Biochimica et Biophysica Acta, 1107 (1992) 111–118

BBAMEM 75647

The effect of cholesterol and epicholesterol on the activity and temperature dependence of the purified, phospholipid-reconstituted ($Na^+ + Mg^{2+}$)-ATPase from *Acholeplasma laidlawii* B membranes

Rajan George and Ronald N. McElhaney

Department of Biochemistry, University of Alberta, Edmonton, Alberta (Canada)

(Received 5 December 1991)
(Revised manuscript received 4 March 1992)

Key words: Cholesterol; Epicholesterol; Lipid fluidity; Enzyme modulation; ATPase, ($Na^+ + Mg^{2+}$)-; (*A. laidlawii*)

The ($Na^+ + Mg^{2+}$)-ATPase purified from *Acholeplasma laidlawii* B membranes was reconstituted into large, unilamellar vesicles formed from dimyristoylphosphatidylcholine (DMPC) and varying amounts of cholesterol or epicholesterol. The ATP hydrolytic activity of the reconstituted enzyme was then determined over a range of temperatures and the phase state of the DMPC in the ATPase-containing vesicles was characterized by high-sensitivity differential scanning calorimetry. In the vesicles containing only DMPC, the ATPase activity is higher in association with lipids in the liquid-crystalline state than with gel-state phospholipids, resulting in a curvilinear, biphasic Arrhenius plot with a pronounced change in slope at the elevated gel to liquid-crystalline phase transition temperature of the DMPC. The incorporation of increasing amounts of cholesterol into the DMPC vesicles results in a progressively greater degree of inhibition of ATPase activity at higher temperatures but a stimulation of activity at lower temperatures, thus producing Arrhenius plots with progressively less curvature and without an abrupt change in slope at physiological temperatures. As cholesterol concentration in the ATPase-DMPC vesicles increases, the calorimetric phase transition of the phospholipid is further broadened and eventually abolished. The incorporation of epicholesterol into the DMPC proteoliposomes results in similar but less pronounced effects on ATPase activity, and its effect on the phase behavior of the DMPC-ATPase vesicles is also similarly attenuated in comparison with cholesterol. Moreover, cholesterol added to the purified enzyme in the absence of phospholipid does not show any significant effect on either the activity or the temperature dependence of the detergent-solubilized ATPase. These findings are consistent with the suggestion that cholesterol exerts its effect on the ATPase activity by altering the physical state of the phospholipid, since the ordering effect of cholesterol (or epicholesterol) on liquid-crystalline lipid results in a reduction of ATPase activity while the disordering of gel-state lipid results in an increase in activity.

Introduction

Acholeplasma laidlawii is a cell wall-less eubacterium which contains only a single membrane system, the cell surface membrane. This organism, like several other mycoplasmas, has been widely used to study membrane-associated phenomena, since the lipid fatty acid and polar headgroup composition of the membrane lipids can be dramatically altered and highly pure preparations of membranes can be easily prepared. Moreover, although *A. laidlawii* B does not synthesize or require cholesterol for growth, it can incorporate substantial amounts of this and other sterols into the membrane from the growth medium. The ability to greatly manipulate the fluidity and phase state and the thickness and surface charge density of the lipid bilayer, by altering the fatty acid and polar headgroup composition and the cholesterol content of the *A. laidlawii* membrane, has made this organism an ideal one for studying the role of lipids in biological membranes (for reviews, see Refs. 1 and 2).

The Na^+-stimulated, Mg^{2+}-dependent ATPase of *Acholeplasma laidlawii* B is the major ATPase present in the membrane of this microorganism [3–5]. This enzyme is a tightly bound, lipid-requiring, integral transmembrane protein [5–12] that functions in vivo and in vitro as an ATP-driven, sodium-extruding pump [13,14] and that is involved in regulating the intra-

Correspondence: R.N. McElhaney, Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada T6G 2H7.

cellular osmolarity and thus the cell volume of this mycoplasma [3,13]. The *A. laidlawii* ($Na^+ + Mg^{2+}$)-ATPase has been isolated and purified to homogeneity and shown to consist of five subunits (α, β, γ, δ and ϵ) ranging in apparent molecular weight from 68 000 to 16 000 with a probable subunit stoichiometry of $\alpha_3\beta_3\gamma\delta_2\epsilon_3$ [5,15]. All of the subunits of the purified, lipid-reconstituted enzyme [16] can be labeled by water-soluble, amino acid side chain-specific reagents, suggesting that at least a portion of each subunit is exposed to the aqueous phase [17,18]. However, the α-subunit, which also bears the nucleotide binding site [17,18], and possibly also the ϵ-subunit, can be photolabeled by phospholipids containing a photosensitive fatty acyl group, suggesting that a portion of this subunit (or subunits) penetrates into or traverses the hydrophobic core of the lipid bilayer [11]. The intimate association of the reconstituted ATPase with its host phospholipid bilayer has been confirmed by differential scanning calorimetry (DSC) studies, which show that this enzyme interacts with both the polar headgroups and the hydrocarbon chains of adjacent phospholipid molecules [12].

The effect of variations in temperature and in membrane lipid fatty acid composition on the activity of the *A. laidlawii* ($Na^+ + Mg^{2+}$)-ATPase in native membranes have been extensively studied in this laboratory [3,4] and in others [10,19,20]. In these studies the relationship between ATPase activity and membrane lipid phase state and fluidity was investigated by comparisons of Arrhenius plots of the rates of ATP hydrolysis versus reciprocal temperature with DSC or electron paramagnetic spin resonance (EPR) spectroscopic determinations of lipid phase transition temperatures and relative fluidities. It was initially reported that Arrhenius plots of ATPase activity are linear in isolated membranes whose lipids were enriched in low-melting *cis*-unsaturated fatty acids but exhibit a biphasic linear shape in membranes enriched in higher-melting *trans*-unsaturated or saturated fatty acids. More specifically, a 2.5-fold increase in the Arrhenius plot slope was reported to occur at temperatures between the lower boundary and the midpoint of the lipid gel to liquid-crystalline phase transition. However, a later study [4], utilizing a much larger number of temperature points and considering the marked increase in the K_m values for ATP with increasing temperature [3,21], revealed that in membranes containing exclusively liquid-crystalline lipid in the physiological temperature range, Arrhenius plots of ($Na^+ + Mg^{2+}$)-ATPase activity are clearly nonlinear (slope gently downward). This latter study also established that the temperature dependence of the ATPase activity is not dependent on membrane lipid fatty acid composition as long as the lipids exist in the fluid state. The absolute activity of this enzyme, however, does vary significantly with fatty acid composition, but there is no discernible relation between enzyme activity and lipid fluidity per se. If a gel to liquid-crystalline phase transition occurs within the physiological range, however, a gently curving biphasic Arrhenius plot is observed, in which the ($Na^+ + Mg^{2+}$)-ATPase activity falls off more steeply with decreasing temperature than would otherwise be the case. No effect of the lipid phase transition on the ATPase activity is noted until about half of the membrane lipid is converted to the gel state, and some ATPase activity remains at temperatures considerably below the lower boundary of the lipid phase transition, although eventually all ATPase seems to be lost. These results suggest that the *A. laidlawii* ($Na^+ + Mg^{2+}$)-ATPase is active only in association with liquid-like boundary lipids, and that the ATPase hydrolytic reaction exhibits a significant heat capacity of activation in this case. This enzyme appears to become progressively inactivated when its boundary lipids undergo a liquid-like to solid-like 'phase transition' that is driven by the liquid-crystalline to gel phase transition of the bulk membrane lipid phase, but that is less cooperative and that takes place over a lower temperature range than does the bulk lipid transition. Very similar results have recently been obtained when the purified enzyme is reconstituted with phosphatidylcholines (PC's) containing a wide variety of different fatty acids [22,23]. However, in contrast to isolated membranes, the absolute activity of the ATPase in reconstituted proteoliposomes does not vary significantly with fatty acid structure at temperatures above the lipid phase transition temperature. This result indicates that changes in membrane lipid fluidity in the liquid-crystalline state have only a small effect on enzyme activity. This result also suggests that the wide variations in ATPase activity observed at 37°C in isolated *A. laidlawii* membranes of differing fatty acid composition are due primarily to variations in the number of ATPase molecules present rather than to variations in the specific activity of the native enzyme.

Although the variation of the *A. laidlawii* ($Ca^{2+} + Mg^{2+}$)-ATPase activity with changes in membrane lipid fatty acid composition has been extensively studied, the effect of the presence of sterols on the absolute activity and temperature dependence of this enzyme has received relatively little attention. De Kruyff et al. [19] reported that the incorporation of cholesterol into *A. laidlawii* membranes enriched in elaidic acid results in a modest decrease in absolute activity at higher temperatures, an increase in absolute activity at lower temperatures, and a downward shift in the Arrhenius plot break temperature by 6–7 C°. These workers also reported that cholesterol incorporation decreases the lipid phase transition temperature of the isolated membranes. Moreover, treatment of the cholesterol-enriched membranes with the polyene antibiotic filipin,

which specifically complexes cholesterol, reverses the effect of cholesterol incorporation on both the ATPase activity and the lipid phase transition. Finally, the incorporation of epicholesterol, the α-epimer of cholesterol, was reported to have no effect on either ATPase activity or the membrane lipid phase transition. This latter result seemed consistent with the much weaker effect of epicholesterol as compared to cholesterol in condensing phospholipid monolayers and in reducing the permeability and increasing the order of phospholipid bilayers (see Ref. 26).

Although the reported effects of cholesterol and epicholesterol incorporation on the ATPase activity on isolated membranes may well be correct, it should be pointed out that in this previous study the Na^+ and ATP concentrations were not adjusted so as to optimize ATPase activity over the entire physiological temperature range. As well, since changes in membrane lipid composition are now known to effect the number of functional ATPase molecules present in isolated membranes, it is possible that the results reported above could reflect sterol effects on ATPase number rather than on ATPase specific activity. Finally, in *A. laidlawii* membranes enriched in elaidic acid, a maximum of only about 15 mol% of cholesterol or epicholesterol can be incorporated. For these reasons, we have decided to reinvestigate the effect of cholesterol and epicholesterol on the absolute activity and temperature dependence of the purified *A. laidlawii* ATPase reconstituted into dimyristoylphosphatidylcholine (DMPC) vesicles, since in this model membrane system the possibly confounding effects described above can be overcome and the incorporation of much higher levels of sterol can be obtained. As before, DSC analysis of the reconstituted vesicles was also performed, so that the effect of cholesterol and epicholesterol incorporation on the thermotropic phase behavior of DMPC molecules in the presence of the incorporated ATPase could be determined. The results of this study are reported below.

Materials and Methods

Materials. L-α-Dimyristoylphosphatidylcholine (DMPC) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Steraloids Inc. (Wilton, NH, USA) supplied epicholesterol. Cholesterol was purchased from Aldrich Chemical Co. (Milwaukee, WI, USA) and recrystallized from a mixture of methanol and chloroform before use.

Methods. The $(Na^+ + Mg^{2+})$-ATPase was purified from *A. laidlawii* B membranes according to the method of Lewis and McElhaney [5]. Reconstitution of the purified ATPase with lipids and estimation of the ATPase activity were done as described by George and McElhaney [17]. Essentially this reconstitution involves a cholate solubilization and dialysis procedure in which almost all of the residual native lipid and most of the detergent is removed by exchange with a large excess of phospholipid, in this case DMPC. Generally 50–100 μg of detergent-solubilized ATPase was reconstituted with 2.5 mg of phospholipid, with or without various quantities of cholesterol or epicholesterol. Although there was little effect of cholesterol or epicholesterol on the efficiency of reconstitution, the specific activity of the enzyme was calculated from the amount of protein actually present in the reconstituted proteoliposomes, which was determined for each sample [17]. Phospholipid was quantitated by the procedure of Raheja et al. [24] and cholesterol by the method of Watson [25]. High-sensitivity DSC analyses were done using a Microcal MC-2 scanning calorimeter (Microcal Inc. Amherst, MA, USA) as described previously [12].

Data analysis and presentation. The experiments described here were done three times. However, because the activity of the purified, lipid-depleted ATPase decreases with time, each independent set of experiments was performed with a different, freshly prepared enzyme preparation. Since the absolute activity (but not the temperature dependence) of these three ATPase preparations varied considerably, we did not consider that a pooling of these experimental results was appropriate. Thus, the data to be presented below are those from a single set of experiments on the same ATPase preparation. However, the results presented are fully representative of those obtained in the other two sets of experiments.

Results

Effect of cholesterol on the activity and temperature dependence of the purified ATPase prior to phospholipid reconstitution

The Arrhenius plot of the purified, detergent-solubilized ATPase, before reconstitution with phospholipid, is a smooth, downward-curving line (Fig. 1A) showing no abrupt change in slope. This curve resembles the Arrhenius plots of the ATPase activity in native *A. laidlawii* membranes enriched in low-melting fatty acids [4]. This was expected, since the small amounts of native membrane lipids and detergent still associated with the purified protein [16] do not undergo any change in phase state within the temperature range studied [12].

Cholesterol is known to compete with phospholipids for binding to the hydrophobic regions of several membrane-bound proteins and to effect the activity of some membrane-associated enzymes, transporters and receptors (see Ref. 26 for a recent review). In order to determine if added cholesterol can directly effect the *A. laidlawii* B $(Na^+ + Mg^{2+})$-ATPase, a sonicated suspension of cholesterol in the assay medium was incu-

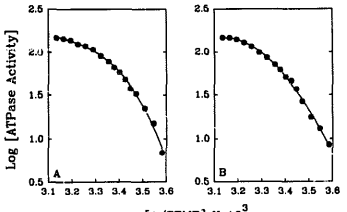

Fig. 1. Temperature dependence of purified ATPase in the absence and presence of exogenous cholesterol. ATPase activity was estimated at various temperatures and the log specific activity (μmoles P_i released per h per mg protein) is plotted against the reciprocal of absolute temperature. Cholesterol was added by mixing the purified enzyme with cholesterol. The final concentration in the mixture was 530 moles of cholesterol/mol of ATPase. (A) Purified ATPase; (B) Purified ATPase + cholesterol.

bated with purified, detergent-solubilized enzyme at 37°C for 15 min and aliquots of this mixture were used to determine the ATPase activity at various temperatures. The Arrhenius plot in Fig. 1B shows that exogenously added cholesterol does not produce any change in the temperature dependence of the purified ATPase. At this concentration of cholesterol (0.51 mg/mg of ATPase protein), there is also no effect on the absolute activity of purified ATPase when assayed at 37°C, the optimal growth temperature of this organism (Table I). Therefore, cholesterol molecules either do not physically associate with the purified ATPase or, if they do associate, do not directly alter the absolute activity or temperature dependence of this enzyme.

TABLE I

Effect of cholesterol and epicholesterol incorporation on the ATPase activity of enzyme-containing proteoliposomes at 37°C

ATPase reconstituted with	Mol percent sterol	ATPase specific activity (μmol P_i per h per mg protein	Percent control
None	0	124 [a]	60
Cholesterol (530 mol/mol ATPase)	~100	124	60
DMPC (2.4 mg/ml)	0	208	100
DMPC + cholesterol	13	196	94
DMPC + cholesterol	24	181	87
DMPC + cholesterol	33	137	66
DMPC + cholesterol	45	65	31
DMPC + epicholesterol	50	171	82

[a] Replicate determinations of ATPase specific activity at 37°C normally agree to within ±1–2%.

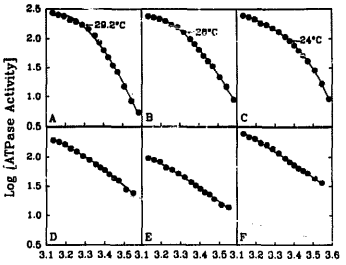

Fig. 2. Temperature dependence of purified ATPase reconstituted with DMPC and various concentrations of cholesterol or epicholesterol. Purified ATPase was reconstituted with either DMPC (2–3 mg/ml) alone or in combination with varying concentrations of cholesterol. ATPase activity was estimated at specified temperatures and the results represented as Arrhenius plots. Arrows indicate the DMPC phase transition temperature determined by DSC. (A) DMPC alone; (B) DMPC + cholesterol (13 mol%); (C) DMPC + cholesterol (24 mol%); (D) DMPC + cholesterol (33 mol%); (E) DMPC + cholesterol (45 mol%); (F) DMPC + epicholesterol (50 mol%).

The effect of cholesterol and epicholesterol incorporation on the absolute activity and temperature dependence of the phospholipid-reconstituted ($Na^+ + Mg^{2+}$)-ATPase

The purified ATPase was reconstituted with DMPC alone, or with DMPC and various concentrations of cholesterol, and the ATPase hydrolytic activity was determined over a range of temperatures. The temperature dependence of the ATPase activity in the presence of increasing cholesterol concentrations in the DMPC vesicles are presented as Arrhenius plots in Figs. 2A–E. In the absence of cholesterol, the Arrhenius plot of ATPase activity is biphasic, consisting of two gently sloping lines of different average slopes, with the average slope of the lower-temperature line being about 2.5-times that of the upper-temperature line. The change in slope occurs at about 29°C, which corresponds to the gel to liquid-crystalline phase temperature of the reconstituted vesicles as determined by DSC (see below). As the cholesterol concentration in the DMPC proteoliposomes increases, the slope in the higher-temperature region remains nearly constant but that in the lower-temperature region progressively decreases until, at the two highest cholesterol concentrations, the Arrhenius plots lose their biphasic character. Moreover, the change in slope persisting at the two lowest cholesterol concentrations decreases slightly in temperature with increases in cholesterol content. It is also clear from this series of Arrhenius plots that the presence of increasing levels of cholesterol in the

DMPC proteoliposomes decreases the absolute activity of the ATPase at higher temperatures (see Table I), while increasing it at lower temperatures. This inhibitory effect of cholesterol on enzyme activity at 37°C, the optimal growth temperature of this organism, is relatively modest at cholesterol concentrations of 13 and 24 mol% but becomes progressively greater at higher cholesterol concentrations.

The effect of the incorporation of a high level of epicholesterol on the temperature dependence of the phospholipid-reconstituted ($Na^+ + Mg^{2+}$)-ATPase is illustrated in Fig. 2F. The Arrhenius plot of the DMPC-epicholesterol reconstituted ATPase is similar to that of the DMPC proteoliposomes containing relatively high levels of cholesterol, in that only a single line without an abrupt change in slope is observed. Moreover, the presence of epicholesterol decreases the absolute activity of this enzyme at higher temperatures (see Table I) while increasing activity at lower temperatures, just as is observed for cholesterol. However, at 37°C, the inhibitory effect of epicholesterol on ATP hydrolytic activity is much less than that observed for cholesterol at a comparable level of incorporation. Interestingly, at lower temperatures the presence of epicholesterol actually increases ATPase activity to a greater extent than does the presence of cholesterol, implying that epicholesterol is more disruptive of hydrocarbon chain packing in gel state bilayers than is cholesterol.

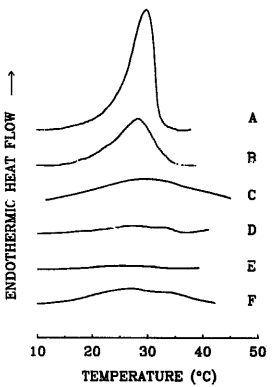

Fig. 3. Effect of cholesterol and epicholesterol incorporation on the thermotropic phase behavior of DMPC-ATPase proteoliposomes as determined by DSC. (A) DMPC alone; (B) DMPC+cholesterol (13 mol%); (C) DMPC+cholesterol (24 mol%); (D) DMPC+cholesterol (33 mol%); (E) DMPC+cholesterol (45 mol%); (F) DMPC+epicholesterol (50 mol%).

The effect of cholesterol and epicholesterol incorporation on the thermotropic phase behavior of DMPC in ATPase-containing proteoliposomes

The thermotropic phase behavior of DMPC vesicles containing comparable amounts of the ATPase protein and various quantities of cholesterol or epicholesterol were studied by high-sensitivity DSC. A typical set of DSC thermograms are presented in Figs. 3A–E and the thermodynamic parameters derived from these thermograms are presented in Table II. In the absence of any sterol (Fig. 3A), the incorporation of the enzyme into the DMPC vesicles results in an increase in the temperature, a decrease in the calorimetric enthalpy and a decrease in the cooperativity of the gel to liquid-crystalline phase transition of the DMPC molecules, indicating that this integral, trans-membrane protein interacts with the DMPC bilayer by both electrostatic and hydrophobic interactions (for a more complete calorimetric study of ATPase-DMPC interactions, see Ref. 12). The incorporation of 13 or 24 mol% cholesterol into the ATPase-containing proteoliposomes decreases the phase transition midpoint temperature

TABLE II

The thermodynamic properties of the gel to liquid-crystalline phase transition of DMPC vesicles containing roughly comparable amounts of the purified A. laidlawii ($Na^+ + Mg^{2+}$)ATPase and variable amounts of cholesterol or epicholesterol

Vesicle lipid composition	ATPase concentration (μg protein/mg DMPC)	T_m [a] (°C)	ΔH [a] (kcal/mol) [b]	$\Delta T_{1/2}$ [a] (C°)
DMPC	208	29.2	4.2	5
DMPC+13 mol% cholesterol	192	28.0	2.0	7
DMPC+24 mol% cholesterol	187	~30	~0.2	~12.5
DMPC+33 mol% cholesterol	159	~29	<0.15	>15
DMPC+45 mol% cholesterol	177	n.m.	n.m.	n.m.
DMPC+50 mol% epicholesterol	194	~29	~0.3	~13

[a] T_m is the gel to liquid-crystalline phase transition midpoint temperature, ΔH is the phase transition enthalpy change, and $\Delta T_{1/2}$ is a measure of the cooperativity of the phase transition, with progressively larger $\Delta T_{1/2}$ values indicating a progressively broadened and less cooperative transition. n.m., not measurable.

[b] 1 kcal = 4.1868 kJ.

slightly and progressively reduces both the enthalpy and cooperativity of the DMPC chain-melting phase transition still further. Cholesterol incorporations of 33 and 45 mol% essentially abolish the cooperative gel to liquid-crystalline phase transition of the DMPC molecules, or at least reduce the cooperativity of this transition to such an extent that it can not be detected by high-sensitivity DSC. Interestingly, the incorporation of a high amount of epicholesterol into the ATPase-containing proteoliposomes also markedly reduces the enthalpy and cooperativity of the gel to liquid-crystalline phase transition of the DMPC proteoliposomes without altering the midpoint temperature. However, epicholesterol is somewhat less effective than cholesterol in this regard (see Fig. 3F), since at a comparably high cholesterol concentration, a cooperative DMPC chain-melting transition is completely abolished.

Discussion

In the present study we find a generally good correlation between the effect of cholesterol incorporation on the activity and temperature dependence of the *A. laidlawii* B ($Na^+ + Mg^{2+}$)ATPase and the fluidity and phase state of the DMPC bilayer into which this enzyme is incorporated. In the absence of cholesterol, most of the DMPC molecules in the ATPase-containing proteoliposomes undergo a gel to liquid-crystalline phase transition at an elevated temperature compared to DMPC alone, and this is reflected in a break in the slope of the Arrhenius plot of ATPase activity which also occurs at the same elevated lipid phase transition temperature (about 29°C). As increasing quantities of cholesterol are incorporated into the proteoliposomes, the enthalpy and cooperativity of the DMPC chain-melting phase transition are reduced considerably, and the phase transition temperature is reduced slightly, while the magnitude of the change in slope of the Arrhenius plots of ATPase activity is also decreased. At higher incorporations of cholesterol, the cooperative gel to liquid-crystalline phase transition of the DMPC molecules in the ATPase-containing liposomes is abolished, as is the break in the slope of the Arrhenius plot of enzyme activity. It thus appears that the overall temperature dependence of the ATPase activity reflects the thermotropic phase properties of its lipid environment, as previously reported for both the native [3,4,10,19,20] and for the purified lipid-reconstituted [22,23] enzyme.

The absolute activity of this ATPase also appears to depend on the fluidity or degree of organization of the DMPC bilayer into which it is incorporated. As mentioned earlier, it is now well established that the incorporation of cholesterol into liquid-crystalline phospholipid bilayers results in a tighter packing of the melted hydrocarbon chains and an increase in hydrocarbon chain order, while having an opposite effect on gel state phospholipid bilayers (see Ref. 26). If the absolute activity of this enzyme reflects the fluidity or degree of organization of its lipid matrix, such that a decrease in fluidity results in a reduction in activity, we would predict that the incorporation of increasing quantities of cholesterol at temperatures above the chain-melting phase transition temperature would result in a progressive decrease in ATPase activity, while below the DMPC phase transition the presence of increasing quantities of cholesterol should increase enzyme activity. In fact, this is exactly what is observed experimentally, indicating that this ATPase is sensitive to the degree of organization of its host bilayer. Interestingly, in bilayers of synthetic phospho- or glycolipids which do not contain cholesterol, fairly substantial changes in fatty acid chain length and structure have relatively little effect on ($Na^+ + Mg^{2+}$)ATPase activity, as long as the lipid bilayers remains in the liquid-crystalline state [22,23]. This result may imply that the effect of cholesterol incorporation on lipid hydrocarbon chain packing in fluid bilayers is greater in magnitude than that produced by physiologically relevant variations in the chain length and chemical structure of the membrane lipid fatty acyl groups themselves (see Refs. 1 and 2 for a further discussion of this point).

As discussed earlier, De Kruyff and co-workers [19] found that the incorporation of cholesterol into *A. laidlawii* isolated membranes decreases the ($Na^+ + Mg^{2+}$)-ATPase activity at higher temperatures (temperatures above the Arrhenius plot break temperature) while increasing ATP hydrolytic rates at lower temperatures. These workers also reported that the presence of cholesterol shifts the Arrhenius plot break temperature downward by 6–7 C°. Our studies with the purified, DMPC-reconstituted enzyme fully confirm the former observations, and also reveal that the cholesterol affect on ATPase activity depends on the amount of cholesterol present. The incorporation of smaller amounts of cholesterol affects ATPase activity only modestly, while the incorporation of larger amounts of cholesterol produces major reductions in ATP hydrolytic rates. Although De Kruyff et al. [19] report a larger decrease in the Arrhenius plot break temperature upon cholesterol incorporation into *A. laidlawii* membranes than we find for ATPase-containing DMPC vesicles, this probably represents a difference in the effect of cholesterol on the lipid thermotropic phase behavior of the two systems, since cholesterol incorporation lowers the phase transition midpoint temperature of *A. laidlawii* membranes and isolated membrane lipids (Ref. 19 and unpublished results from this laboratory) to a greater degree than observed with the ATPase-containing DMPC vesicles (present study). Thus both studies are in agreement on the central

point that the characteristic effect of cholesterol incorporation on the temperature dependence of the ATPase accurately reflects its effect on the gel to liquid-crystalline phase transition of the host lipid bilayer. The only significant difference in results between the native and lipid-reconstituted ATPase is quantitative. At comparable cholesterol levels, De Kruyff and coworkers [19] report a larger reduction in ATPase activity in native membranes than we observe in our model membrane system.

The major difference in the results of our studies and those of De Kruyff and co-workers [19] concerns the effect of epicholesterol on ATPase activity and on the calorimetric phase transition observed in isolated membranes and in DMPC-ATPase proteoliposomes. De Kruyff et al. [19] report that the incorporation of epicholesterol instead of cholesterol has no effect on either the temperature-dependence of the *A. laidlawii* ATPase or on the membrane lipid phase transition. However, we find that cholesterol and epicholesterol show similar qualitative effects on both the ATPase activity and on the thermotropic phase behavior of the reconstituted proteoliposomes, although epicholesterol was found to be less effective than cholesterol at decreasing enzyme activity at 37°C and broadening and abolishing the lipid phase transition. Moreover, conventional [27] and high-sensitivity DSC (unpublished data from this laboratory) studies of the effect of the incorporation of epicholesterol on the thermotropic phase behavior of pure PC vesicles show that epicholesterol behaves much like cholesterol itself in phospholipid bilayers, at least at low to moderate sterol levels. It is therefore probable that the relatively small amounts of epicholesterol taken up by the *A. laidlawii* cells in the previous study may not have appeared to significantly affect the properties of the enzyme or the lipid bilayer simply because its effects may have been too small to detect. However, our results clearly show that cholesterol and epicholesterol at comparable levels of incorporation have qualitatively similar effects on both phospholipid thermotropic phase behavior and ATPase activity, although the effects due to epicholesterol do appear to be quantitatively less than those due to cholesterol.

The effect of pressure and of pentanol on the activity and temperature dependence of the $(Na^+ + Mg^{2+})$-ATPase of the *A. laidlawii* plasma membrane was studied by MacNaughton and Macdonald [28]. Increases in hydrostatic pressure increase the temperature at which the break in the Arrhenius plot of ATPase activity versus temperature is observed as well as the gel to liquid-crystalline phase tansition temperature of the membrane lipids [29]. Since this Arrhenius plot break is known to be induced by a chain-melting phase transition of the membrane lipids, this implies that the effect of hydrostatic pressure on this enzyme is mediated predominantly through its action on the phase state of membrane lipid bilayer. Moreover, the increases in the Arrhenius plot slopes (apparent activation energies) observed at temperatures above the break temperature with increases in hydrostatic pressure can also be explained by changes in the physical properties of the lipid bilayer, since hydrostatic pressure increases the order of liquid-crystalline lipid bilayers. Pentanol, however, which is known to decrease the lipid phase transition temperature and to increase the fluidity of liquid-crystalline bilayers of this organism, inhibits ATPase activity without affecting the Arrhenius break temperature at 37°C and atmospheric pressure, suggesting that it can act directly on the enzyme to inhibit its function. However, pentanol can partially offset the inhibitory effects of high hydrostatic pressure, which is consistent with a bilayer-fluidizing effect of this alcohol. These workers concluded from their study that the absolute activity and the temperature dependence of this enzyme are determined primarily by the fluidity and phase state of the membrane lipid bilayer, a conclusion further supported by the results of the present study. However, we find that cholesterol does not increase the slope of the Arrhenius plot above the break temperature as was observed for increases in hydrostatic pressure. This apparent difference in results could be due to a direct effect of pressure on the ATPase superimposed on its lipid-mediated effects.

In recent years it has been shown that a number of membrane-associated phenomena are modulated by the cholesterol content of the membrane (see Ref. 26). Considering only the membrane-bound ATPases, alterations in cholesterol levels appear to affect the activity of these enzymes in different ways, depending on the membrane system and organism studied. In *Mycoplasma mycoides*, a cholesterol-requiring mycoplasma, the activity and temperature dependence of the Mg^{2+}-ATPase are little affected by even large variations in the cholesterol content of the cell membrane [30], and similar results have been reported from the Ca^{2+}-ATPase of the rabbit muscle sarcoplasmic reticulum [31,32], although cholesterol incorporation has been reported to inhibit Ca^{2+}-ATPase activity in some preparations [33,34]. In contrast, the Na^+/K^+-ATPase activity of the rat kidney outer medulla [26] appears to be stimulated by low levels of cholesterol incorporation and inhibited by high levels. On the other hand, the incorporation of cholesterol into membranes containing Na^+/K^+-ATPases from other sources results in a progressive inhibition of ATP hydrolytic activity [35–39]. Thus cholesterol may either inhibit, stimulate, or have no effect on various ATPases. In *A. laidlawii* B membranes, however, it seems clear, both from the previous in vivo studies and from the present in vitro study, that in the biologically relevant liquid-crystalline phase state, cholesterol in-

corporation progressively inhibits ($Na^+ + Mg^{2+}$)-ATPase activity, most probably by its ordering of the host phospholipid bilayer rather than by interacting directly with enzyme.

Acknowledgements

Financial support for these studies was from the Medical Research Council of Canada and the Alberta Heritage Foundation for Medical Research. We thank Dr. Ruthven N.A.H. Lewis for valuable assistance with data analysis and figure preparation, and for helpful discussions.

References

1 McElhaney, R.N. (1984) Biochim. Biophys. Acta 779, 1–42.
2 McElhaney, R.N. (1989) CRC Crit. Rev. Microbiol. 17, 1–32.
3 Jinks, D.C., Silvius, J.R. and McElhaney, R.N. (1978) J. Bacteriol. 136, 1027–1036.
4 Silvius, J.R. and McElhaney, R.N. (1980) Proc. Natl. Acad. Sci. USA 77, 1255–1259.
5 Lewis, R.N.A.H. and McElhaney, R.N. (1983) Biochim. Biophys. Acta 735, 113–122.
6 Pollack, J.D., Razin, S. and Cleverdon, R.C. (1965) J. Bacteriol. 90, 617–622.
7 Rottem, S. and Razin, S. (1966) J. Bacteriol. 92, 714–722.
8 Ne'eman, Z., Kahane, I. and Razin, S. (1971) Biochim. Biophys. Acta 249, 169–176.
9 Ne'eman, Z., Kahane, I., Kovartovsky, J. and Razin, S. (1972) Biochim. Biophys. Acta 266, 255–268.
10 Bevers, E.M., Snoek, G.T., Op den Kamp, J.A.F. and Van Deenen, L.L.M. (1977) Biochim. Biophys. Acta 467, 346–356.
11 George, R., Lewis, R.N.A.H. and McElhaney, R.N. (1985) Biochim. Biophys. Acta 821, 253–258.
12 George, R., Lewis, R.N.A.H. and McElhaney, R.N. (1990) Biochem. Cell Biol. 68, 161–168.
13 Mahajan, S., Lewis, R.N.A.H., George, R., Sykes, B.D. and McElhaney, R.N. (1988) J. Bacteriol. 170, 5739–5746.
14 Mahajan, S., Lewis, R.N.A.H. and McElhaney, R.S. (1990) Zbt. Bakt. Suppl. 20, 670–673.
15 Chen, J.-W., Sun, Q. and Huang, F. (1984) Biochim. Biophys. Acta 777, 151–154.
16 George, R., Lewis, R.N.A.H. and McElhaney, R.N. (1987) Biochim. Biophys. Acta 903, 283–291.
17 George, R. and McElhaney, R.N. (1985) Biochim. Biophys. Acta 813, 161–166.
18 Lewis, R.N.A.H., George, R. and McElhaney, R.N. (1986) Arch. Biochem. Biophys. 247, 201–210.
19 De Kruyff, B., Van Dijck, P.W.M., Goldbach, R.W., Demel, R.S. and Van Deenen, L.L.M. (1973) Biochim. Biophys. Acta 330, 269–282.
20 Huang, J.C., Huang, L., Hoy, D.J. and Huang, A. (1974) Can. J. Biochem. 52, 974–980.
21 Silvius, J.R., Read, B.D. and McElhaney, R.N. (1978) Science 199, 902–904.
22 George, R., Lewis, R.N.A.H. and McElhaney, R.N. (1987) Israel J. Med. Sci. 23, 374–379.
23 George, R., Lewis, R.N.A.H., Mahajan, S. and McElhaney, R.N. (1989) J. Biol. Chem. 264, 11598–11604.
24 Raheja, R.K., Kaur, C., Singh, A. and Bhatia, I.S. (1973) J. Lipid Res. 14, 695–697.
25 Watson, D. (1960) Clin. Chim. Acta 5, 637–643.
26 Yeagle, P.L. (1988) in Biology of Cholesterol (Yeagle, P.L., ed.), Chapter 6, pp. 121–145, CRC Press, Boca Raton, FL.
27 De Kruyff, B., Demel, R.A. and Van Deenen, L.L.M. (1972) Biochim. Biophys. Acta 255, 331–347.
28 MacNaughton, W. and Macdonald, A.G. (1982) Comp. Biochem. Physiol. 72A, 405–414.
29 Macdonald, A.G. and Cossins, A.R. (1983) Biochim. Biophys. Acta 730, 239–244.
30 Le Grimellec, C. and Leblanc, G. (1980) Biochim. Biophys. Acta 599, 639–651.
31 Johannsson, A., Keightley, C.A., Smith, G.A. and Metcalfe, J.C. (1981) Biochem. J. 196, 505–511.
32 Madden, T.D., King, M.D. and Quinn, P.J. (1981) Biochim. Biophys. Acta 641, 265–269.
33 Madden, T.D., Chapman, D. and Quinn, P.J. (1979) Nature 279, 538–541.
34 Ortega, A. and Mas-Oliva, J. (1984) Biochim. Biophys. Acta. 773, 231–236.
35 Yeagle, P.L. (1983) Biochim. Biophys. Acta 727, 39–44.
36 Kimelberg, H.K. and Papahadjopoulos, D. (1974) J. Biol. Chem. 249, 1071–1080.
37 Claret, M., Garay, R. and Giraud, F. (1978) J. Physiol. 274, 247–263.
38 Sinensky, M., Pinkerton, F., Sutherland, E. and Simon, F.R. (1979) Proc. Natl. Acad. Sci. USA 76, 4893–4897.
39 Yoda, S. and Yoda, A. (1987) J. Biol. Chem. 262, 103–109.