate buffer pH 7.0, and stored in $N_{2(l)}$ (−196°C). Particles were resuspended in 0.1 M phosphate buffer pH 7.0 using a tissue grinder before use.

Respiration. Succinate oxidase activity was determined by polarographic measurement of O_2 uptake, using a Rank Oxygen Electrode (Rank Bros. Bottisham, U.K.). The reaction mixture consisted of 2.4 ml phosphate buffer, (0.1 M KH_2PO_4/0.1 M Na_2HPO_4, pH 7.0), 0.05–0.1 ml of respiratory particles containing an appropriate amount of protein (0.5 to 2.5 mg/ml^{-1}) and 50 μmol of succinate as substrate, in a final volume of 2.5 ml [6].

A standard oxygen-electrode plug was modified to hold a thermistor probe, which allowed temperature measurement in the electrode chamber to within ±0.5°C. This direct measurement was essential because the temperature difference between the electrode chamber and the thermostated water bath supply increased from 1°C at 30°C to 5°C for a 90°C supply.

Electron spin resonance experiments. ESR spectra were recorded on a Varian E104A spectrometer, operating at approx. 9.5 GHz, equipped with Varian E257 variable temperature accessory. Samples were contained in sealed capillary tubes and temperatures were monitored (±1°C using a copper-constantan thermocouple attached to the sample tube. The spin probe Tempo (2,2,6,6-tetramethylpiperidine-1-oxyl) was purchased from Syva (NZ) Ltd., Wellington, New Zealand.

For measurements with lipids, 8 μl of Tempo ($5 \cdot 10^{-3}$ M) in water was added to 0.8 ml of lipid suspension (26.5 mg dry wt./ml^{-1}) to give a final Tempo concentration of $5 \cdot 10^{-5}$ M [8]. For respiratory particles, 1 ml of suspension (32 mg protein/ml^{-1}) was added to 10 μl of spin label ($5 \cdot 10^{-3}$ M) in a test-tube, the final Tempo concentration being $5 \cdot 10^{-5}$ M. For whole cells, 0.9 ml of cells (38 mg cell protein/ml^{-1}) was added to 30 μl $K_3Fe(CN)_6$ (100 μM) and shaken in a vortex mixer. After one minute, 30 μl Tempo ($5 \cdot 10^{-3}$ M) in water was added, and the sample again shaken. Final Tempo concentration was $1.6 \cdot 10^{-4}$ M.

The partitioning parameter *f* is a measure of the relative concentrations of the probe in hydrophobic and aqueous phases and was derived from the relative amplitudes of the peaks on the high field hyperfine line of the nitroxide spectrum (see, for example, Ref. 9).

Results and Discussion

The Arrhenius plots for succinate oxidase activity from both whole cells and respiratory particles (Figs. 1 and 2) are very similar. Breaks in slope denoting activation energy changes occur at about 65, 40 and 20°C. The middle break is oblique and might conceivably represent more than one change. The nature of the break at about 20°C is quite unexpected, in that it is concave upwards indicating an increase in activation energy with increased temperature.

Initial ESR experiments using Tempo with either whole cells or respiratory particles exhibited spectra which rapidly decreased in magnitude as the temperature was raised towards 75°C, apparently due to reduction of the spin label by the electron transport system. This general phenomenon has been observed by other workers [10]. This reduction in respiratory particles was halted by repeated washing (×4) [3]. However, this was ineffective in whole cells. For whole *Thermus*, addition of ferricyanide at a low concentration followed by incubation, halted reduction of the spin label up to about 65°C.

The size of the partitioning parameter '*f*' in the ESR results (Figs. 3–5) varies with the changing solubility of the probe in the hydrophobic phase as the fluidity alters. In the absence of phase transitions this will vary smoothly with temperature, while phase changes will show up as discontinuities. The ESR results presented in Figs. 3 and 4

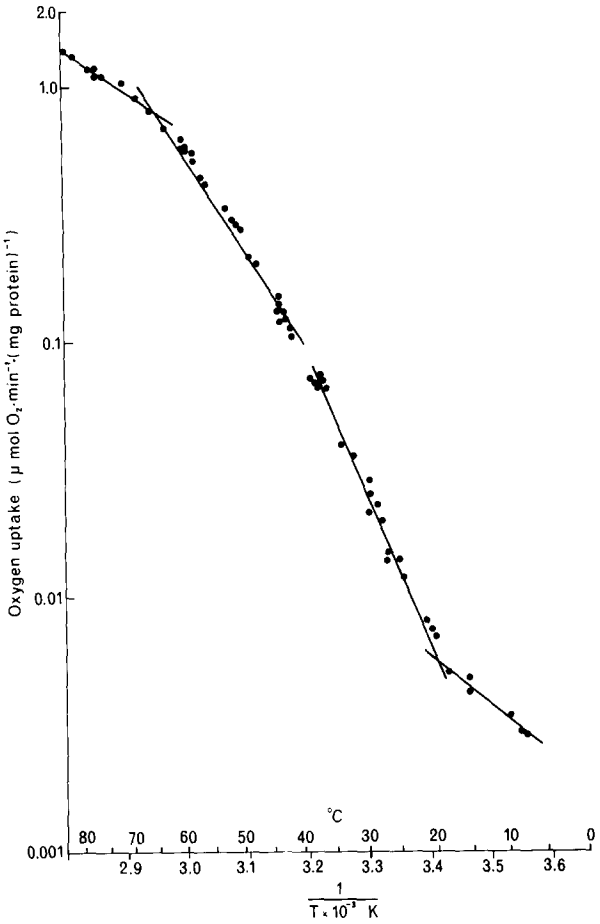

Fig. 1. Effect of temperature on the succinate oxidase activity of intact cells of *Thermus* T351. Assays were conducted as described in Materials and Methods. The graph shows results from five different batches of cells. To compensate for variations in activity the oxygen uptake rate was adjusted to a common value at 45°C. The adjustment factors used were 0.87, 0.98, 1.13, 1.19. To maintain the sensitivity and accuracy of the assay at low temperatures the amount of preparation used was increased in two steps between 85 and 5°C. Several overlapping assays were carried out at each step point, and no significant difference due to protein concentration was found.

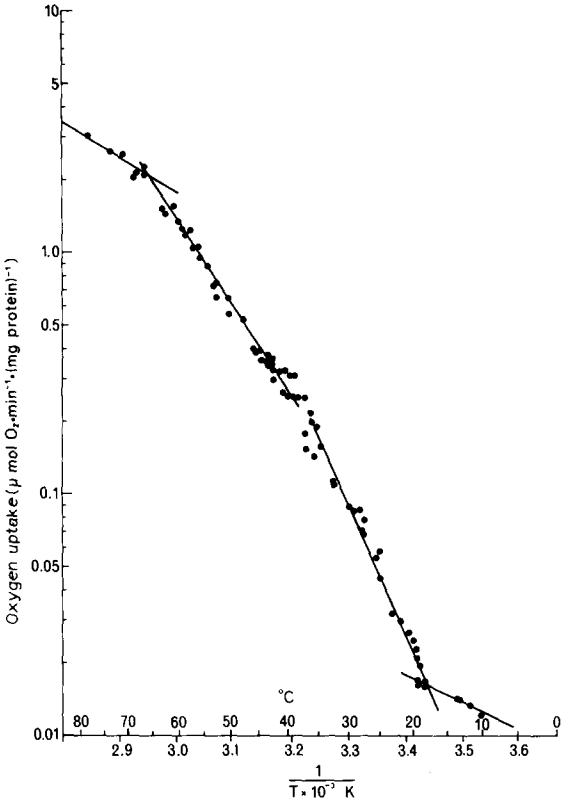

Fig. 2. Effect of temperature on the succinate oxidase activity of respiratory particles from *Thermus* T351. Assays were conducted as described in Materials and Methods. The graph shows results from two different preparations. To compensate for variations in activity the oxygen uptake rate was adjusted to a common value to 45°C. The adjustment factor was 0.92. To maintain the sensitivity and accuracy of the assay at low temperatures the amount of preparation used was increased in two steps beween 85 and 5°C. Several overlapping assays were carried out at each step point, and no significant different due to protein concentration was found.

indicate that the phase changes in respiratory particles and extracted lipid, as reflected by the partitioning of Tempo, occur at about the same temperatures as do the activation energy changes for succinate oxidase. The results for whole cells (Fig. 5) are similar, except that because of enzymic reduction of the spin probe it was not possible to obtain data above 66°C. Overall these results, together with those obtained using a spin probe M12NS (results not shown), show an increase in lipid fluidity with increasing temperature as might be expected.

It is clear from the data summarised in Table I that three distinct phase changes occur in the membranes of *Thermus* T351 between 0 and 85°C. There is a good correspondence between the results for succinate oxidase activity with both whole cells and respiratory particles, and those obtained using ESR to measure lipid fluidity. Although there are other possible reasons for the discontinuities in the succinate oxidase Arrhenius plots, we

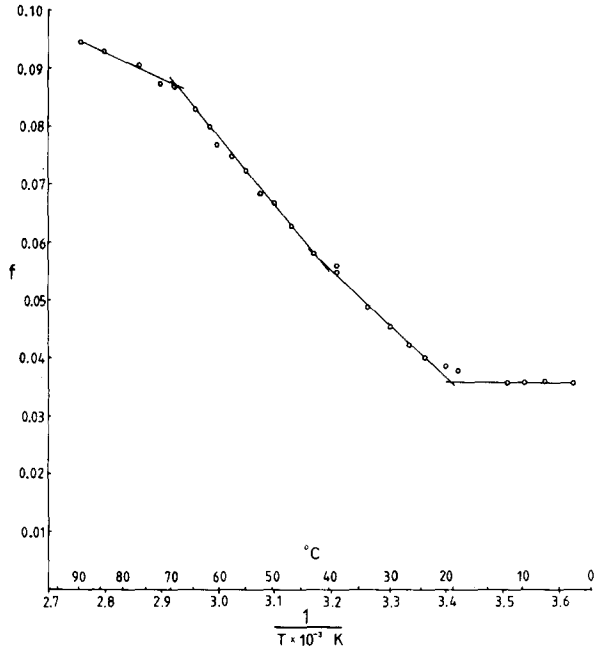

Fig. 3. Effect of temperature on the partitioning of Tempo in respiratory particles from *Thermus* T351.

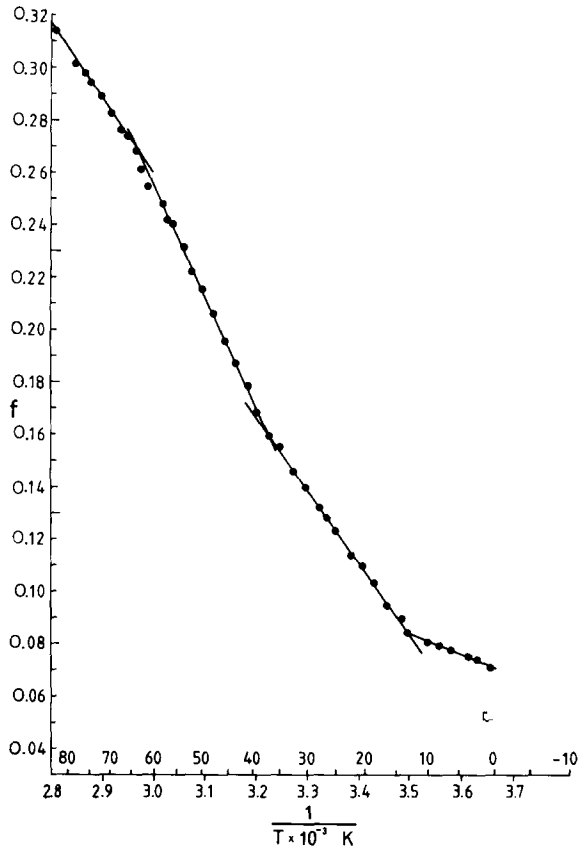

Fig. 4. Effect of temperature on the partitioning of Tempo in an aqueous dispersion of lipids extracted from *Thermus* T351.

believe that this correspondence shows that they are due to membrane phase changes. Further confirmation has been provided by a separate study of the NADH dehydrogenase from this organism. The Arrhenius plot for this enzyme in respiratory particles, over the 20–80°C range, shows discontinuities at about 35°C and about 62°C whereas

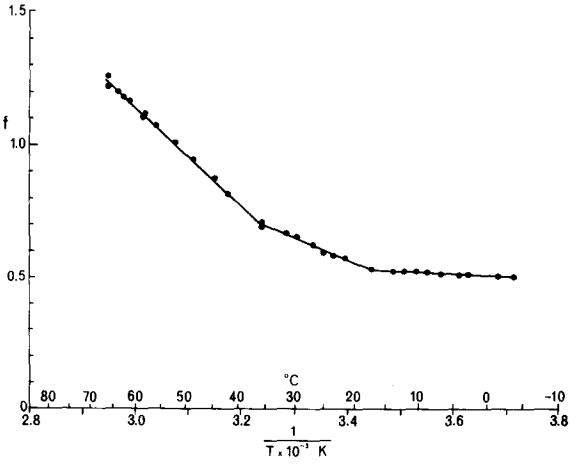

Fig. 5. Effect of temperature on the partitioning of Tempo in intact cells of *Thermus* T351.

TABLE I

TRANSITION TEMPERATURES IN THE EXTREME THERMOPHILE, *THERMUS* T351

	Transition temperatures (°C)		
	Lower	Middle	Upper
ESR			
Tempo Extracted lipid	13.5	37	63
Tempo Respiratory particles	20	42	69
Tempo Whole cells	17	38	n.d. [a]
Succinate oxidase			
Whole cells	20	37	62
Respiratory particles	18	39	66

[a] n.d., not determined.

the soluble enzyme does not (Walsh, Daniel and Morgan, personal communication).

The lowest membrane transition occurs at about 19°C, and may mark the onset of flexing of individual fatty acyl chains. The fact that the Tempo results show this transition as occurring at 13.5°C in extracted lipid suspensions is probably due to a loss of the in vivo ordering effect of proteins [10], since membrane proteins increase the transition temperature by modulating the motional freedom of lipids in their immediate environment [11]. This effect is also seen for the middle and upper transitions.

The middle transition, at about 39°C, is less marked than the other transitions in the sense that the change in slope is smaller. Between 39 and 66°C, an intermediate phase probably exists, with extensive areas of phase separation, i.e. with the co-existence of both liquid and solid (gel) phase. The presence of 0.1% Tween 80 abolishes this transition (as determined by Tempo partitioning) in extracted lipids, but does not affect the upper or lower transition. In a similar study of *Thermus thermophilus* membranes Oshima and Miyagawa (1974) [7] found transitions occurring at 77 and 48°C. These workers concluded that the membrane was fluid above 77°C and solid below 48°C, with phase separation occurring between these temperatures.

Above 66°C, the membrane of *Thermus* T351 is presumably in the liquid-crystalline state, with the majority of lipids being fluid. It is clear from other studies that this liquid-crystalline state is necessary for most membrane processes (see, for example, Ref. 3).

The lowest phase transition is clearly an extraordinary one, in that the Arrhenius plot for succinate oxidase is concave upwards. No plot of this type has ever been reported for a membrane-bound enzyme [2]. The only instance of this type of Arrhenius plot [2] is that of fumarate dehydratase reported by Massey (1953) [12]. The implication of such a plot is that a rise in temperature through this transition has resulted in an increase in the activation energy, E_a, of the system. For fumarate dehydratase, the change was interpreted as a moving apart of the enzyme subunits leading to an increase in E_a. This could also explain the change in E_a in our experimental system. The phenomenon of rafting in membrane systems, i.e. formation of protein aggregates as lipid fluidity decreases, is well known [1,13]. Such aggregation might well lead to closer association of the various components of the succinate oxidase system and hence cause a decrease in E_a. This decrease in E_a seems unlikely to be of any physiological significance, since the catalytic rates involved are less than 1% of those at the growth temperature, although it is conceivable that it might facilitate energy provision for survival or maintenance functions.

It must be borne in mind that succinate oxidase is a multi-enzyme system, and that the activation energy changes shown by Arrhenius plots are presumably those of the rate limiting step rather than of the system as a whole. We have found that the K_m for succinate of respiratory particles is 25 μM at 75°C, but is 500 μM at 55°C and it may well be the succinate dehydrogenase which is that rate-limiting step.

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