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Membrane lipid physical state and modulation of the Na^+,Mg^{2+}-ATPase activity in *Acholeplasma laidlawii* B

(membrane enzymes/freeze-fracture electron microscopy/Arrhenius plot/lipid–protein interactions/differential thermal analysis)

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ABSTRACT Careful analysis of the Arrhenius plot of the Na^+,Mg^{2+}-ATPase (ATP pyrophosphohydrolase, EC 3.6.1.8) activity in *Acholeplasma laidlawii* B membranes of varying fatty acid composition has been combined with differential thermal analysis of the membrane lipid phase transitions to evaluate the effects of membrane lipid properties on the enzyme activity. Our results indicate that the enzyme is active only in association with liquid-crystalline lipids, exhibiting a significant heat capacity of activation, $\Delta C_p{}^{\ddagger}$, for the ATP hydrolytic reaction in this case. Quantitative analyses of Arrhenius plots for the enzyme activity in membranes whose lipids exhibit a gel-to-liquid-crystalline phase transition in the physiological temperature range suggest that the ATPase is inactivated when its boundary lipids undergo a phase transition that is driven by the bulk lipid phase transition but is less cooperative than the latter. Our results suggest that the familiar "biphasic linear" Arrhenius plots obtained for many membrane enzymes may in fact have a more complex shape, analysis of which can furnish useful information regarding the behavior of the enzyme molecule.

Nonlinear Arrhenius plots are commonly obtained when the temperature dependences of membrane-associated enzyme and transport activities are studied (1–3). Such plots usually are analyzed as sets of straight-line segments whose slopes are thought to correspond to the reaction activation enthalpies for distinct states of the enzyme. The apparently sharp transitions of the plots from one slope to another are often attributed to "lateral phase separations" (4–6), "lipid phase transitions" (1, 7, 8), or other types of membrane structural changes (9, 10). Recently, Thilo *et al.* (11) have presented a more sophisticated and quantitative analysis of the temperature dependence of the transport of β-glucosides and β-galactosides in *Escherichia coli* based on a physically plausible model of membrane protein partitioning between coexisting lipid phases. The success of these authors' analysis suggests that Arrhenius plots for membrane enzyme or transport activities in general may furnish more detailed and useful molecular information than has previously been extracted from them. We have recently developed a series of quantitative theoretical descriptions of the temperature dependence of a membrane-associated rate process, using various models for the physical basis of the temperature effects on the process of interest (unpublished data).

In the present study, we have used this theoretical framework to evaluate quantitatively and systematically the temperature dependence of a membrane enzyme activity, the Na^+,Mg^{2+}-ATPase (ATP, pyrophosphohydrolase, EC 3.6.1.8) (12, 13) activity of the simple prokaryote *Alcholeplasma laidlawii* B, whose membrane lipid composition and physical properties can be extensively manipulated *in vivo* (14–16). Although the results of our analysis agree in some aspects with the model of Thilo *et al.* (11) for the *E. coli* sugar transporters, our findings lead us to more specific and, in some respects, rather different conclusions regarding the behavior of the *A. laidlawii* B ATPase.

MATERIALS AND METHODS

Cell Growth and Membrane ATPase Activity Measurement. *A. laidlawii* B was grown statically on an undefined but lipid-depleted medium at 36°C essentially as described (16). To produce "fatty acid-homogeneous" membranes whose lipids have essentially only one type of acyl chain, cells were grown in the presence of avidin (2 mg/ml) in addition to the normal fatty acid growth supplement, which was added to a concentration of 0.12 mM. Cells were harvested in late logarithmic phase, membranes were prepared, and the ATPase activity in isolated membranes was assayed as described (17). Because K_m for ATP is a function of temperature but not of the lipid composition and phase transition temperature of the membrane lipids (13), a number of determinations of the temperature dependence of K_m from experiments using fatty acid-heterogeneous and fatty acid-homogeneous membranes were combined to produce a pooled estimate of K_m at each assay temperature. These best estimates were then used to convert the activity of the enzyme at 1 mM ATP (determined in duplicate) to a V_{max} value (the correction required was never more than 30% at any temperature, and that at temperatures below 25°C was never more than 15%). The V_{max} data obtained from these experiments were analyzed by direct fitting of the derived Arrhenius plots by the appropriate theoretical equations.

Differential Thermal Analysis (DTA) of Membrane Lipids. Total membrane lipids were extracted, purified, and prepared for DTA as described (18, 19). The hydrated lipid dispersions were subjected to DTA on a DuPont model 900 thermal analyzer, with sample heating rates of 3–5°C/min and glass beads as an inert reference. The resulting thermograms, which are comparable in position and shape to those measured for packed membranes (15, 20), were numerically integrated to determine the midpoint (t_c) of the lipid phase transition and the temperature range [$\delta t(10 \rightarrow 90)$] required for the transition to pass from 10% to 90% of completion. Because lipids melting out at different temperatures may have different heats of transition, some small errors in estimation of these values from differential thermograms are undoubtedly present. However, these errors are not of such a magnitude that they could affect the nature of the conclusions that we draw from the DTA results in this paper.

Freeze-Fracture Electron Microscopy. The conditions used for sample preparation, freeze-fracturing, and the preparation and electron microscopic examination of the replicas were essentially as described by Wallace and Engelman (21), but whole cells rather than isolated membranes were used.

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Abbreviations: DTA, differential thermal analysis; t_c, midpoint of the lipid phase transition; $\delta t(10 \rightarrow 90)$, temperature required for transition to pass from 10% to 90% of completion.

RESULTS AND ANALYSIS

Temperature Dependence of the ATPase Activity in a Liquid-Crystalline Lipid Environment. To study the ATPase activity in a membrane whose lipids are all in the liquid-crystalline state, *A. laidlawii* B was grown with avidin plus *cis*-vaccenic or anteisopentadecanoic acid to produce membranes whose lipids were homogeneous in these fatty acids and exhibited phase transitions well below 0°C, and the ATPase activity in isolated membranes was analyzed as described above. In Fig. 1, Arrhenius plots for the ATPase activity in the two membrane preparations are shown together, demonstrating that the lipid fatty acyl chain structure does not affect the temperature dependence of the ATPase activity so long as the lipids surrounding the enzyme are in the liquid-crystalline state. This conclusion is supported by the finding that the temperature dependence of the ATPase activity is essentially identical in other membrane preparations above the upper temperature limit of the calorimetrically determined lipid phase transition (data not shown), regardless of the position of the lipid phase transition midpoint.

As can be seen in Fig. 1, the temperature dependence of the ATPase activity in the presence of liquid-crystalline lipids cannot be described by a simple Arrhenius equation with a constant activation energy. To describe the Arrhenius plot more satisfactorily, we considered two types of models for the enzyme behavior: (*i*) the enzyme-catalyzed reaction has a finite heat capacity of activation, $\Delta C_p^{\ddagger}$, as suggested by Sturtevant and Mateo (22) for certain other enzymes, and (*ii*) the enzyme slowly changes its conformation, or its rate-limiting step, over the temperature range studied. The equations appropriate to these models will be described elsewhere. We found that model *i* gives a better fit to the data than does model *ii*, although the latter cannot be absolutely ruled out. Significantly, the measured slope of the Arrhenius plot is nearly a linear function of temperature, as model *i* would predict but not model *ii*. The data of Fig. 1 can be well fitted by the equation appropriate to model *i*, with $\Delta C_p^{\ddagger}$ −515 cal/mol-deg, a value of the same order of magnitude as that suggested by Sturtevant and Mateo (22) for D amino acid oxidase.

Effect of a Lipid Phase Transition on the ATPase Activity. To study the effect of the lipid phase state on the activity of the ATPase, we exploited our ability to produce membranes of homogeneous lipid fatty acyl composition, which exhibit lipid phase transitions considerably sharper than those normally observed in the membrane of *A. laidlawii* B (19). An Arrhenius plot for the ATPase activity in membranes of cells grown with avidin plus isoheptadecanoic acid, whose glycerolipids contain 98% isoheptadecanoyl chains, is shown in Fig. 2.

Three points are immediately obvious. First, the Arrhenius plot is clearly triphasic. Such plots are typical of preparations whose lipid phase transitions are fairly sharp and centered at more than ≈25°C, but when the transitions occur at lower temperatures or are less sharp, biphasic plots are observed. Second, the "breaks" in the plot are not sharp but are quite noticeably curved. Third, t_c, determined by DTA, lies only slightly above the center of the upper break region.

Taken together, the last two points (which are generally valid for preparations that show a lipid phase transition in the temperature range covered by the experimental Arrhenius plot) suggest that the high-slope region of the Arrhenius plot represents a transition of the ATPase from a high-activity state at higher temperatures to a low-activity state at low temperatures, which is induced by a change in the phase state of the membrane lipids and which creates the steeply sloping phase of the Arrhenius plot. This behavior is generally consistent with that reported by Thilo *et al.* (11) for sugar transport in *E. coli*. The low-temperature, shallow-slope phase of the Arrhenius plot shown in Fig. 2 could represent either the activity of the ATPase associated with gel-state lipids or a distinct (but low) ATPase activity that is "unmasked" only when the major ATPase is virtually completely inactivated. Because the high- and low-temperature activities in isoheptadecanoate-homogeneous membranes differ appreciably in their stability to brief incubation at increased temperatures (data not shown), we favor the latter suggestion. Since the low-temperature activity becomes an appreciable fraction of the total ATPase activity only

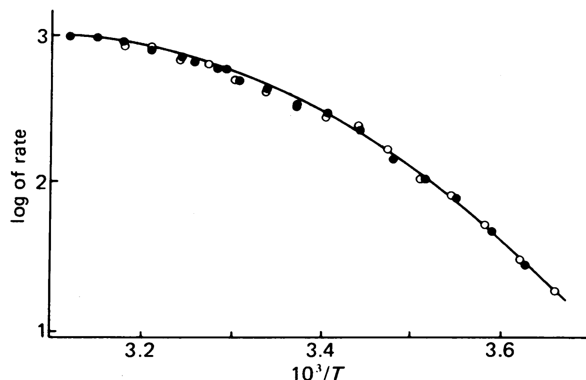

FIG. 1. Arrhenius plot of the Na^+,Mg^{2+}-ATPase activity in membranes of *A. laidlawii* B grown with avidin plus anteisopentadecanoic acid (●) or *cis*-vaccenic acid (○). The experimental points are fitted (solid line) to an equation of the form: log rate = $(2.303)^{-1}(K - \Delta H_0^{\ddagger}/R + (\Delta C_p^{\ddagger}/R)(\ln T) - (\Delta C_p^{\ddagger}/R)(1 - T_0/T))$, setting $\Delta H_0^{\ddagger}$ = 13.8 kcal/mol at T_0 = 300 K and heat capacity of activation $\Delta C_p^{\ddagger}$ = −515 cal/mol-deg. The constant K has been adjusted to scale the theoretical curve (solid line) to the experimental data along the *y* axis. The deviations of the data points from the theoretical curve are not simply attributable to random error but have been treated as such in our analysis.

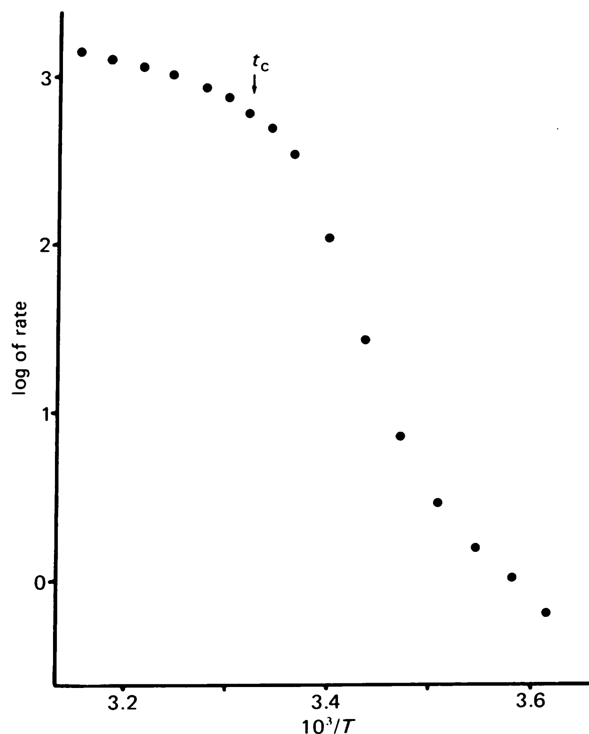

FIG. 2. Experimental Arrhenius plot for the Na^+,Mg^{2+}-ATPase in membranes whose lipids are made homogeneous in isoheptadecanoic acid. The lipid phase transition midpoint (t_c) of 28.8°C is indicated.

when the total activity is less than roughly 3–5 nmol/mg·min, this range of activities was ignored in subsequent analyses of the ATPase Arrhenius plots.

Theoretical Analysis of the ATPase Arrhenius Plots. In the light of the conclusions discussed above, we turned to a more quantitative analysis of the effect of a lipid phase transition on the ATPase activity. If we ignore the low-temperature break region in plots where such a break is observed, and if we assume that the Na^+,Mg^{2+}-ATPase exhibits a negligible activity when it enters its low-temperature (gel-phase lipid-associated) state, the appropriate equation for the ATPase Arrhenius plot is:

$$\ln \text{rate} = K + F(T) - \ln\{1 + \exp[(\Delta H_{21}/R)(1/T - 1/T_0)]\}. \quad [1]$$

In this equation, K is a scaling constant to correct for small differences in the activities of different preparations, $F(T)$ is the expression describing the temperature dependence of the enzyme's activity in its high-temperature state (see legend to Fig. 1), and the fractions f_1 of the enzyme in high- and low-temperature states (termed 2 and 1, respectively) are given by:

$$f_2/f_1 = \exp[(-\Delta H_{21}/R)(1/T - 1/T_0)]. \quad [2]$$

In this expression, ΔH_{21} is an effective enthalpy of transition between the two states (and may not necessarily be equal to the calorimetric enthalpy of transition); T_0 is the temperature at which the enzyme is equally distributed between the two states. In Fig. 3, the Arrhenius plot for the ATPase in membranes whose lipids are highly enriched (91%) in *cis*-15-octadecenoyl chains is shown along with the best-fit curve generated from Eq. 1, using $K = 0.37$, $\Delta H_{21} = 36$ kcal/mol, and $T_0 = 293.3$ K (20.3°C; the calorimetrically determined lipid phase transition midpoint is 27.6°C). The deviations of the data points from the theoretical curve at higher temperatures are quite reproducible and are not simply due to experimental error. However, because we have not been able to explicitly incorporate this aspect of the enzyme behavior into our physical models, we have treated such deviations as random errors in our analysis.

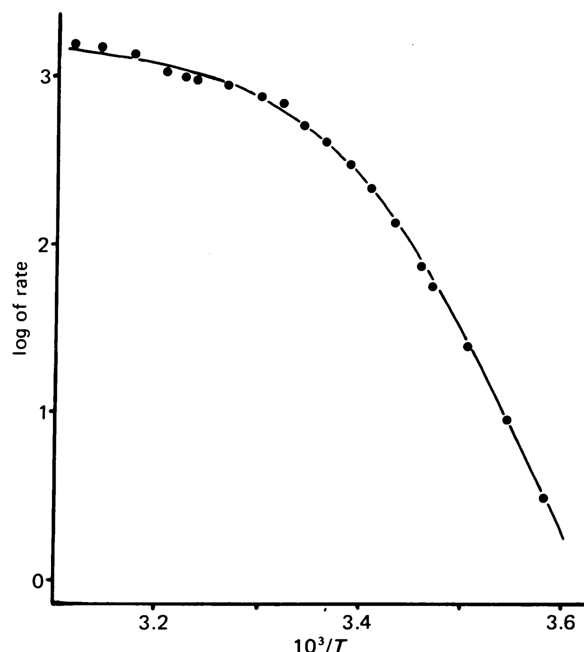

FIG. 3. Description of the Arrhenius plot for the ATPase activity in *cis*-15-octadecenoate-homogeneous membranes in terms of a temperature-dependent transition of the ATPase to an inactive form at low temperatures. The experimental data (circles) have been fitted with a theoretical curve (solid line) generated from Eq. 1 by using $K = 0.37$, $\Delta H_{21} = 36$ kcal/mol, and $T_0 = 293.3$ K (20.3°C).

Table 1. Lipid phase transition midpoint temperatures (t_c) and ATPase equidistribution temperatures (t_0)

Growth supplement	t_c, °C	t_0 °C
None	33.8	30.9
Palmitate	31.2	27.7
Palmitate/cholesterol	30.7	25.3
Isopalmitate	25.8	22.3
Elaidate	28.7	22.4
Anteisopentadecanoate/avidin	−14.5	—*
cis-Vaccenate/avidin	−8.5	—*
Isomyristate/avidin	10.1	7.2
Isopentadecanoate/avidin	14.8	13.5
trans-Vaccenate/avidin	20.0	14.0
Elaidate/avidin	20.1	14.9
Isopalmitate/avidin	21.8	20.9
Palmitate/elaidate/avidin	26.9	24.8
cis-15-Octadecenoate/avidin	27.6	20.7
Isoheptadecanoate/avidin	28.8	25.3
Palmitate/myristate/avidin	32.9	30.7

Values were determined for various membrane preparations by DTA and by Arrhenius plot analysis, respectively. Fatty acid growth supplements were added to the culture medium to a total concentration of 0.12 mM; cholesterol was at 25 mg/liter and avidin was at 2 mg/liter.

* No break was seen above 0°C in these Arrhenius plots.

Table 1 summarizes our determinations of the parameter T_0 (or t_0 for °C) in various experiments using membranes with different lipid fatty acyl compositions and phase transition temperatures. The calorimetrically determined t_c values are also given for the various membrane preparations studied. It is evident that t_c and t_0 are systematically related, with t_0 consistently lying 1–8°C below t_c. When t_c was less than 0°C, no Arrhenius plot break could be detected. As noted above, this t_c/t_0 correlation suggests that a thermotropic phase change of the membrane lipids drives a transition of the enzyme from an active state to an inactive one.

The observed effects of the lipid physical state on the membrane ATPase activity could be mediated in two distinct but related ways. Let us assume that the progress of the thermotropic bulk lipid phase transition can be approximately described by:

$$f_l/f_g = \exp[-(\Delta H_{\text{eff}}/R)(1/T - 1/T_c)] \quad [3]$$

in which f_l and f_g are the fractions of the total bulk lipids in the liquid-crystalline and gel states, respectively, and ΔH_{eff} is an empirical value that corresponds to the van't Hoff transition enthalpy in the case of a single pure lipid. Let us also assume that a similar equation describes a change in state of the ATPase boundary lipids, driven by the bulk phase transition (23) but with possibly different characteristic temperatures and effective enthalpy values (we will call the latter ΔH_{bnd}). Theoretical (23) and experimental results (24, 25) suggest that ΔH_{bnd} will in general be smaller than ΔH_{eff}. Then, if the ATPase activity is directly determined by the phase state of the surrounding bulk lipid phase, we predict that $\Delta H_{21} = \Delta H_{\text{eff}} + \Delta H_p$, in which ΔH_{21} is defined in Eq. 2 and ΔH_p is the enthalpy change of the enzyme molecule between the active and inactive states (and, in this model, between the liquid-crystalline and gel lipid phases as well). If, however, it is the state of the ATPase boundary lipids that directly determines the ATPase activity, we predict that $\Delta H_{21} = \Delta H_{\text{bnd}} + \Delta H_p$.

In Fig. 4, we have plotted values of ΔH_{21}, determined for various membrane preparations by fitting Eq. 1 to the experi-

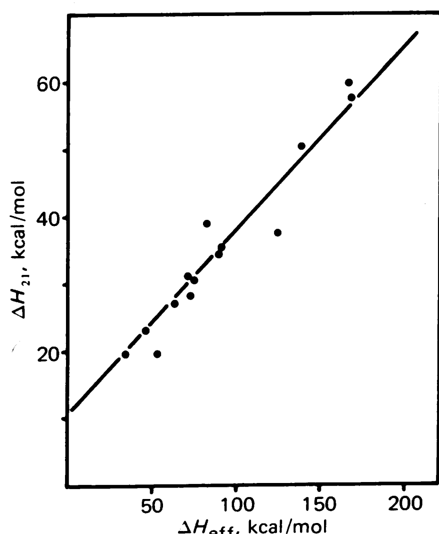

FIG. 4. Correlation of the effective enthalpy of the temperature-dependent transition of the ATPase, ΔH_{21}, with the effective lipid phase transition enthalpy, ΔH_{eff}, for a number of membrane preparations of varying fatty acyl composition. The measured correlation coefficient is 0.90 and the coefficient of regression of ΔH_{21} on ΔH_{eff} is 0.27.

mental data, against the values of ΔH_{eff} for the corresponding membrane lipid phase transitions, determined by analysis of the membrane lipid phase transition and by use of:

$$\Delta H_{\text{eff}} = \frac{[\ln(81)] \cdot R \cdot T_c^{\,2}}{\delta t(10 \rightarrow 90)} \quad [4]$$

in which $\delta t(10 \rightarrow 90)$ is the temperature range required for the transition to pass from 10 to 90% of completion, T_c is given in K, and R is the gas constant. This equation is readily derived from Eq. 3. It is evident that ΔH_{21} and ΔH_{eff} are well-correlated ($r = 0.90$), but the measured regression coefficient of 0.27 is clearly too far from unity to justify the conclusion that $\Delta H_{21} = \Delta H_{\text{eff}} + \Delta H_p$. It is more reasonable to accept the alternative hypothesis, that $\Delta H_{21} = \Delta H_{\text{bnd}} + \Delta H_p$. The finite y intercept in Fig. 4 provides a rough estimate of ΔH_p, whose positive value ($\approx$10 kcal/mol) is not unreasonable if the van der Waals' interactions between the enzyme and its boundary lipids are weakened when the latter enter the liquid-crystalline-like state. Detailed interpretations of the results shown in Fig. 4, such as this last point, must be regarded as highly speculative at present, although they are consistent with our current understanding of enzyme–lipid interactions.

The "boundary lipid" fraction whose thermotropic transition is suggested to regulate the ATPase activity could correspond to the lipid annulus immediately adjacent to the ATPase or to lipid nonspecifically trapped in protein-rich patches that form as the bulk lipids enter the gel state and freeze out membrane proteins (21, 26, 27). The former possibility is strongly supported by the following observation. *A. laidlawii* B membranes whose lipids are highly enriched in *iso*-acyl chains show no significant aggregation of intramembrane particles below the lipid phase transition by freeze-fracture electron microscopy, in agreement with previous findings (28), whereas membranes containing mainly *n*-acyl lipids show extensive aggregation of intramembrane particles below t_c (Fig. 5). In spite of this difference, *n*-acyl and *iso*-acyl lipid-containing membranes give ATPase Arrhenius plots that are indistinguishable in their basic form and in the relationships of t_0 to t_c and of ΔH_{21} to ΔH_{eff}. Although aggregation of integral membrane proteins into small oligomeric clusters of a few molecules may still occur in membranes containing diisoacyl glycerolipids, a major aggregation of such molecules clearly does not occur in such membranes. Therefore, the boundary lipid fraction whose physical state regulates the ATPase activity would seem to correspond to the lipid directly associated with the enzyme molecule, not to lipid trapped in large protein aggregates which form below the lipid phase transition temperature.

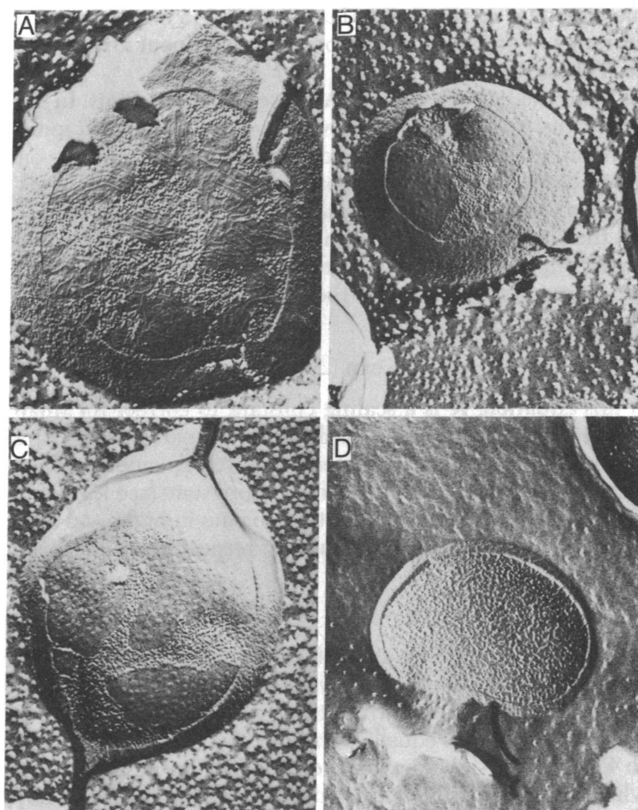

FIG. 5. Electron micrographs (× 15,200) of freeze-fracture replicas of cell membranes whose lipids contain equimolar amounts of palmitoyl and myristoyl chains (*A*), elaidoyl chains (*B*), *cis*-15-octadecenoyl chains (*C*), or isopalmitoyl chains (*D*). The cells were held at 4°C, a temperature well below the lipid phase transition lower boundary, for 1 hr prior to rapid freezing.

CONCLUSIONS

The analysis presented above yields results that are basically consistent with our understanding of lipid–protein interactions in membranes. The rather high calculated value of the apparent $\Delta C_p^{\ddagger}$ for the ATPase reaction suggests that the $\Delta C_p^{\ddagger}$ values for other enzymic reactions may be sufficiently large to cause marked nonlinearities in the Arrhenius plots for such reactions, as Sturtevant and Mateo (22) have suggested. The apparent inactivation of the ATPase when its boundary lipids enter a low-temperature, presumably gel-like, state suggests that the overall enzyme-catalyzed reaction involves at least one step that requires a transient displacement or deformation of the lipid molecules around the enzyme, because such a step would be hindered by the more rigid gel-state lipids. This possibility is reasonable in view of the probable function of this enzyme as a transmembrane alkali cation pump (13) and generally agrees with the findings of Therisod *et al.* (29), who reported a similar inactivation of the *lac* carrier protein in *E. coli* membranes as the lipid phase is cooled through the phase transition. However, when the lipids around the enzyme are in a liquid-crystalline state, their fatty acyl chain composition and physical properties do not significantly affect the temperature dependence of the

enzyme activity. This finding could indicate either that the rate-limiting step for catalysis by the enzyme in a liquid-crystalline lipid environment does not involve the lipid molecules or that the lipids around the enzyme do not appreciably hinder a transient enzyme conformational change within the membrane bilayer so long as they are in the liquid-crystalline state.

The results reported here, along with those recently described by Thilo *et al.* (11), indicate that careful analyses of Arrhenius plots for membrane enzyme activities can furnish a considerable amount of useful information regarding the behavior of various membrane-bound enzymes. By combining such approaches with manipulation of membrane lipid composition (30, 31), it may be possible to learn a good deal about the behavior of many membrane enzymes which cannot at present be isolated and reconstituted with lipids in well-defined model membranes. Such studies will require more sophisticated types of theoretical analyses than those commonly used at present; in particular, the common practice of dissecting Arrhenius plots into a set of straight-line segments will have to be evaluated more critically than it is currently.

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