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CHARACTERIZATION OF THE PLASMA MEMBRANE OF *MYCOPLASMA LAIDLAWII*

VII. PHASE TRANSITIONS OF MEMBRANE LIPIDS

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SUMMARY

Use of a very sensitive differential calorimeter has revealed two major regions of heat absorption in purified plasma membranes of *Mycoplasma laidlawii*. These consist of a rapidly increasing rate of heat absorption showing an inflection point at approx. 40°; this is followed at higher temperatures by a slightly decreased rate of heat uptake. Within this latter region it is barely possible to distinguish a second inflection point. These transitions were found also to be properties of whole cells.

Experimental results presented in this paper support the conclusion that the first region of heat absorption is due to a reversible transition in the lipid component of the membrane, and that the second region is due to an irreversible denaturation of the protein component. These results also provide evidence that the lipid transition is essentially unaffected by the protein component of the membrane.

The calorimetric transition curves indicate that the apparent heat capacity of the membrane lipids increases during the transition. This most likely arises from new modes of freedom available to the fatty acid hydrocarbon chains in the melted state.

By means of a viable cell titer taken before and after cells were submitted to a calorimetric run extending to 40°, the lipid transition was demonstrated unambiguously to be a property of living cells.

The T_m of the lipid transition was found to be a function of the conditions of growth of the cells. In all cases studied the temperature at which the cells grew lies within the range of the lipid transition. Results suggest that the cells attempt to regulate their position in the lipid transition.

INTRODUCTION

The use of differential scanning calorimetry has revealed the existence of endothermic transitions in phospholipids which have been interpreted as phase

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changes[1]. STEIM *et al.*[2] have demonstrated by the same method the occurrence of similar transitions in membranes from *M. laidlawii*. Using X-ray diffraction techniques on the same membrane system, ENGELMAN[3] has provided evidence that the transition corresponds to a change from hexagonal close packing of the hydrocarbon chains in the lipid to a liquid paraffin-like structure.

We have employed a very sensitive differential calorimeter[4,5] in a detailed calorimetric investigation of the transition in dilute aqueous suspensions of purified membranes and in relatively dilute suspensions of living cells of *M. laidlawii*. The sensitivity of the calorimeter has allowed us to study quantitatively the heat of transition as a function of membrane parameters and to demonstrate unambiguously that the transition is a property of the living cells.

MATERIALS AND METHODS

Organism

These studies were carried out on *M. laidlawii* B.

Medium and growth

The following media were used: (1) Tryptose (Fisher), 20 g; water, 900 ml. After autoclaving, 100 ml of 10 % sterile glucose solution were added. (2) Heart Infusion Broth (Fisher), 25 g; water, 900 ml; supplemented with 100 ml of 10 % glucose. (3) Heart Infusion Broth (Fisher) was extracted with chloroform. After drying, 20 g were autoclaved in 900 ml of water. Sterile 10 % glucose (100 ml) was added. (4) The above medium was supplemented with various fatty acids according to the procedures of TOURTELLOTTE AND MCELHANEY[6].

For cell growth, 1 l of medium was innoculated with 20 ml of a 24-h culture which was then incubated at the desired temperature. The cells were grown until late log phase when they were harvested.

Membrane preparation

Cells were lysed in deionized water and membranes were prepared by the method of ENGELMAN *et al.*[7].

Pronase digestion

Membranes at a concentration of 5 mg protein/ml in distilled water were mixed with pronase (Calbiochem) at a concentration of 100 μg/ml and incubated at 45° for 2 h. The resulting suspension was centrifuged and the partially digested membranes were resuspended in distilled water.

Lipid extraction

Membranes were suspended in dilute buffer (0.01 M NaCl, 0.0025 M Tris (pH 7.4)) at a concentration of 2 mg of membrane protein per ml. 9 vol. of acetone per volume of membrane suspension were added with vigorous stirring which was continued at room temperature for 1 h. The resulting suspension was centrifuged at 14000 $\times$ g for 30 min and the supernatant decanted and dried in a Buhl rotary evaporator. The material was resuspended in water by vigorous pipetting.

Analytical methods

Dry weighths were obtained by evaporating to constant weight at 105°. Nitrogen was determined by the Kjeldahl method[8] and protein concentration was

calculated from the nitrogen value using the reported amino acid composition of the membrane[9] and correcting for the membrane hexosamine. Viable cell titers were performed by colony count on agar plates (water, 20 g; Bacto Tryptose, 10 g; NaCl, 5 g; Tris, 1.5 g; glucose, 10 g; Bacto agar, 10 g; Difco PPLO Serum Fraction, 20 ml).

Calorimetry

A highly sensitive differential calorimeter[4,5] was employed to obtain heating curves on suspensions of purified membranes, suspensions of membrane lipids, and suspensions of whole cells. In all cases other than cells the suspending medium was distilled water. Whole cells were suspended in a buffer containing 1000 ml water, 10 g NaCl, and 6 g Tris adjusted to pH 7.4 with HCl. The detailed calorimetric method as well as the procedures for data reduction are the same as those described earlier[4,5].

RESULTS

Typical calorimetric experiments are illustrated in Fig. 1. Curve A was obtained with a suspension of the lipid extracted from the membranes of cells grown at 37°

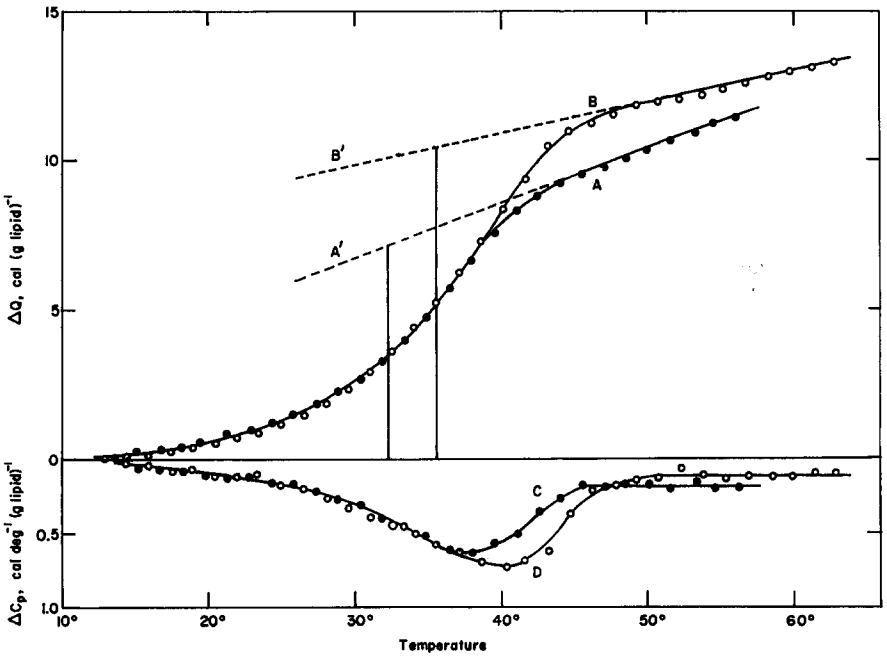

Fig. 1. Absorption of heat, in excess of that absorbed by the solvent, during the heating of components of the membranes of *M. laidlawii* cells grown in Medium 3 (see text) at 37°. The upper curves record the apparent excess enthalpies, in cal·(g of lipid)$^{-1}$, as functions of temperature for an aqueous suspension of the lipids extracted from the membranes (Curve A) and a suspension of pronase-treated membranes (Curve B). The lower curves, which are the derivatives of the upper curves, record the apparent heat capacities of the materials in suspension relative to the values at 0–10°. The lipid suspension contained 2.4 mg/ml of lipid with less than 1% protein content. The pronase-treated membranes were used at a concentration of 6.4 mg/ml, and contained 41% protein.

in Medium 3 described above; Curve B was obtained with pronase-treated membranes from the same source. Curves C and D are the graphically evaluated derivatives of Curves A and B, respectively, and thus represent the apparent heat capacity of the material in suspension relative to the value at low temperature (0–10°).

As in the case of the protein and polynucleotide transitions previously studied with this calorimeter[4,5,10,11] the continuing heat absorption observed above the main transition region is attributed to an increase in the apparent heat capacity of the suspended material. In the experiments shown in Fig. 1 the final heat absorption is linear, indicating a temperature-independent apparent heat capacity for the high temperature form of the suspended material. If this indication is taken at face value the linear portion of each curve may be extrapolated back into the transition region (for example, Line A′ in Fig. 1) to establish a high temperature baseline, and the hypothetical isothermal heat of the transition at a specified temperature estimated from the length of a vertical line from the low temperature base line to the extrapolated line at this temperature. As discussed in earlier publications[4,5], in our calorimetric equipment, instrument adjustments are made in the pretransition region in such a way that the low temperature baseline is always horizontal.

We have arbitrarily chosen to characterize the transition curves by the isothermal enthalpy increase, ΔQ in cal·(g lipid)$^{-1}$, evaluated at the 'melting temperature', T_m, defined as the temperature at which the vertical line referred to above is bisected by the experimental transition curve.

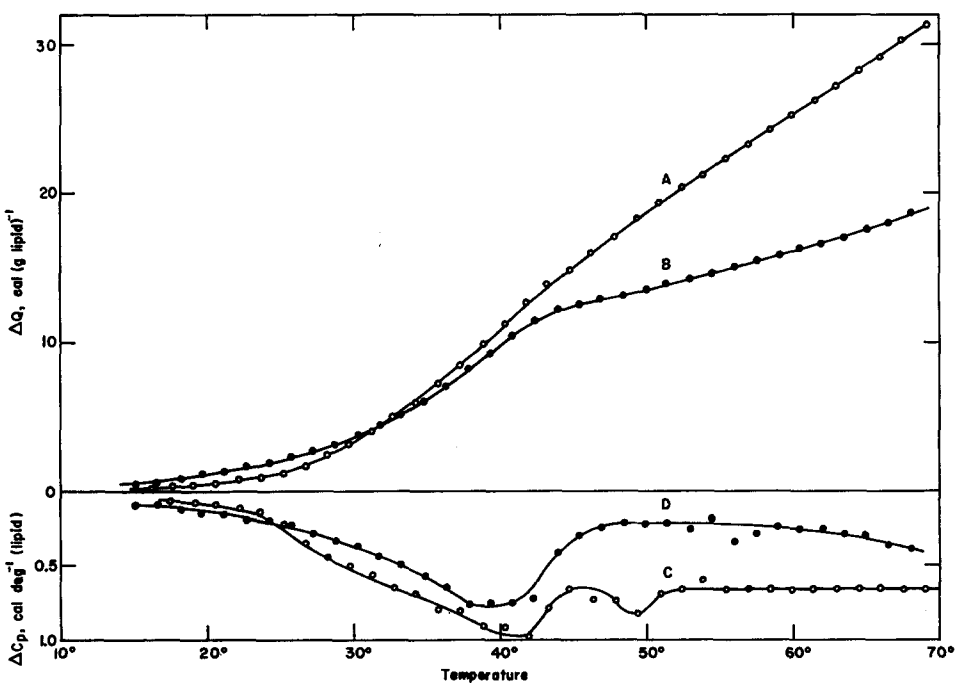

Fig. 2. Apparent excess enthalpy in cal·(g of lipid)$^{-1}$ as a function of temperature for a suspension of whole membranes of *M. laidlawii* grown on Medium 3 (see text) at 37°. Curve A, first heatings; Curve B, second heating of the same material. Curves C and D are the derivatives of Curves A and B respectively, and represent the apparent heat capacity of the suspended material relative to the value at 0–10°. Concentration 13.4 mg/ml; protein content, 70%.

Fig. 2 presents experiments performed with a suspension of whole membranes. Curve A records the heat absorbed during the first heating of the suspension. At the conclusion of this experiment, the calorimeter and its contents were cooled and a second heating was carried out on the following day. As in Fig. 1, graphically evaluated derivative curves, C and D, are given. Curve A and its derivative Curve C give an indication of a heat-absorbing process centered at roughly 40°; this is followed by continuing rapid heat absorption, with a suggestion of a second transition range at about 50°. It is obvious that any attempt to utilize the data in Curve A to estimate enthalpy changes for individual transitions in the manner outlined in the preceeding paragraph will be subject to very large uncertainties.

Curve B, for the second heating of the membrane suspension, is similar in shape to the curves in Fig. 1, except for the fact that the high temperature portion of the curve is slightly concave upward, presumably due to a small residual heat absorption by the protein component of the membrane. This curvature, which was encountered in all experiments involving the second heating of whole membranes or of whole cells, obviously renders the estimation of T_m and ΔQ considerably more uncertain than in cases having linear heat uptakes in the high temperature region. It is important to note that the heat absorption in Curve A below about 40° is similar to that in Curve B, suggesting that the process occurring in the low temperature range is essentially independent of the additional processes giving rise to the excess of heat absorption in Curve A over that in Curve B at higher temperatures.

The data obtained in experiments with membranes subjected to various treatments and obtained from cells grown under various conditions are summarized in

TABLE I

ENTHALPY INCREASE ACCOMPANYING THE LIPID CONFORMATIONAL TRANSITION IN *M. laidlawii*

Growth media are described in MATERIALS AND METHODS. Medium 3: these preparations were all from the same batch of cells. Medium 4: Medium 3 supplemented with myristic acid.

Growth temp.	*Growth medium*	*Concn. of suspended material (mg/ml)*	*Protein (%)*	T_m	ΔQ *(cal·g of lipid)*
Native membranes					
37°	2	11.0	66	~26°	~ 7
25°	2	25.1	44	~23°	~ 3
37°	3	13.4	70	~30°	~ 6
37°	4	31.4	67	~36°	~11
Heat-denatured membranes					
37°	1	30.8	47	24°	4.9
37°	2	11.0	66	26°	7.7
25°	2	25.1	44	23°	3.4
37°	3	13.4	70	32°	9.6
37°	4	31.4	67	36°	12.6
Pronase-digested membranes					
37°	2	7.9	34	26°	4.5
37°	2*	7.9	34	25°	5.0
37°	3	6.4	41	36°	10.4
37°	4	11.7	29	36°	7.8
Extracted lipid					
37°	3	2.4	<1	32°	7.1

* Reheat of the material on preceeding line.

Table I. It is important to keep in mind that the data for native membranes are of a very low order of accuracy, while those for pronase-treated membranes or for extracted lipids are considerably more reliable; in the latter cases the values for ΔQ are estimated to have an uncertainty of approx. 10%. The accuracy of the data for heat-denatured membranes is intermediate between these limits.

Calorimetric experiments were also carried out with whole cells. The results of such an experiment with cells grown on Medium 3 at 37° are shown in Fig. 3. In one of these experiments, viability was determined by plaque count before and after a calorimetric run which was interrupted at 40°. The final viable count was 83% of the original count. The upper dashed curve in the figure is a reheating of the material which had been heated to 70° as shown in the upper solid curve. Both the integral and the derivative curves (lower curves) show evidence of the transition centered at approx. 35° which are seen in Figs. 1 and 2. A rough estimate of the enthalpy change in this transition is consistent with the values in Table I.

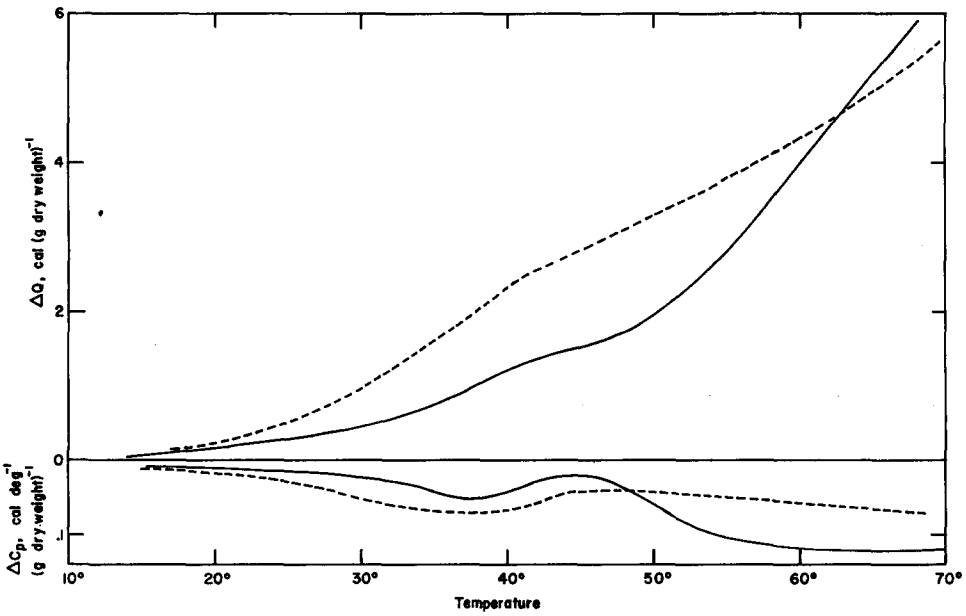

Fig. 3. Excess absorption of heat, in cal·(g of cells)$^{-1}$ (dry weight), by whole *M. laidlawii* cells as they are heated from 10 to 70°. Solid line, first heating; dashed line, second heating of the same material. The curves in the lower part of the figure are the corresponding derivative curves, and give the apparent heat capacity of the suspended material relative to the value at 0–10°, Concentration, 35.0 mg (dry weight)/ml. The cells were grown in Medium 3 at 37°.

DISCUSSION

When membranes of *M. laidlawii* are heated in dilute aqueous suspension, two regions of heat absorption can be distinguished (Curve A, Fig. 2). In the temperature range 20–40° there is a rapidly increasing rate of heat absorption showing an inflection point at approx. 40°; this is followed at higher temperatures by a slightly decreased rate of heat uptake. Within this latter region it is barely possible to distinguish a second inflection point. The experimental results presented in this paper

support the conclusion that the first region of heat absorption is due to a reversible transition in the lipid component of the membrane, and that the second region is due to an irreversible denaturation of the protein component. Briefly summarized the evidence pointing in this direction is as follows: (a) The heat absorption in the low temperature region is essentially unaffected by the process which takes place at higher temperatures; (b) the transition curve observed with membranes in which the protein content has been reduced by treatment with pronase is very similar to that observed with membranes in which the protein has presumably been denatured by heating; and (c) lipid extracted from the membranes and resuspended in water also shows transition behavior on being heated very similar to membranes in which the protein component has been denatured. The similarities extend to quantitative aspects, in that the heat absorbed in the low temperature transition is roughly constant regardless of changes made in the protein component of the membranes. It thus appears that the protein component has essentially no effect on the lipid transition, as expected on the basis of membrane models composed of relatively large separate domains of lipid and protein.

As pointed out above in connection with the data in Fig. 1, the calorimetric transition curves indicate that the apparent heat capacity of the suspended lipid material increases during the transition. The increase shown by Curve A, Fig. 1, is 0.18 cal·degree^{-1}·(g of lipid)$^{-1}$ and that by Curve B is 0.12 cal·degree^{-1}·(g of lipid)$^{-1}$. These values are similar to those which have been found to result from the thermal unfolding of bovine pancreatic ribonuclease[5] and chymotrypsinogen[12] and they are much larger than those found in the transitions of polynucleotides and nucleic acids. In the case of proteins, these heat capacity increases are generally considered to be due largely to the exposure of hydrophobic groups to the aqueous medium, with resultant formation of structured shells of water molecules of higher heat capacity than that of bulk water. In membranes, on the other hand, since it appears that the lipid transition involves a disordering of the hydrocarbon chains without

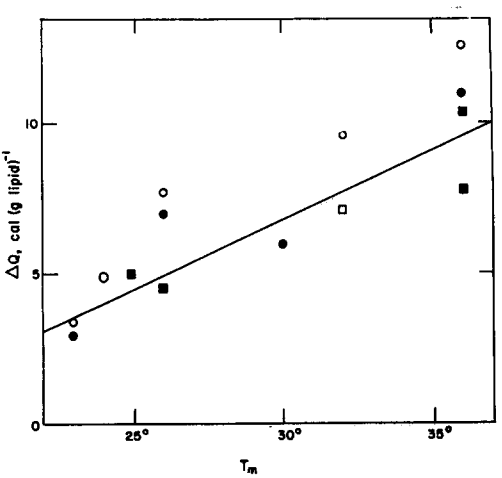

Fig. 4. Variation with melting temperature, T_m, of the excess enthalpies at the T_m of native membranes of *M. laidlawii* (●), heat-denatured membranes (○), pronase-treated membranes (■) and the lipid extracted from the native membranes (□). Enthalpies in cal·(g of lipid)$^{-1}$.

the gross alteration of the membrane structure which would be required to expose the hydrocarbon chains to the aqueous medium, the large increases in heat capacity presumably arise from new degrees of freedom available to the hydrocarbon chains.

Another indication of an increase in heat capacity is given in Fig. 4, where the values of ΔQ given in Table I are plotted as a function of T_m. The slope of the plot corresponds formally to a value for ΔC_p of approx. 0.46 cal·degree^{-1} (g of lipid)$^{-1}$. If the only influence of lipid composition on the enthaply of transition were exerted indirectly by the influence of composition on the melting temperature, a slope of approx. 15 cal·degree^{-1}·(g of lipid)$^{-1}$ would be expected for the plot in Fig. 4. The fact that the observed slope is 3 times larger shows that lipid composition has a pronounced direct effect on the energetics of the transition.

If it is assumed that the increase in enthalpy during a transition, after due allowance is made for the change in apparent heat capacity, affords a linear measure of the extent of conversion, α, between the initial and final states of the suspended lipid, it is possible to construct a curve of α as a function of temperature. It is interesting to compare the slope of this curve with what might be expected if the transition were a simple two-state, all-or-none process involving a single pure substance. In the case of a two-state process, the standard enthalpy change, ΔH°_{vH}, is given by the van 't Hoff equation in the form

$$\frac{d\alpha}{dT} = \alpha(1-\alpha)\frac{\Delta H^\circ_{vH}}{RT^2} \qquad (1)$$

Although it is obvious that we are not dealing with a single pure substance in the present experiments, we may nevertheless apply Eqn. 1 in a purely formal manner. If this is done, the curves in Fig. 1 as well as several of our other transition curves lead to values of ΔH°_{vH} clustering around $4 \cdot 10^4$ cal·mole^{-1} ($d\alpha/dT$ evaluated at $\alpha = 1/2$). Taking the molecular weight of a typical lipid molecule to be about 700, the calorimetrically determined transition enthalpy is approx. $3.6 \cdot 10^3$ cal·(mole of lipid)$^{-1}$ at 33°. The fact that this figure is much smaller than ΔH°_{vH} shows that the transition, if it does indeed involve only the lipid material, is cooperative. The measure of the cooperativity afforded by this comparison is probably a gross underestimate because of the broadening of the transition curve by the heterogeneity of the lipid in the membranes.

Inspection of the data listed in Table I shows that T_m is a function of the conditions of growth of the cells, presumably because of a dependence of lipid composition on the conditions of growth. In all cases studied, the lipid transition extended above the temperature at which the cells were grown, that is, the cells grow at a temperature within the range of the lipid transition. In all cases except those in which the medium was supplemented with fatty acids, the cells grew at a temperature within the latter part of the lipid transition. The decrease in T_m for cells grown at 25° from the value for cells grown in the same medium at 37° suggests that the cells attempt to regulate their position in the transition.

Since the lipid transition was shown to be a property of living cells and since the cells appear to grow slightly above the T_m, the transition may be expected to exert some physiological effects on the cells. Work currently under way in this laboratory suggests that there is an effect of the transition on ion transport by the membranes.

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