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THE INFLUENCE OF BRANCHED-CHAIN AND ω-ALICYCLIC FATTY ACIDS ON THE TRANSITION TEMPERATURE OF *BACILLUS SUBTILIS* LIPIDS

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Summary

The influence of branched-chain and ω-alicyclic fatty acids on the transition temperature of *Bacillus subtilis* lipids was studied by measuring the fluorescence depolarisation of the probe 1,6-diphenyl-1,3,5-hexatriene incorporated into lipid bilayers. Only anteiso-C_{15} and C_{17} fatty acid-enriched lipids showed no transition in the observed temperature range. Compared to the transition of normal lipids iso-fatty acid-enriched lipids have a slightly higher transition temperature. The incorporation of ω-alicyclic fatty acids with increasing size of the alicycle leads to a decrease in the transition temperature. A possible role of ω-cyclohexane fatty acids in *Bacillus acidocaldarius* is proposed.

Introduction

The thermophilic, acidophilic bacterium *Bacillus acidocaldarius* contains a high percentage of ω-cyclohexane fatty acids [1]. In comparison to membranes of mesophilic organisms, a higher stability is one requirement for membranes of organisms living at high temperatures. How could this adaptation be achieved? One factor surely is by a suitable structure of the fatty acid moiety in the lipids, as could be shown for *Escherichia coli* [2] and *Acheloplasmas* [3].

We studied the influence of different fatty acid patterns using a mutant *Bacillus subtilis* auxotroph for short branched-chain fatty acids [4] and alicyclic carboxylic acids [5]. After feeding, this *B. subtilis* mutant synthesizes cyclohexane carboxylic acid ω-cyclohexane fatty acids to somewhat the same extent as *B. acidocaldarius*. Besides this the mutant is able to synthesize other

Abbreviation: DPH, 1,6-diphenyl-1,3,5-hexatriene.

ω-alicyclic fatty acids when fed with the appropriate precursor. We first tested the influence of the changed fatty acid pattern on growth at different temperatures [5]. For information of physical membrane properties, we now examined the influence of ω-alicyclic and branched-chain fatty acids on the transition temperature of *B. subtilis* lipids. This has been tested using the fluorescence polarisation of the probe 1,6-diphenyl-1,3,5-hexatriene (DPH) embedded in the bilayer as an indicator for phase transition.

Materials and Methods

Preparation of lipids

The incorporation experiments with isobutyrate, isovalerate, 2-methylbutyrate, cyclopropane carboxylate, cyclobutane carboxylate, cyclopentane carboxylate, and cyclohexane carboxylate as precursors for fatty acid biosynthesis have been done as described previously [5]. Cells were grown up to an extinction of 1.2 at $\lambda = 578$ nm (1 cm light path). Lipids were extracted according to 6 at room temperature, hydrolyzed, and the fatty acids extracted and methylated [5]. The fatty acids were identified and quantitatively determined by gas-liquid chromatography [5].

Fluorescence polarisation measurements

For the determination of the phase transition temperature of aqueous dispersions of the extracted lipids, the temperature dependance of the fluorescence polarisation of the probe DPH was measured using a Zeiss PMQ II spectrophotometer with a xenon arc lamp, second monochromator M4 Q III, sample holder ZMF 4 and Zeiss polarisation filters. The sample holder was thermostated using a Lauda K2R thermostat.

Shinitzky et al. [7,8] used the Perrin equation

$$r_0/r = 1 + C(r)\frac{T \cdot \tau}{\overline{\eta}}$$

to determine 'microviscosities', where r_0 is the fluorescence anisotropy of the totally immobilized probe, $r = (I_{\parallel} - I_{\perp})/(I_{\parallel} + 2I_{\perp})$ the measured anisotropy, T the absolute temperature, τ the exited state life time and $\overline{\eta}$ the 'microviscosity'. $C(r)$ contains the volume and shape factors of the fluorophore. As the decays of the emission anisotropy as well as the total emission are not monoexponential, and the emission anisotropy of DPH embedded in phospholipid bilayers does not decay to zero in the time scale of the emission decay, $C(r)$ is not constant, but depends on the used solvent, which imposes different restrictions to the rotation of the probe [9—12]. Therefore it is not possible to calculate absolute values of $\overline{\eta}$ from steady state anisotropy measurements. This can only be done using nanosecond fluorescence techniques [9—11] or ESR measurements with biradicals as probes [13].

From the Perrin equation one can derive the expression $(T \cdot \tau)/(r_0/r - 1)$, which is proportional to $\overline{\eta}$. For the determination of the transition temperature T_m it is convenient to plot the logarithm of this expression versus $1/T$, because this plot gives straight lines at high temperatures, from which the fusion activation energies can be calculated, and the phase transitions can be

distinguished by deviations from this linearity. The exited state life time as a function of temperature was calculated from the temperature dependance of the emission intensity according to Shinitzky et al. [7,8], using a value of 11.4 ns for τ_0. As only relative values are reported there is no need for the experimental determination of r_0; therefore the theoretical value of 0.4 was used for the calculations.

Lipid dispersions were prepared by sonication with a MSE Ultrasonic Disintegrator, equipped with a microtip. 5 ml of a handshaken dispersion in bidistilled water (c = 1 mg/ml) were sonicated at room temperature for 3—5 min till the absorbance at 430 nm (1 cm light path) was below 0.2. To 3 ml of this dispersion 3 μl of a 10 mM DPH solution in dioxane was added at a temperature above the phase transition of the dispersed lipid. For measurements starting below 0°C 20% ethyleneglycol was added to prevent freezing. This did not lead to a significant change of the polarization of DPH. The emission intensities $I_{\parallel}$ and $I_{\perp}$ were determined during the same heating run, changing the polarization direction of the analyzer every degree. From $I_{\parallel}$ and $I_{\perp}$ the anisotropy r was calculated. The errors in r for different runs of the same lipid dispersions were within ±2%. The expression $(T \cdot \tau)/(r_0/r - 1)$ was calculated and plotted as a function of $1/T$ with the help of a Wang computer, model 720 C, with x-y-plotter 720. In these computer diagrams the phase transition temperature T_m was determined graphically according to ref. 14. The error for the determination of T_m was ±1°C.

Results and Discussion

The percentage of synthesized fatty acids after feeding the precursors is shown in Table I. In each case a fairly good incorporation was obtained.

Figs. 1 and 2 show the dependance of lg $[(T \cdot \tau)/(r_0/r - 1)]$ on temperature for the various lipid dispersions with different fatty acids. At high temperatures all curves show a linear dependance of this expression vs. $1/T$. The calculated fusion activation energies are 7—9 kcal/mol. All lipid dispersions, with one exception only, clearly show a phase transition, indicated by the deviation from linearity in the plots. The only exception, the lipid extracts with incorparated anteiso-C_{15} and C_{17} fatty acids, shows no transition even at —5°C. The apparent fusion activation energies below the phase transition are always

TABLE I

RELATIVE AMOUNT AND CHAIN LENGTH OF BRANCHED-CHAIN AND ω-ALICYCLIC FATTY ACIDS SYNTHESIZED BY *BACILLUS SUBTILIS* bfm 49 WHEN GROWN ON RESPECTIVE PRECURSORS

Precursor added	Fatty acid synthesized	Relative amount (%)
Isobutyrate	iso-C_{14}, C_{16}	86
2-Methylbutyrate	anteiso-C_{15}, C_{17}	86
Isovalerate	iso-C_{15}, C_{17}	78
Cyclopropane carboxylate	ω-cyclopropane-C_{16}, C_{18}	52
Cyclobutane carboxylate	ω-cyclobutane-C_{15}, C_{17}	75
Cyclopentane carboxylate	ω-cyclopentane-C_{16}, C_{18}	75
Cyclochexane carboxylate	ω-cyclohexane-C_{17}, C_{19}	70

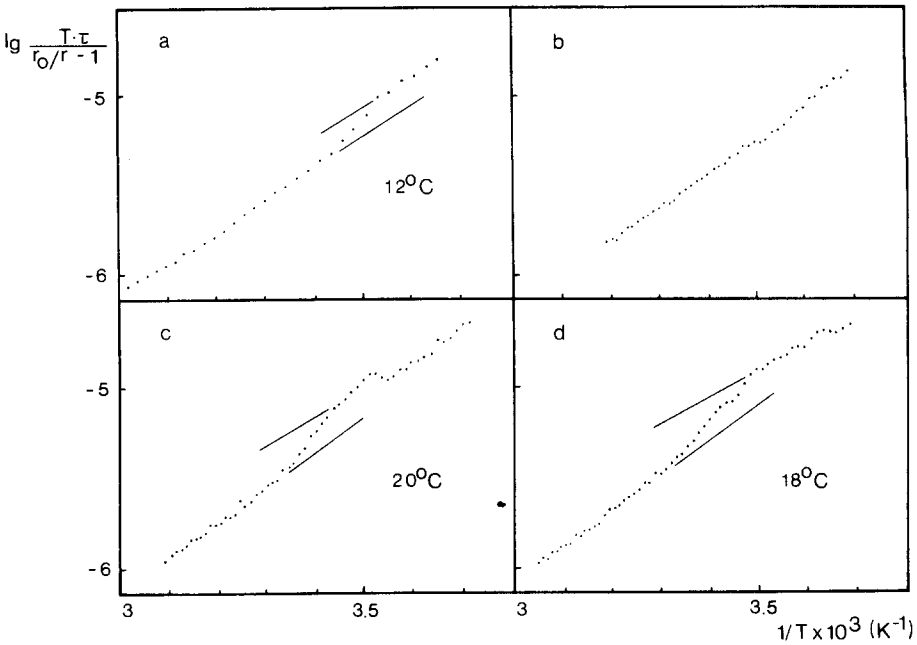

Fig. 1. Plot of the logarithm of $[(T \cdot \tau)/(r_0/r - 1)]$ vs. $1/T$ for dispersions of lipids of *B. subtilis* containing the following fatty acids predominantly: (a) normal pattern of the wild type, (b) anteiso-C_{15}, C_{17}, (c) iso-C_{14}, C_{16}, (d) iso-C_{15}, C_{17}. Numbers designate the transition temperature. The uncertainty of these values is ±1°C.

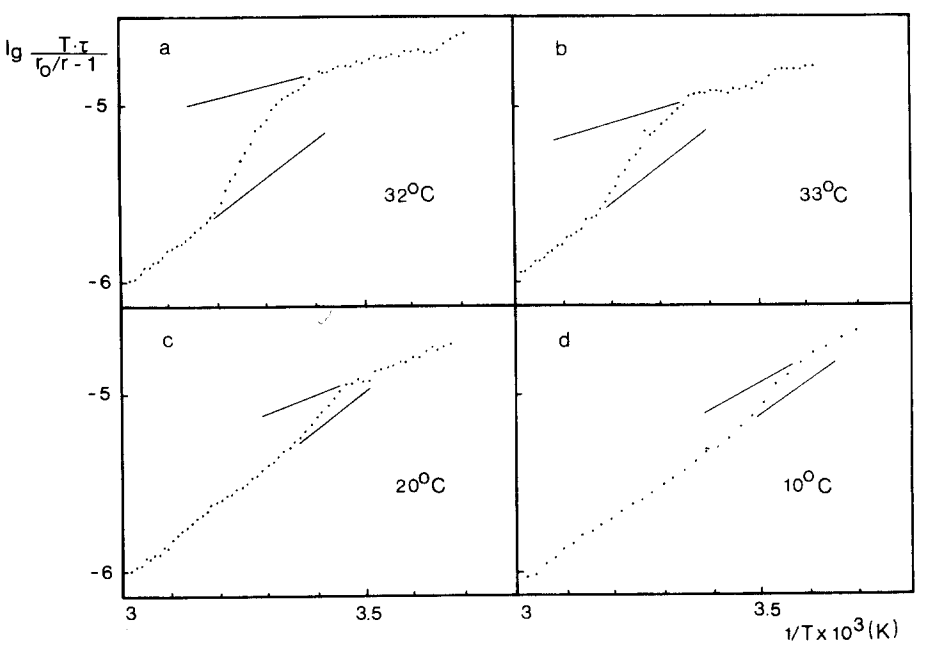

Fig. 2. Plot of the logarithm of $[(T \cdot \tau)/(r_0/r - 1)]$ vs. $1/T$ for dispersions of lipids of *B. subtilis* containing ω-alicyclic fatty acids predominantly: (a) ω-cyclopropane-C_{16}, C_{18}, (b) ω-cyclobutane-C_{15}, C_{17}, (c) ω-cyclopentane-C_{16}, C_{18}, (d) ω-cyclohexane-C_{17}, C_{19}. Numbers designate the transition temperature. The uncertainty of these values is ±1°C.

smaller, as already known for experiments with other lipid dispersions [15,16]. Whereas the transition temperature for lipids with normal fatty acid pattern is about 12°C, incorporation of iso-C_{14}, C_{16} and iso-C_{15}, C_{17} fatty acids leads to an increase in transition temperature to approximately 18—20°C. The transitions for these lipids are not very pronounced, though. As mentioned above, the change from iso to anteiso fatty acids either shifts the transition to very low temperatures or completely abolishes it. The relative values of lg $[(T \cdot \tau)/(r_0/r - 1)]$ of these lipid dispersions are lower at low temperatures compared to the iso-C_{14}, C_{16} and iso-C_{15}, C_{17} fatty acid enriched lipids, indicating a higher degree of disturbance of the packing of the hydrocarbon chains in the middle of the bilayer.

Comparing the transitions of lipid extracts with incorporated ω-alicyclic fatty acids a distinct dependence of the transition temperature on the size of the alicycle can be detected (Fig. 2). Lipids with ω-cyclopropane and ω-cyclobutane fatty acids give almost the same transition temperature of about 33°C. The transition of lipids with ω-cyclopropane fatty acids is more distinct, i.e. the change of lg $[(T \cdot \tau)/(r_0/r - 1)]$ is larger, despite the fact that only 52% of the total fatty acids are cyclopropane fatty acids.

Introducing larger and bulkier alicycles leads to a decrease in the transition temperature and a decrease in the value of lg $[(T \cdot \tau)/(r_0/r - 1)]$ at the range of the growth temperature (37°C) as well. This is in accordance with the observed values for lecithins of different chain lengths [16]. The transitions are far less pronounced for lipids with bulkier alicycles, indicating a high degree of disturbance in the packing of the hydrocarbon chains due to the crowding of the alicycles in the middle of the bilayer.

For *B. subtilis* there is no indication for a special advantage of ω-cyclohexane fatty acids for growth at higher temperatures. The function of these unusual fatty acids in their natural environment in *B. acidocaldarius* might be to provide a good interaction with the tetrahydroxypentane substituted pentacyclic triterpene occurring in the membrane of this organism [17]. This compound resembles cholesterol in its ring system. But it has in contrast no hydroxy group substituted on the ring system. The four hydroxy groups are located in the side chain. One has to assume that the hydrophilic side chain of this polyol is in contact with the water at the bilayer surface. This leads to an arrangement of the ring system just the opposite way around compared to cholesterol. As a consequence, the bilayer becomes less dense in the middle region. The incorporation of ω-cyclohexane fatty acids would give the organism the opportunity to fill these less dense areas with bulky residues, in order to stabilize the membrane. A membrane of this special composition might thus give the organism the possibility to grow at high temperatures, where membranes with other fatty acids normally become very fragile.

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