ELSEVIER

Biochimica et Biophysica Acta 1283 (1996) 45–50

BBA Biochimica et Biophysica Acta

Cholesterol increases the thermal stability of the Ca^{2+}/Mg^{2+}-ATPase of cardiac microsomes

Alicia Ortega [a,b,1], Juan Santiago-García [c], Jaime Mas-Oliva [c], James R. Lepock [a,*]

[a] *Departments of Biology and Physics, University of Waterloo, Waterloo, Ontario N2L 3G1, Canada*
[b] *Departamento de Bioquimica, Facultad de Medicina, Universidad Nacional Autonoma de México, UNAM, A.P. 70-159, México, 04510, D.F., México*
[c] *Departamento de Bioenergetica, Instituto de Fisiologia Celular, UNAM, México, México*

Received 18 April 1995; revised 14 March 1996; accepted 26 March 1996

Abstract

The effect of membrane cholesterol on the thermal inactivation of Ca^{2+}/Mg^{2+}-ATPase activity of bovine cardiac microsomes was measured and compared to the thermal denaturation profiles of the microsomes as measured by differential scanning calorimetry (DSC). Inactivation, defined as loss of activity, and denaturation, defined as conformational unfolding, were irreversible under the conditions used. Both thermal inactivation of Ca^{2+}/Mg^{2+}-ATPase activity and thermal denaturation were shifted to higher temperatures in microsomes enriched with cholesterol (37 ± 5 μg cholesterol/mg protein, cholesterol/phospholipid molar ratio 0.31) compared to control microsomes (15 ± 3 μg cholesterol/mg protein, molar ratio 0.12). Thermal inactivation was measured by two methods: first, measuring activity at room temperature as a function of heating to elevated temperatures at 1 K/min, where inactivation temperatures (T_I, temperature of half activity) were 58.9 ± 0.3°C for control membranes and 59.9 ± 0.1°C for cholesterol-enriched membranes, respectively. Second, measuring ATPase activity as a function of time at constant temperature, where T_I values of 57.6 ± 0.5°C and 59.2 ± 0.5°C were determined for control and cholesterol-enriched membranes, respectively. DSC profiles of microsomal membranes consisting of a number of overlapping peaks were obtained. A well resolved component (transition C) was observed with a transition temperature ($T_{1/2}$) of 58.2°C. This $T_{1/2}$, which is a measure of conformational stability, correlates with the T_I for Ca^{2+}/Mg^{2+}-ATPase activity and is 1.9 ± 0.6 K higher in cholesterol-enriched membranes. Thus, the increased resistance to inactivation appears to be due to increased conformational stability of the protein induced by cholesterol, demonstrating that a change in lipid composition can influence the stability of an integral membrane protein in a natural membrane. The increased stability is of sufficient magnitude to account for the previously observed correlation between cholesterol content and resistance to heat shock in several cell lines.

Keywords: Membrane protein thermostability; Cholesterol; ATPase, Ca^{2+}/Mg^{2+}-; DSC; Heat shock

1. Introduction

The properties and effects of cholesterol on the physical behavior of membrane lipids has been extensively documented. Cholesterol decreases the enthalpy of transition between the gel and liquid crystalline phases [1,2], increases the orientational order of the acyl chains of fatty acids [3,4], and changes membrane thickness [5]. Bloom and Mouritsen [6] have proposed that integral membrane proteins operate in an optimal manner in membranes whose natural hydrophobic thickness, which is influenced by cholesterol, matches the hydrophobic thickness of the protein. Cholesterol apparently is required at a higher concentration in some membrane systems, such as the t-tubule membrane of skeletal muscle, where cholesterol/phospholipid ratios of 0.5 [7] to 0.9 [8] have been measured.

There is evidence that the cholesterol level of cellular membranes is a factor influencing the sensitivity of cells to heat shock. For a number of cells there is a linear relationship between cholesterol content and resistance to heat

Abbreviations: ADP, adenosine diphosphate; ATP, adenosine triphosphate; ATPase, adenosine triphosphatase; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; NADH, nicotinamide adenine dinucleotide (reduced form); DSC, differential scanning calorimetry; E_A, activation energy; T_I, inactivation temperature; $T_{1/2}$, transition temperature; Tris, tris(hydroxymethyl)aminomethane.

* Corresponding author. Fax: +1 519 7468115.
[1] Present address: Departamento de Bioquimica, Facultad de Medicina, Universidad Nacional Autonoma de México, UNAM, A.P. 70-159, México, 04510, D.F., México.

PII S0005-2736(96)00072-7

shock [9,10]. CHO cells appear to be able to express a limited degree of homeoviscous adaptation by altering cholesterol levels, with those cells having a higher ratio of cholesterol to phospholipids more resistant to heat stress [11]. A potential explanation for these observations is that cholesterol influences the thermal stability of some integral membrane proteins.

In the present work, we used thermal analysis and differential scanning calorimetry (DSC) to determine the influence of varying concentrations of cholesterol on the thermal stability of the plasma membrane Ca^{2+}/Mg^{2+}-ATPase present in isolated cardiac microsomes [12] as a measure of lipid–protein interactions.

2. Materials and methods

2.1. Chemicals

ADP, ATP, cholesterol, DTT, EDTA, EGTA, Hepes, L-malic acid, Mops, NADH and Tris were purchased from Sigma (St. Louis, MO). All other chemicals were analytical grade.

2.2. Cardiac microsome isolation

Cardiac microsomes were obtained from bovine heart. The heart was cleaned of connective tissue and the ventricles minced. The minced tissue was homogenized in 5 volumes of 10 mM sodium bicarbonate, 0.5 mM sodium azide (pH 7.0) with an Ultraturrax for five cycles of 5 s each. The homogenate was filtered through four layers of gauze and centrifuged at $8700 \times g$ for 30 min. The pellet was rehomogenized and the above steps were repeated twice. The supernatants obtained were collected and centrifuged at $22\,000 \times g$ for 60 min. The resulting pellet was resuspended and homogenized in 5 volumes of 20 mM Tris-malate, 0.6 M KCl (pH 6.8) and centrifuged at $33\,000 \times g$ for 60 min. The pellet was then resuspended in 10 mM Hepes-KOH EDTA (pH 7.4), stirred for 10 min at 4°C, and centrifuged at $50\,000 \times g$ for 30 min. The final pellet was saved and resuspended in 50 mM Tris-malate, 0.5 mM $MgCl_2$, 0.05 mM $CaCl_2$, and 2 mM DTT (pH 7.4). The resuspended membranes were rapidly frozen and stored in liquid nitrogen. This membrane fraction is highly enriched in Ca^{2+}/Mg^{2+}-ATPase activity.

2.3. Cholesterol enrichment of cardiac microsomes

Changes in the concentration of cholesterol in cardiac microsomes were achieved by incubating the membranes in a plasma medium where cholesterol exchange between plasma lipoproteins and biological membranes occurs as was done previously for cardiac sarcolemma [13]. Briefly, approximately 10 ml of blood were centrifuged, and the resulting blood plasma heated for 30 min at 56°C. The inactivated plasma was diluted 1:10 in 10 mM NaH_2PO_4 (pH 7.1) and further diluted 10:1 with 10 ml of NaH_2PO_4 containing 30 mg of cholesterol previously dissolved in 1 ml of 2% DMSO. Plasma without cholesterol was prepared by the above procedure for use as a control. The cardiac microsomes were incubated with the plasma media for 5 h at 25°C, followed by three washes with a solution containing 50 mM Tris-malate, 0.5 mM $MgCl_2$, 0.05 mM $CaCl_2$ and 2 mM DTT (pH 7.4). The microsomes were finally resuspended in 20 mM Mops and 0.1 M KCl (pH 7.0). The cholesterol concentration of cardiac microsomes was determined by a colorimetric method provided by Biochemical Analysis (Boehringer Mannhein).

2.4. Thermal inactivation

Isolated cardiac microsomes at 1.5–2 mg/ml were heated in 20 mM Mops and 0.1 M KCl (pH 7.0) in a water bath. The temperature of the bath was increased so that the temperature of the microsome suspension increased at a heating rate of 1 K/min. Samples were removed at time intervals of one minute from 30°C to 70°C and placed on ice. The Ca^{2+}/Mg^{2+}-ATPase activity was then determined at room temperature by the coupled enzyme assay described by McClure [14]. An inactivation curve was generated by plotting enzymatic activity as a fraction of control activity (no heating) vs. temperature. The inactivation temperature (T_I) is defined as the temperature of half inactivation. The first derivative of the inactivation curve was calculated and compared to the apparent excess specific heat (C_p) profiles of cardiac microsomes obtained by DSC. For all inactivation experiments, the derivative of the inactivation curves was not symmetrical; therefore, T_I does not correspond to the peak temperature but to the temperature of half inactivation.

Inactivation was also determined by measuring activity as a function of time at constant temperature from 51 to 60°C. The rate constants for inactivation were determined from plots of ln(activity) vs. time, and Arrhenius plots were constructed from which the activation energy (E_A) and intercept (A) were calculated. Fractional inactivation or denaturation (f_D) as a function of temperature increased at 1 K/min. was calculated using the method described in Ref. [15].

2.5. Differential scanning calorimetry

Calorimetric experiments were performed with a high resolution Microcal MC2 differential scanning calorimeter (DSC) interfaced to a DEC 380 computer with an automatic data collection system as previously described [15]. For DSC experiments, cardiac membranes were suspended in the same solution used for thermal inactivation. An initial DSC scan of cardiac membranes (10 mg/ml protein) was obtained at a scan rate of 1 K/min from 10 to 100°C. The sample was immediately cooled to 10°C fol-

lowed by a rescan to 100°C. Denaturation was completely irreversible after scanning to 100°C. Scans were also made to 49, 56, 63, and 70°C to check for partial reversibility. The intrinsic baseline curvature was corrected by subtracting the rescan, and the shift in specific heat on denaturation (ΔC_p) was corrected as previously described [15]. The transition temperature (T_m) of each peak is defined as the temperature at which the apparent C_p reaches a maximum. The DSC scans were deconvoluted assuming irreversible denaturation as previously described [15].

3. Results

3.1. Thermal inactivation of the Ca^{2+}/Mg^{2+}-ATPase from cardiac membranes

Using the in vitro incorporation procedure, the cholesterol concentration of microsomal membranes was increased from 15 ± 3 μg (n = 10) for control membranes to 37 ± 5 μg (n = 10) cholesterol/mg protein for enriched membranes which correspond to cholesterol/phospholipid molar ratios of 0.12 and 0.31, respectively. The thermal stability of the Ca^{2+}/Mg^{2+}-ATPase of microsomal membranes with these two cholesterol concentrations was determined by thermal inactivation and thermal denaturation.

For thermal inactivation utilizing the first method, the Ca^{2+}/Mg^{2+}-ATPase activity in control membranes (C) and membranes enriched (E) with cholesterol was measured at room temperature after heating, as a function of temperature increased at a rate of 1 K/min. These results

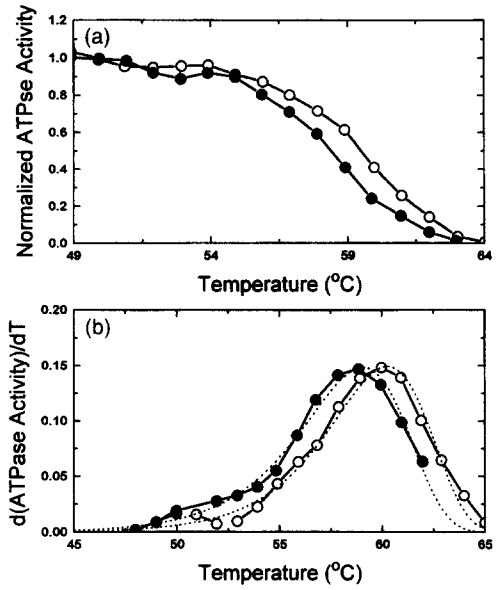

Fig. 1. Thermal inactivation of the Ca^{2+}/Mg^{2+}-ATPase from cardiac microsomes. (A) ATPase activity at room temperature after heating to temperatures of 49 to 64°C is shown for control (●) and cholesterol-enriched membranes (○). (b) The first derivative of the data shown in Fig. 1a (solid lines) and the best fit theoretical curves (dotted lines).

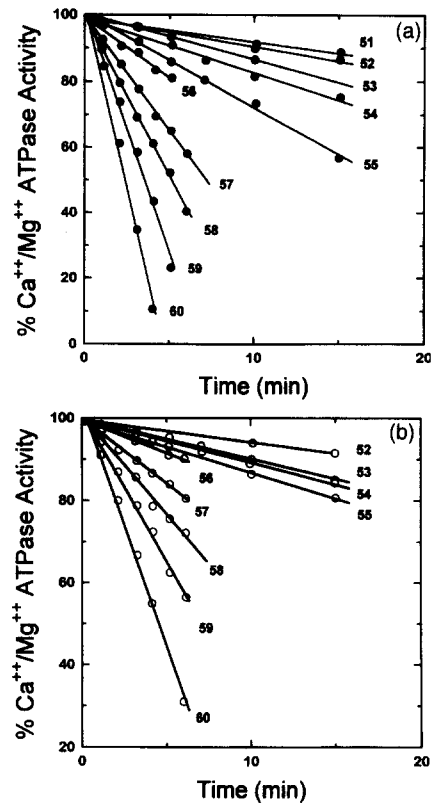

Fig. 2. Thermal inactivation of Ca^{2+}/Mg^{2+}-ATPase activity as a function of time at constant temperatures of 51–60°C for control (a) and cardiac microsomes enriched in cholesterol (b).

are presented in Fig. 1a. Activity is lost in membranes following heating to temperatures of 55–60°C. The inactivation temperature (T_I) was determined from the first derivative of the curves given in Fig. 1a which was fit with the best theoretical curve [15]. The theoretical fits for irreversible inactivation were calculated assuming the presence of one component. The first derivative curves for two-state inactivation

$$N \xrightarrow{k} D$$

are skewed towards the low temperature side [16]. The experimental curves in Fig. 1b are of this form and are fit well by the theoretical curves. This supports the use of the two-state model for inactivation given above. The activation energy for the rate constant k was obtained directly from the fit. Control membranes have a T_I of 58.9 ± 0.3°C and an E_A = 374 kJ/mol. In contrast, membranes enriched in cholesterol have a higher T_I of 59.9 ± 0.1°C and E_A not significantly different from that for control membranes. These results are the average of eight experiments. A 1.0 ± 0.3°C difference in T_I appears small but corresponds to a two-fold difference in the rate of inactivation following heating to 60°C.

The T_I was also calculated by measuring inactivation as a function of time at constant temperature (Fig. 2), generating Arrhenius plots, and using E_A and A to calculate T_I as

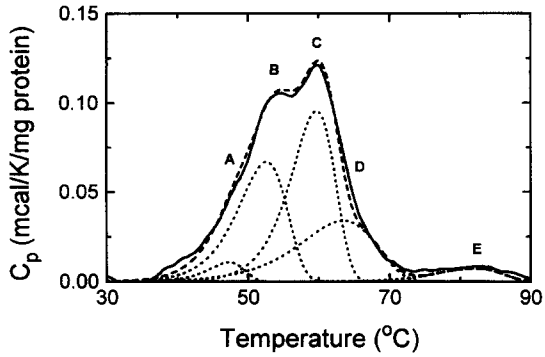

Fig. 3. Differential scanning calorimetry profile (C_p[exess] vs. temperature, scan rate 1 K/min) of control cardiac microsomes (solid line) and the best theoretical fit (dashed line) which is the summation of the five independent components (labeled A–E, dotted lines) obtained by deconvolution of the original scan. This scan and deconvolution is representative of six experiments.

previously described [15]. This method gave comparable results with a T_1(control) = 57.6 ± 0.5°C and T_1(cholesterol enriched) = 59.2 ± 0.5°C for a ΔT_1 = 1.6 ± 0.7 K.

3.2. Thermal denaturation of cardiac microsomal membranes

Two questions arise from these results: (1) Is thermal inactivation of ATPase activity due to denaturation of the Ca^{2+}/Mg^{2+}-ATPase as is true for the Ca^{2+}-ATPase from sarcoplasmic reticulum [15]? (2) Is the increased thermal stability in cholesterol-enriched membranes due to increased conformational stability of the enzyme or simply to a change in the reversibility of unfolding?

To investigate if the T_1 values obtained for the thermal inactivation of Ca^{2+}/Mg^{2+}-ATPase activity corresponds to the thermal denaturation of a specific membrane component in cardiac microsomes with low and high cholesterol content, DSC profiles were obtained from control and cholesterol-enriched microsomes which are shown in Figs.

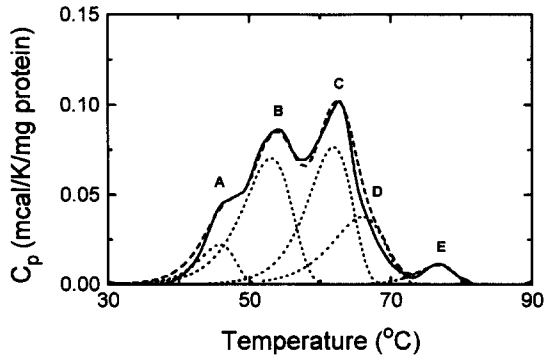

Fig. 4. Differential scanning calorimetry profile (C_p[excess] vs. temperature, scan rate 1 K/min) of cardiac microsomes enriched in cholesterol (solid line) and the best theoretical fit (dashed line) which is the summation of the five independent components (labeled A–E, dotted lines) obtained by deconvolution of the original scan. This scan and deconvolution is representative of four experiments.

Table 1
Transition temperatures ($T_{1/2}$) of the components of control and cholesterol-enriched cardiac microsomes

Component	$T_{1/2}$ (mean ± S.E.) (°C)	
	control	enriched
A	45.9 ± 0.4	45.3 ± 0.2
B	51.5 ± 0.2	53.0 ± 0.4
C	58.2 ± 0.3	60.1 ± 0.5
D	64.3 ± 0.1	64.5 ± 0.4
E	77.8 ± 0.4	75.2 ± 0.7

S.E., standard error from six and four experiments for control and enriched membranes, respectively.

3 and 4, respectively. Both profiles have similar shape and consist of at least two strong peaks with peak transition temperatures ($T_{1/2}$) of about 54 and 60°C (peaks B and C). In addition, in most but not all scans a much weaker peak at 75–85°C and a partially resolved peak between 40 and 50°C are observed (peaks E and A). In order to get a good fit, another component at approximately 65°C (peak D) is required. Thus, the DSC profiles in Figs. 3 and 4 were deconvoluted assuming five components or transitions. The transition temperature of the components, defined as the temperature of half denaturation ($T_{1/2}$), not the peak temperature, are given in Table 1.

In order to make a direct comparison between conformational denaturation measured by DSC and inactivation, denaturation must be highly irreversible and obey the irreversible model

$$N \xrightarrow{k} D$$

Partial scans to 49, 56, 63, and 70°C for both control and cholesterol-enriched membranes demonstrate almost complete irreversibility of protein denaturation below these temperatures when scanning to each temperature (results not shown), supporting the use of the irreversible model for denaturation and validating a direct comparison between inactivation and denaturation.

A comparison of the $T_{1/2}$ values for control and enriched membranes (Table 1) shows that $T_{1/2}$ for the two well-resolved transitions B and C is increased in enriched membranes. Transition C ($T_{1/2}$ = 58.2°C) corresponds closely to the inactivation temperature of Ca^{2+}/Mg^{2+}-ATPase activity (T_1 = 57.6–58.9°C in control membranes). The $T_{1/2}$ of transition C is increased to 60.1°C in cholesterol-enriched membranes. This increase of 1.9 ± 0.6°C is close to the increase of 1.0–1.3 K for T_1. Thus, inactivation is due to denaturation of component C which is stabilized by cholesterol.

4. Discussion

As shown in this study, a high concentration of cholesterol in cardiac microsomes reduces the rate of thermal

inactivation of the Ca^{2+}/Mg^{2+}-ATPase. Membrane cholesterol also increases the thermal stability of the ATP hydrolytic function of other membrane ATPases including the Ca^{2+}-ATPase of sarcoplasmic reticulum [17] and cardiac sarcolemma [18]. The ATPase hydrolytic function of the Ca^{2+}-ATPase is located in a protein domain outside the membrane core in a hydrophilic environment [19,20] while cholesterol is present in the lipid bilayer which contacts only the transmembrane region. Since ATPase activity is affected by cholesterol [4,21–23], this must occur through the influence of cholesterol on the transmembrane region of the protein.

Inactivation must result from an irreversible alteration of the protein. The simplest model for inactivation is

$$N \xrightarrow{k} D$$

where N and D are the native and inactive forms, respectively, and k is the temperature dependent rate constant for this pseudo first order process. This two-state model is entirely consistent with the inactivation results; however, if the conformational unfolding of the protein is to be considered explicitly, then the two-state model must be extended to

$$N \underset{k_2}{\overset{k_1}{\rightleftharpoons}} U \xrightarrow{k_3} D$$

where U is the thermally unfolded state and the k_i values are the rate constants for the various steps. The two-state model represents an appropriate simplification if U fails to accumulate to significant amounts (i.e. $k_3 \gtrsim k_1$, k_2 [16]). Inactivation is due to a step subsequent to unfolding in the three-state model which makes refolding impossible. Since unfolding must precede inactivation, decreased inactivation can occur through two mechanisms: an increase in conformational stability (i.e. an increase in the transition temperature of unfolding) or a decrease in the rate constant of inactivation k_3 without any change in conformational stability. The latter possibility has been shown to occur for human and bovine superoxide dismutase where replacement of the free cys residues results in an enzyme with decreased conformational stability but increased resistance to thermal inactivation, which can only be explained by a large decrease in k_3 [24].

The DSC scans of control and cholesterol-enriched membranes have a peak at the temperature predicted from inactivation ($T_{1/2} \approx 59$°C) which is shifted 1.9 ± 0.6 K higher in the cholesterol-enriched membranes. This shift is highly significant ($P < 0.01$ by an unpaired t-test) and indicates that the increased conformational stability of the Ca^{2+}/Mg^{2+}-ATPase is sufficient to account for the increased resistance to inactivation. There is no evidence that the step or process leading to irreversibility is influenced by cholesterol.

What is the mechanism by which cholesterol increases the conformational stability of the Ca^{2+}/Mg^{2+}-ATPase? This cannot be answered with certainty, but there are several possibilities. Cholesterol (at 43 mol%) has been shown to increase the transition temperature of the erythrocyte band 3 protein in reconstituted dioleylphosphatidylcholine vesicles by 6 K [25]. In addition, stability increased as the chain length of the phospholipids comprising the lipid bilayer increased. The T_m of the band 3 protein increased from 47 to 65°C as the fatty acyl chain length of monounsaturated, symmetric phosphatidylcholines increased from 14 to 24. Since cholesterol is known to increase the thickness of the lipid bilayer [5], this led to the suggestion that cholesterol might stabilize by a similar mechanism [25]. However, natural membranes differ from one-component lipid bilayers in that a variety of lipids are present. Thus, one must consider that any overall change in bilayer thickness might be minimized by preferential binding of specific lipid components to the Ca^{2+}/Mg^{2+}-ATPase.

Another mechanism of stabilization follows from the effect of cholesterol on the order and packing characteristics of unsaturated bilayer lipids. Any conformational change in an intrinsic membrane protein, of which denaturation is an extreme example, requires a compensatory reorganization of the lipid bilayer. The fractional volume (f_v), which is a bulk lipid bilayer parameter related to the acyl chain packing free volume, characterizes the volume available for membrane conformational changes that occur with a positive volume change [26]. A greater degree of lipid acyl saturation, lower temperature, and increased cholesterol decrease f_v and reduce the likelihood of membrane protein conformational changes [27]. By similar reasoning, a decrease in f_v caused by increased cholesterol should decrease the probability of unfolding with a resulting increase in T_m.

The increase in stability of the Ca^{2+}/Mg^{2+}-ATPase has direct physiological relevance. Numerous factors, including cholesterol, influence the response of cells to heat shock. The rates of killing for the rodent cells studied by Cress and Gerner [9] differ by about a factor of three for a less than a two-fold change in cholesterol content. A factor of three change in the rate of killing corresponds to a 1–2 K shift in inactivation temperature at the high activation energies for the thermal killing of mammalian cells (400–600 kJ/mol). For the Ca^{2+}/Mg^{2+}-ATPase, we found a 1.0–1.6 K increase in inactivation temperature (T_I) and a 1.9 K increase in denaturation temperature ($T_{1/2}$) for a 2.5-fold increase in cholesterol content. Thus, the magnitude of the shift in T_I and $T_{1/2}$ is sufficient to account for the increased resistance to heat shock of cells with greater cholesterol content and indicates that a relatively small change in thermal stability has physiological relevance. Although the inactivation temperature of the Ca^{2+}/Mg^{2+}-ATPase is too high for this protein to be a critical target for heat killing, these results illustrate that proteins in a natural membrane can be stabilized by increasing cholesterol content.

Acknowledgements

Partial financial support was obtained from DGAPA, National University of Mexico (Grants IN-213495 and IN-209492), CONACYT (Grant ICSAXNA1872) and USPHS Grant CA40251 awarded by the National Cancer Institute.

References

[1] Cullis, P.R., Van Dijck, P.W.M., De Kruijff, B. and De Gier, J. (1978) Biochim. Biophys. Acta 513, 21–30.
[2] Estep, T.N., Mountcastle, D.B., Biltonen, P.L. and Thompson, T.E. (1978) Biochemistry 17, 1984–1989.
[3] Presti, F.T. (1985) in Membrane Fluidity in Biology, Vol. 4 (Aloia, R.C. and Boggs, J.M., ed.), pp. 97–146, Academic Press, Orlando FL.
[4] Yeagle, P.L. (1985) Biochim. Biophys. Acta 822, 267–287.
[5] McIntosh, T.J. (1978) Biochim. Biophys. Acta 513, 43–58.
[6] Bloom, M. and Mouritsen, O.G. (1988) Can. J. Chem. 66, 706–712.
[7] Rosemblatt, M., Vergara, C., Hidalgo, C. and Ikemoto, N. (1981) J. Biol. Chem. 256, 8140–8148.
[8] Sabbadini, R. and Dhams, S. (1989) J. Bioenerg. Biomembr. 21, 163–212.
[9] Cress, A.E. and Gerner, E.W. (1980) Nature (London) 283, 677–679.
[10] Anderson, R.L., Lunec, J. and Cresswell, S.R. (1985) Int. J. Hyperthermia 1, 337–347.
[11] Anderson, R.L., Minton, K.W., Li, G.C. and Hahn, G.M. (1981) Biochim. Biophys. Acta 641, 334–348.
[12] Zao, D., Makinu, N. and Dhalla, N.S. (1988) Arch. Biochem. Biophys. 268, 40–48.
[13] Ortega, A. and Mas-Oliva, J. (1984) Biochim. Biophys. Acta 773, 231–236.
[14] McClure, W.R. (1969) Biochemistry 8, 2782–2786.
[15] Lepock, J.R., Rodahl, A.M., Zhang, C., Heynen, M.L., Waters, B. and Cheng, K.-H. (1990) Biochemistry 29, 681–689.
[16] Lepock, J.R., Ritchie, K.P., Kolios, M.C., Rodahl, A.M., Heinz, K.A., and Kruuv, J. (1992) Biochemistry 31, 12706–12712.
[17] Cheng, K.-H., Hui, S.W. and Lepock, J.R. (1987) Cancer Res. 47, 1255–1261.
[18] Mas-Oliva, J. and Santiago-Garcia, J. (1990) Biochem. Int. 21, 233–241.
[19] MacLennan, D.H., Brandl, C.J., Korzcak, B., and Green, N.M. (1985) Nature (London) 316, 696–700.
[20] Serrano, R. (1988) Biochim. Biophys. Acta 947, 1–28.
[21] Ortega, A. and Mas-Oliva, J. (1986) Biochem. Biophys. Res. Commun. 139, 868–874.
[22] Warren, G.B., Housley, M.D., Metcalfe, J.C. and Birsau, N.J.M. (1975) Nature (London) 255, 684–687.
[23] Cheng, K.H., Lepock, J.R., Hui, S.W. and Yeagle, P.L. (1986) J. Biol. Chem. 261, 5081–5087.
[24] Lepock, J.R., Frey, H.E. and Hallewell, R.A. (1990) J. Biol. Chem. 265, 21612–21618.
[25] Maneri, L.R. and Low, P.S. (1988) J. Biol. Chem. 263, 16170–16178.
[26] Straume, M. and Litman, B.J. (1988) Biochemistry 27, 7723–7733.
[27] Mitchell, D.C., Straume, M., Miller, J.L. and Litman, B.J. (1990) Biochemistry 29, 9143–9149.