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Plasma membrane rafts of rainbow trout are subject to thermal acclimation

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Summary

Rafts are cholesterol- and sphingolipid-enriched microdomains of the plasma membrane (PM) that organize many signal transduction pathways. Interactions between cholesterol and saturated lipids lead to patches of liquid-ordered membrane (rafts) phase-separating from the remaining PM. Phase behavior is temperature sensitive, and acute changes in temperature experienced by poikilotherms would be expected to perturb raft structure, necessitating an acclimatory response. Therefore, with thermal acclimation, we would expect compositional changes in the raft directed to offset this perturbation. Using differential and density gradient centrifugation, we separated PM from the livers of rainbow trout acclimated to 5°C and 20°C into raft-enriched (raft) and raft-depleted PM (RDPM). Compared with RDPM, the raft fractions were enriched in cholesterol, the β_2-adrenergic receptor and adenylyl cyclase, which are commonly used markers for this microdomain. Furthermore, cholesterol was enriched in all fractions from warm- compared with cold-acclimated animals, but this increase was 3.4 times greater in raft than in PM. We developed a novel approach for measuring membrane molecular interaction strength (and thus the tendency to stabilize raft structure) based on the susceptibility of membranes to detergent. Specifically, studies with model vesicles demonstrated that the capacity of a membrane to accommodate detergent prior to solubilization (saturation point) was a good index of this property. The saturation point of the isolated membrane preparations was temperature sensitive and was significantly different in 5°C- and 20°C-acclimated RDPM when assayed at 5°C and 20°C, respectively. By contrast, this comparison in rafts was not significantly different, suggesting compensation of this property. These data suggest that compositional changes made in the PM during thermal acclimation act to offset thermal perturbation of the raft but not the RDPM structural integrity.

Key words: raft, plasma membrane, raft-depleted plasma membrane, temperature, cholesterol, detergent, thermal acclimation, rainbow trout, *Oncorhynchus mykiss*.

Introduction

During thermal acclimation, poikilotherms must make substantial biochemical changes to their plasma membranes (PMs) to preserve physiological function. These changes include increases in PM cholesterol concentration and acyl chain saturation with acclimation or adaptation to elevated temperatures (Hazel et al., 1991). The maintenance of membrane order/fluidity (homeoviscous adaptation) has classically been invoked to rationalize these modifications (Sinensky, 1974). However, these compositional adjustments would also be expected to stabilize the structure of rafts, microdomains of the PM implicated in organizing diverse signaling pathways (Brown and London, 1998). Therefore, we have investigated the possibility that the thermal acclimatory response of the PM is directed to maintain raft structure.

Favorable interactions among cholesterol and the saturated hydrocarbon chains of lipids, especially sphingomyelin, result in patches of liquid-ordered (L_o) phase membrane (raft) separating from the remaining liquid-disordered (L_d) membrane (Ahmed et al., 1997). The L_o phase is characterized by a highly ordered hydrophobic region compared with the L_d phase (Brown and London, 1998). Specific proteins are targeted to, and concentrated in, rafts based on the greater solubility of their lipid anchors in L_o over L_d phase membrane (Wang et al., 2000). This localization is clearly important since the dissolution of rafts, by stripping the membrane of cholesterol, results in the loss of function of the signaling proteins normally localized there (Pike and Miller, 1998). Raft structure is dependent on lipid phase behavior (Brown and London, 1998), making it temperature sensitive. Therefore, acute increases in temperature could result in raft dissolution while acute decreases could result in inappropriate accretion of membrane components. The resulting disruption of signaling activities would necessitate a homeostatic response to stabilize raft structure and restore appropriate signaling.

The goal of this study was to determine if raft structural integrity is defended during thermal acclimation in the vertebrate poikilotherm rainbow trout (*Oncorhynchus mykiss*). We measured compositional changes in total PM as well as in

the raft and raft-depleted PM (RDPM) sub-domains of hepatocytes from rainbow trout acclimated to 5°C and 20°C. Thermally induced adjustments in membrane lipid composition were greater in raft compared with PM. In addition, we developed a novel approach for measuring membrane molecular interaction strength (and thus the tendency to stabilize raft structure) based on the susceptibility of membranes to detergent. We present evidence that molecular interaction strength is regulated in raft but not in RDPM. These data provide the first evidence for the homeostatic regulation of raft structure and suggest the need to re-examine membrane acclimation from the perspective of a spatially heterogeneous PM.

Materials and methods

Materials

Cholesterol oxidase was purchased from Calbiochem (San Diego, CA, USA). All primary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA) except anti-caveolin, which was purchased from BD Biosciences (San Jose, CA, USA). Goat anti-rabbit peroxidase conjugated secondary antibody was purchased from BioRad (Hercules, CA, USA). Super Signal Enhanced chemiluminescent substrate was purchased from Pierce (Rockford, IL, USA). DNase was type II from Sigma (St Louis, MO, USA). Synthetic lipids were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Other biochemicals were from Sigma, and all other chemicals were of analytical grade.

Animals

Rainbow trout (*Oncorhynchus mykiss* Walbaum) were obtained from the Alchesay National Fish Hatchery in Whiteriver, AZ, USA and were maintained at the Animal Resource Center of Arizona State University. Fish were housed in recirculating freshwater aquaculture systems consisting of circular fiberglass tanks; water temperatures were controlled using flow-through chillers. Animals were acclimated to 5°C or 20°C for at least three weeks before use in experiments. Fish were held under a constant 12 h:12 h L:D cycle and were fed trout food (Rangen Inc., Buhl, ID, USA) to satiation daily.

Plasma and raft membrane isolations

Plasma membranes (PM) were isolated from approximately 14 g of liver according to a modification of the procedure of Armstrong and Newman (1985), as described previously (Hazel et al., 1992). The PM was resuspended in working buffer (WB; 0.25 mol l^{-1} sucrose, 20 mmol l^{-1} tricine, pH 7.8, 1 mmol l^{-1} EDTA) and was separated into raft-depleted PM (RDPM) and raft-enriched PM (raft) using a non-detergent-based method (Smart et al., 1995). The membrane fractions were separated based on the lighter buoyant density of raft, compared with RDPM. Briefly, the PM was sonicated and then adjusted to 23% OptiPrep before layering a 10–20% OptiPrep gradient on top (for a total of 11 ml in each tube). After centrifuging for 90 min at 72 800 ***g*** in a Beckman SW 41-Ti rotor (OP1), the top 5.5 ml (raft) was removed, mixed with 4 ml of 50% OptiPrep in a fresh tube, capped with 250 μl of 5% OptiPrep and centrifuged for 90 min at 72 800 ***g*** (OP2). The raft membrane concentrated at the top of the OP2 tube was collected with a Pasteur pipette, diluted three times with buffered saline and centrifuged to a pellet for 20 min at 20 800 ***g*** in a refrigerated microcentrifuge (Eppendorf 5417 R). The bottom 5.5 ml from OP1 (RDPM) was diluted approximately four times with buffered saline and was centrifuged for 1 h at 23 700 ***g*** in a Beckman JA 30.50 rotor.

Protein, phosphate and cholesterol assays

Total protein was determined colorimetrically by the method of Bradford (1976). Phospholipid concentration was measured by colorimetric phosphate analysis of total lipid extracts after complete hydrolysis (Ames, 1966). An average molecular mass of 750 was assumed for conversion to mass. A coupled cholesterol oxidase fluorometric assay was used to measure cholesterol (Crockett and Hazel, 1995).

Western blots

Western blots were performed to determine the relative abundance of specific proteins in the fractions. Samples (30 μg of total homogenate protein and 15 μg of all other samples) were mixed with an equal volume of 2× Laemmli buffer [62.5 mmol l^{-1} Tris, pH 6.8, 25% glycerol, 2% sodium dodecyl sulfate (SDS), 5% β-mercaptoethanol], boiled for 3 min and subjected to SDS–polyacrylamide gel electrophoresis (12% gel for caveolin, 7.5% gel for all others). The resolved proteins were then transferred to 0.45-μm nitrocellulose membranes and blocked for 1 h in 5% non-fat dry milk (NFDM) in phosphate-buffered saline (PBS). The membranes were immunolabeled for 1 h in 5% NFDM/PBS with an anti-caveolin polyclonal antibody (pAb; 1:2000), anti-β_2 adrenergic receptor pAb (1:250), anti-adenylyl cyclase pAb (1:400), anti-insulin receptor β-subunit pAb (1:1600) or anti-clathrin pAb (1:250). The blots were washed three times in PBS with 0.1% Tween-20 for 5 min and once in PBS for 5 min. The blots were then incubated for 1 h in goat anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (1:12 000 for caveolin, 1:10 000 for all other westerns). The blots were then washed four times in PBS with 0.1% Tween-20 for 5 min, followed by detection using enhanced chemiluminesence and X-ray film. At least two blots were prepared for each experiment, and representative films are shown.

Stable plurilamellar vesicle preparation

Stable plurilamellar vesicles of synthetic lipids were prepared according to the method of Grüner et al. (1985) for use in detergent assays.

Detergent assays

Assay

Detergent assays were performed to measure the molecular interaction strength of membrane samples. Vesicles or biological samples (in 200 μl WB) were dispersed into 2.8 ml

assay buffer (150 mmol l^{-1} NaCl, 20 mmol l^{-1} Hepes, pH 7.4) and maintained in thermally controlled cuvettes. The quantities used for myristoylpalmitoyl phosphatidylcholine (MPPC) vesicles, palmitoyloleoyl phosphatidylcholine (POPC)/cholesterol vesicles and biological samples were 470 nmol of lipid, 270 nmol of lipid and 170 nmol of lipid, respectively. The turbidity of the suspension was measured continuously as the average optical density between 394 nm and 404 nm in a diode array spectrophotometer (8452A; Hewlett Packard, Palo Alto, CA, USA). Triton X-100 was added as serial additions of 12.5 μl and allowed to stabilize for 10–20 min (Fig. 1B). The final five data points before each new detergent addition were averaged as the effect of the previous detergent addition (e.g. Fig. 1B, arrow). The resulting optical density values were normalized as a percentage of the initial optical density and were plotted against detergent/lipid (D/L) ratios, calculated as detergent concentration (% v/v) divided by μmol total lipid (Fig. 1B, inset). The resulting curves were fit with a four-parameter sigmoidal equation using Sigma Plot 2000 (most r^2 values were 0.996 or better, with the lowest r^2=0.987).

Analysis

Detergent–membrane interactions proceed in two phases, both of which are sensitive to molecular interaction strength. Initially, detergent is incorporated into membrane structure until saturation, which is followed by solubilization as the membrane components are incorporated into mixed micelles (Fig. 1A; Lichtenberg et al., 1983). Values reflecting each of these processes were derived from the data. The D/L values corresponding to the peaks of the 2nd derivative of the fitted curve (Fig. 1C, inset, points *a* and *b*) define the onset and completion of the high slope region of the curve (Fig. 1C, points *a* and *b*). The D/L value corresponding to point *a* was chosen as an index of the maximum capacity of the membrane to accommodate detergent (saturation point; Fig. 1C). Further additions of detergent result in membrane dissolution. The slope of a linear regression of the fitted data between points *a* and *b* provides an index of the amount of detergent required for solubilization (solubilization slope; Fig. 1C).

Fourier transform infrared spectroscopy

Infrared spectroscopy was performed to estimate the gel–fluid melting temperature of MPPC vesicles. The MPPC vesicle sample was loaded between two $CaFl_2$ crystals with a 50.8 μm Teflon spacer and placed in a thermally controlled sample block. Using a Fourier transform infrared spectrometer

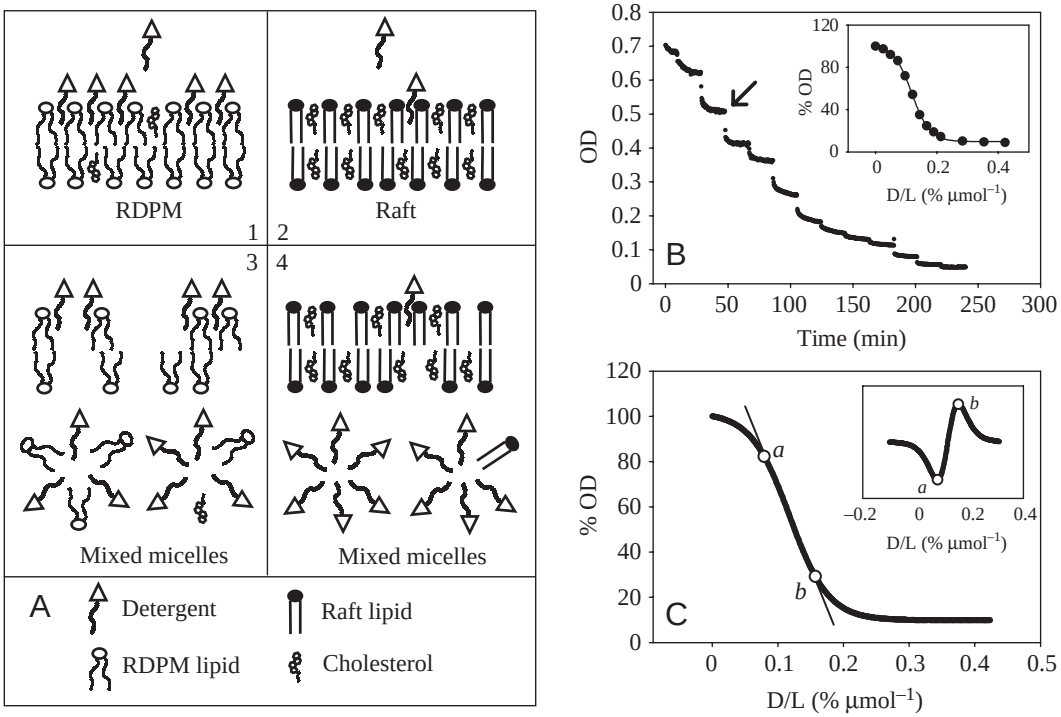

Fig. 1. Detergent analyses. (A) Diagrammatic representation of detergent–membrane interaction. Loosely packed raft-depleted plasma membrane (RDPM) accommodates more detergent prior to saturation (higher saturation point; plate 1) than tightly packed raft (plate 2). After saturation occurs, mixed micelles form, dissolving the membrane. Lipids in RDPM are more readily incorporated into micelles (steeper solubilization slope; plate 3) than are raft lipids (plate 4). (B) Detergent assay. Membrane dissolution is followed as changes in optical density (OD) over time. End points are recorded as optical density just prior to detergent addition (arrow). Inset: normalized end points as a function of detergent/lipid (D/L) ratios with sigmoidal regression. (C) Analysis. Sigmoidal regression with D/L values of 2nd-derivative peaks marked (points *a* and *b*) with the linear regression between points *a* and *b* superimposed. Inset: 2nd derivative with peaks marked (points *a* and *b*). Saturation point (i.e. where solubilization becomes dominant over detergent incorporation) is defined by point *a*. Solubilization slope is defined as the slope of the linear regression between points *a* and *b*.

(Spectrum 2000; Perkin Elmer, Shellon, CT, USA), the mean of 75 scans between the wavenumbers of 370 and 7800 was taken at 2°C intervals between 20°C and 50°C and were background subtracted. The instrument software package was used to find the peaks of the 2nd derivative, identifying the absorption peak corresponding to the methylene symmetric stretching frequencies. This frequency was plotted as a function of temperature. The points were fitted using TableCurve 2D 5.0 (Systat Software, Inc., Richmond, CA, USA), and the gel–fluid melting temperature was found as the peak of the 1st derivative.

Statistics

The protein, phospholipid and cholesterol compositional data were expressed as ratios (e.g. cholesterol/protein). The data were analyzed by two-way analysis of variance (ANOVA) with acclimation group as a factor with two levels (cold and warm) and membrane fraction as a factor with three levels (PM, RDPM and raft). *Post hoc* testing was performed using Tukey's test. The solubilization slopes and saturation points from the detergent experiments were each analyzed using three-way ANOVAs with acclimation group as factor with two levels (cold and warm), assay temperature as a factor with two levels (5°C and 20°C), and fraction as a factor with three levels (PM, RDPM and raft). Where appropriate (significant effect with no interaction), the data were collapsed across one or two factors for multiple comparisons using Tukey's test. The data were also analyzed separately by fraction (PM, RDPM and raft) by one-way ANOVA followed by Tukey's test. In all cases, a P value of ≤ 0.05 was accepted as indicating statistical significance.

Results

Membrane fraction composition

Most cells contain cholesterol- and sphingolipid-enriched microdomains in their plasma membranes. Many cell types, including adipocytes, myocytes and epithelial cells, contain caveolae, which are microdomains with a distinct flask-shaped morphology and a coat made from oligomers of the protein caveolin (Razani and Lisanti, 2001). In rainbow trout, caveolin is expressed in spleen and is abundant in adipose tissue but is absent from kidney and liver tissue (Fig. 2A). In cell types lacking caveolin (and caveolae), microdomains are generally referred to as lipid rafts.

Hepatocyte plasma membranes (PMs) isolated from rainbow trout acclimated to 5°C and 20°C were separated into raft-enriched (raft) and raft-depleted PM (RDPM) by sonication followed by density gradient centrifugation. Based on the yield of protein, cholesterol and phospholipid (estimated as phosphate), and an assumption that the membrane consisted of only these components, we estimated that the fractions taken as raft consisted of 39% and 32% (by mass), respectively, of the total membrane for the cold- and warm-acclimated animals.

Microdomains (rafts and caveolae) have previously been described in numerous systems to be enriched in proteins of many signal transduction cascades, including the β_2 adrenergic receptor (β_2AR; Xiang et al., 2002) and adenylyl cyclase (AC; Ostrom et al., 2001; Rybin et al., 2000). Therefore, we examined the distribution of β_2AR and AC in the crude homogenate, PM, RDPM and raft fractions. Both proteins were depleted in the RDPM fraction, compared with the PM, while they were substantially enriched in the raft fraction (Fig. 2B,C). The insulin receptor has been reported as absent from microdomains in the majority of the literature (Mastick et al., 1995; Ohira et al., 2000), but some researchers have found it to be microdomain associated (Vainio et al., 2002). In our system, the insulin receptor β subunit was depleted in the raft, compared with the PM or RDPM, fractions (Fig. 2D). Clathrin, a protein involved in receptor-mediated endocytosis, is not associated with rafts or caveolae (Razani and Lisanti, 2001). Surprisingly, we found clathrin to be evenly distributed among the PM, RDPM and raft fractions (Fig. 2E).

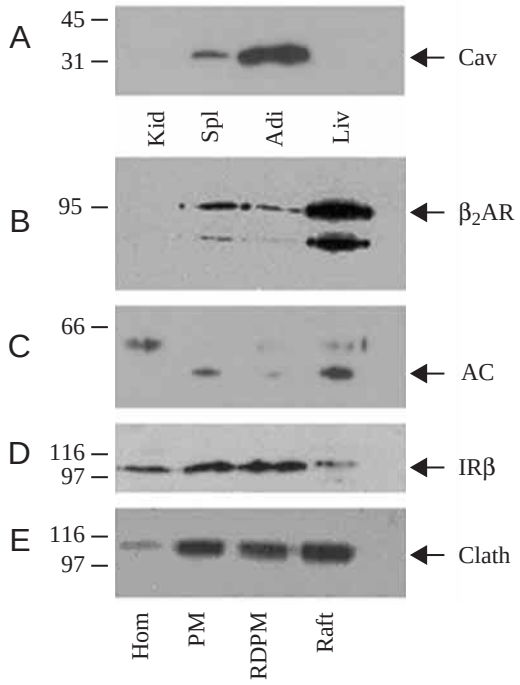

Fig. 2. Western blots. (A) Distribution of caveolin in trout kidney (Kid), spleen (Spl), adipose (Adi) and liver (Liv). All lanes, 15 μg total protein. (B–E) Samples loaded: 30 μg crude homogenate (Hom) and 15 μg plasma membrane (PM), raft-depleted PM (RDPM) and raft-enriched PM (raft). Probed with (B) antibodies against β_2 adrenergic receptor (β_2AR), (C) adenylyl cyclase (AC), (D) insulin receptor β subunit (IRβ) and (E) clathrin (Clath).

Acclimation temperature effects on composition

To further characterize the fractions, we measured the concentrations of cholesterol, phospholipid phosphate and protein in the fractions from cold- and warm-acclimated trout to assess compositional changes associated with thermal acclimation. The data were expressed as mass ratios (Fig. 3A–C) and as mass percentages (Table 1; the table was constructed with the assumption that the membrane consisted

Table 1. *Percentage composition of membrane fractions from cold- and warm-acclimated animals*

Animal	Membrane fraction	% Composition		
		Cholesterol	Phospholipid	Protein
Warm-acclimated	PM	9.5±0.4	23.1±1.1	67.4±0.8
	RDPM	10.0±0.4	23.0±1.5	67.0±1.3
	Raft	16.1±1.0	27.6±1.5	56.3±1.2
Cold-acclimated	PM	8.5±0.3	25.4±0.6	66.1±0.7
	RDPM	7.3±0.2	27.5±1.0	65.2±1.1
	Raft	11.5±1.3	35.9±3.3	52.7±2.2

Values are means ± S.E.M. (N=6) and are based on the assumption that cholesterol, phospholipid and protein content add up to 100% of the membrane.

PM, plasma membrane; RDPM, raft-depleted plasma membrane.

of only protein, phospholipid and cholesterol). Whereas the cholesterol/protein (Ch/Pr) ratios were similar in PM and RDPM in both acclimation groups, this ratio was significantly higher in raft fractions, being elevated 66% and 104% over PM in cold- and warm-acclimated fish, respectively (Fig. 3A). Furthermore, the Ch/Pr of rafts from warm-acclimated fish was significantly greater (P<0.05, N=6) than that from cold-acclimated fish by 33%.

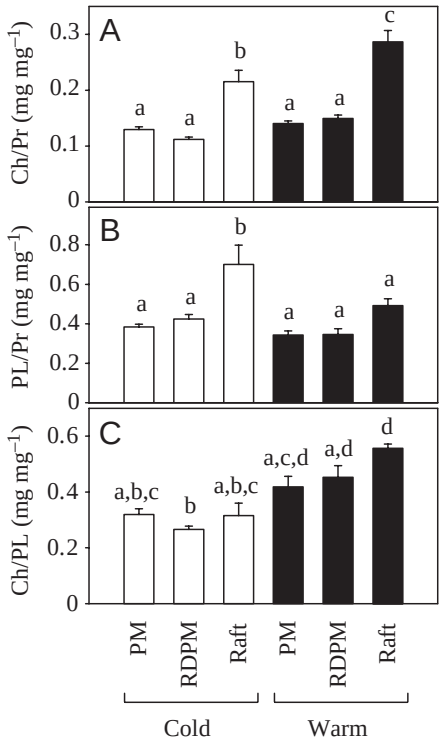

Fig. 3. Compositional analyses of membrane fractions. (A) Compositional comparisons of membrane fractions from cold-acclimated (open bars) and warm-acclimated (filled bars) fish expressed as ratios. (A) Cholesterol/protein (Ch/Pr). (B) Phospholipid/protein (PL/Pr). (C) Cholesterol/phospholipid (Chl/PL). Values are means ± S.E.M., N=6. Bars with a common lower-case letter do not differ significantly. PM, plasma membrane; RDPM, raft-depleted plasma membrane.

Rafts from cold-acclimated fish had significantly higher (P<0.05, N=6) phospholipid/protein (PL/Pr) ratios than all other fractions, which did not differ from one another (Fig. 3B). This represented a 65% increase over the RDPM from cold-acclimated fish. There was a non-significant 42% increase of PL/Pr in raft, compared with RDPM, from warm-acclimated fish.

The Ch/PL ratio was 32% greater in PM from warm- compared with cold-acclimated animals, while this increase was 63% in rafts (Fig. 3C). Furthermore, the increase in cholesterol concentration associated with acclimation to 20°C was 3.4 times greater in raft than in PM (Table 1).

Detergent assays – vesicles

We developed a novel approach for measuring membrane molecular interaction strength based on the susceptibility of membranes to detergent. We followed both the capacity of a membrane to incorporate detergent into the bilayer [saturation point (SP)] and the amount of detergent required to solubilize the membrane (solubilization slope). More tightly packed membranes were expected to saturate with less detergent (low SP) but to require more detergent to solubilize them (shallow solubilization slope) compared with loosely packed membranes (Fig. 1A; see Discussion).

We first analyzed several model vesicle preparations to evaluate the efficacy of this approach. Molecular interaction strength was manipulated by varying assay temperature of myristoylpalmitoyl phosphatidylcholine (MPPC) vesicles and cholesterol content of palmitoyloleoyl phosphatidylcholine (POPC) vesicles.

Fourier transform infrared spectroscopy indicated that MPPC vesicles melted from the tightly packed gel phase to the loosely packed fluid phase at 33°C (T_m; Fig. 4A, circles). SP values for MPPC decreased between 25°C and 30°C as T_m was approached, before increasing above T_m (Fig. 4A, bars). Substantially more detergent was required to reach saturation above the T_m, reflecting the looser packing of the fluid-phase lipids.

Vesicles of POPC alone or with 30 mol% cholesterol were analyzed at 25°C in the fluid phase. Cholesterol has a condensing effect on fluid phase membranes, causing

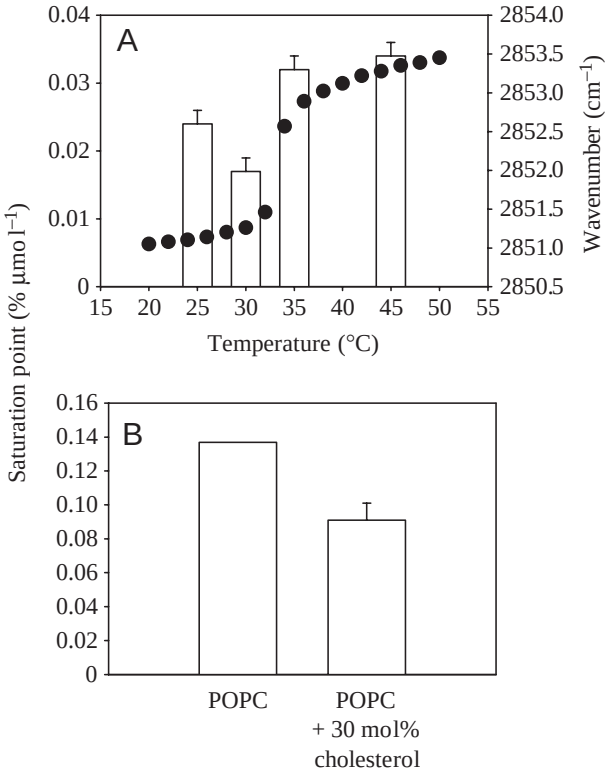

Fig. 4. Effects of temperature and cholesterol content on saturation point. (A) Symmetric methylene vibrational rates of myristoylpalmitoyl phosphatidylcholine (MPPC) vesicles as a function of temperature (circles). Saturation points of MPPC vesicles as a function of temperature (bars). Values are means ± range, *N*=2. (B) Saturation points of palmitoyloleoyl phosphatidylcholine (POPC) and POPC + 30 mol% cholesterol vesicles at 25℃. Values are means ± range, *N*=2.

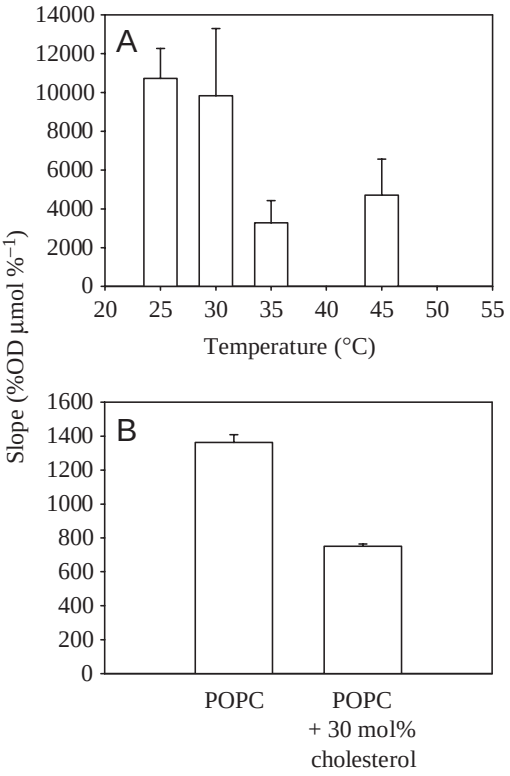

Fig. 5. Effects of temperature and cholesterol content on solubilization slope. Values are means ± range, *N*=2. Absolute values of slopes of near-linear regions between 2nd-derivative peaks of fitted sigmoidal curves for (A) myristoylpalmitoyl phosphatidylcholine (MPPC) vesicles as a function of temperature and (B) palmitoyloleoyl phosphatidylcholine (POPC) and POPC + 30 mol% cholesterol vesicles at 25℃.

membrane lipids to pack more tightly (Phillips, 1972). The addition of 30 mol% cholesterol to the POPC vesicles caused a substantial reduction in the SP, reflecting this tighter packing (Fig. 4B).

In analyzing the solubilization slopes produced from these same vesicle preparations, we expected membranes with tightly packed lipids to produce more shallow slopes (more detergent required to complete solubilization). Contrary to our prediction, solubilization slopes for MPPC vesicles were substantially steeper at temperatures below the T_m compared with those above the T_m (Fig. 5A). However, as expected, POPC vesicles with 30 mol% cholesterol had a more shallow solubilization slope than did POPC vesicles lacking cholesterol (Fig. 5B). These results suggest that SP is a more reliable indicator of molecular interaction strength than is solubilization slope.

Detergent assays – biological membranes

Saturation points

Representative detergent solubility curves of PM, RDPM and raft are presented in Fig. 6. Note that the RDPM curve has a more pronounced sigmoidal shape, reflecting a higher SP,

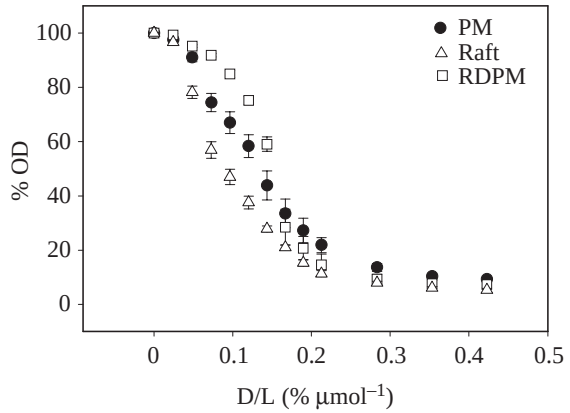

Fig. 6. Detergent solubility curves. Decline in normalized optical density (OD) as a function of detergent/lipid (D/L) ratios. Values are means ± S.E.M., *N*=4. Cold-acclimated samples assayed at 5°C (other data not shown). PM, plasma membrane; RDPM, raft-depleted plasma membrane.

compared with the more flattened curve produced by raft. The SPs for all samples were analyzed by three-way ANOVA (acclimation group × assay temperature × fraction). There was a significant effect of fraction and assay temperature, with no

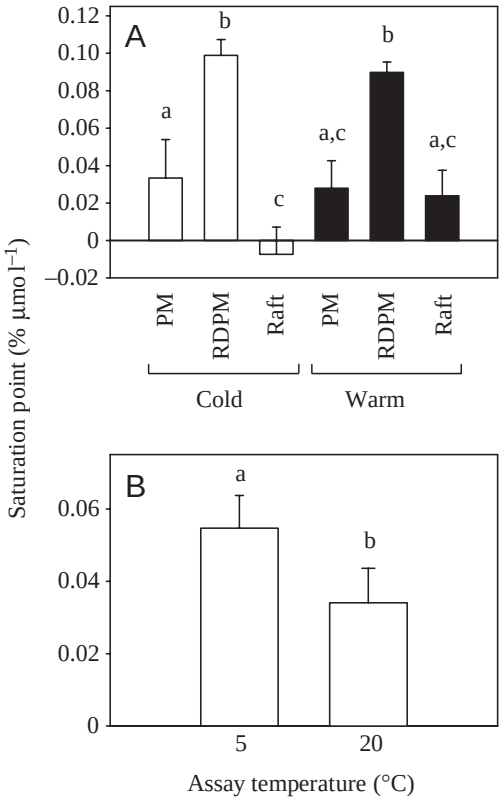

Fig. 7. Saturation points. Bars with a common lower-case letter do not differ significantly. (A) Effects of acclimation group and fraction (data collapsed across assay temperature). Cold-acclimated, open bars; warm-acclimated, filled bars. Values are means ± S.E.M., *N*=8. (B) Effects of assay temperature (data collapsed across fraction and acclimation group). Values are means ± S.E.M., *N*=24. PM, plasma membrane; RDPM, raft-depleted plasma membrane.

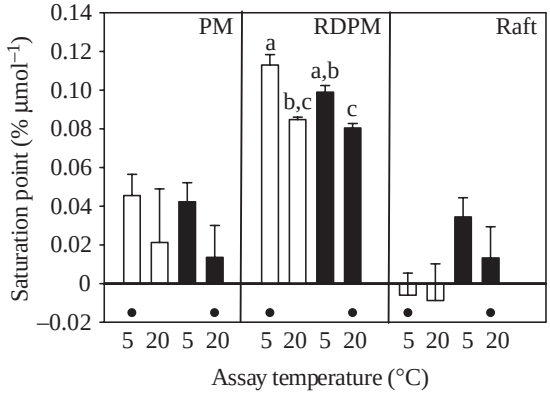

Fig. 8. Saturation points, all comparisons. Values are means ± S.E.M., *N*=4. Cold-acclimated, open bars; warm-acclimated, filled bars. Dots denote measurements made at physiological temperatures. PM, plasma membrane; RDPM, raft-depleted plasma membrane.

effect of acclimation group, and no significant interactions. However, the fraction × acclimation group interaction term was near significance (P=0.075, N=4). Therefore, the data were collapsed across assay temperature for comparisons of fractions from the two acclimation groups (Fig. 7A).

Within acclimation groups, the SPs of the RDPM samples were significantly greater (P<0.05, N=4) than both the PM and raft samples (Fig. 7A). The SP of PM and raft from warm-acclimated fish did not differ significantly. In the samples from cold-acclimated fish the SP of the PM was significantly greater than that for the raft. It should be noted that the SPs for the cold-acclimated raft samples were, on average, negative. The second-derivative peak was chosen as a convenient index of saturation; the negative values are a product of this approach and simply reflect a very low SP. The SPs of all of the fractions were sensitive to assay temperature. Increasing the assay temperature from 5°C to 20°C caused a significant (P=0.011, N=24) mean reduction in SP of 38% (Fig. 7B).

The uncollapsed SP data, grouped and analyzed by fraction, are presented in Fig. 8. The PM and RDPM displayed similar patterns. When assayed at a common temperature, samples had similar SPs, regardless of their acclimation group. As a result, warm-acclimated samples had lower SPs than cold-acclimated samples when assayed at their respective physiological temperatures (Fig. 8, dots). By contrast, the SPs of the raft samples from warm-acclimated fish were up-shifted relative to those from cold-acclimated fish, the source of the near-significant acclimation group × fraction interaction term in the three-way ANOVA. As a result, the SPs of the rafts from the two acclimation groups do not differ at their respective physiological temperatures.

Three of the cold-acclimated raft samples showed a reduced SP when assayed at 20°C compared with at 5°C, while one sample displayed a dramatically higher SP. Based on the data collected on the MPPC vesicles (Fig. 4A), this suggested that 20°C was near the L_o–L_d phase transition temperature for these samples. Therefore, we repeated the assay at 28°C to determine if melting was detectable (Fig. 9). Neither cold- nor warm-acclimated RDPM showed a significant increase in SP when assayed at 28°C. By contrast, the SPs of rafts from both acclimation groups increased significantly at 28°C compared with 20°C.

Solubilization slopes

The region of the detergent solubility curves between points *a* and *b* in Fig. 1C was nearly linear and represented the shift from bilayer morphology to mixed micelles (solubilization). The slopes from linear regressions fitted to these regions were compared with a three-way ANOVA. The analysis found an effect of acclimation group and fraction but no effect of assay temperature. There was a significant interaction between acclimation group and fraction but no other significant interactions. Therefore, the data were collapsed across assay temperature (Fig. 10).

In both acclimation groups, the raft solubilization slopes were intermediate in value between those of the PM and RDPM. The RDPM slopes were significantly greater than those of the PM by 87% and 46% for the cold- and warm-acclimation groups, respectively. In the cold-acclimation group, the slope for the RDPM was significantly greater

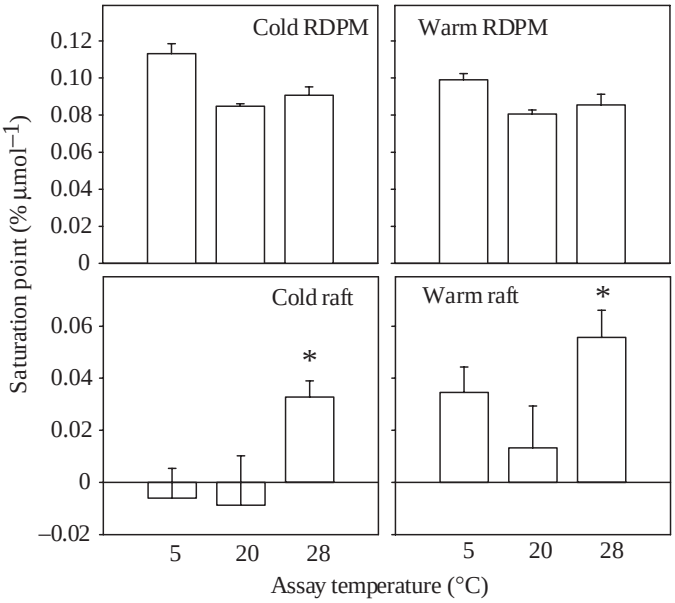

Fig. 9. Saturation points, elevated assay temperature. Values are means ± S.E.M., N=4. Asterisks denote significant difference from values at 20°C (P<0.05). RDPM, raft-depleted plasma membrane.

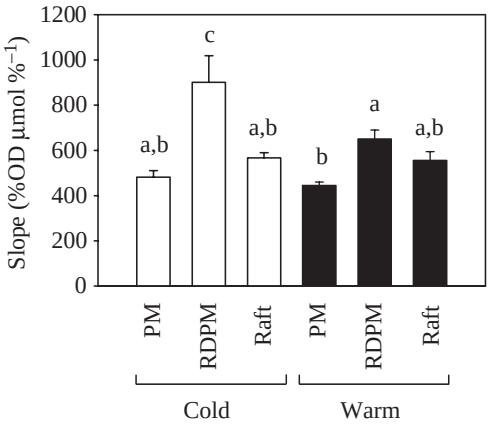

Fig. 10. Slopes. Absolute values of slopes of near-linear regions between 2nd-derivative peaks of fitted sigmoidal curves (data collapsed across assay temperature). Cold-acclimated, open bars; warm-acclimated, filled bars. Values are means ± S.E.M., N=8. Bars with a common lower-case letter do not differ significantly. PM, plasma membrane; RDPM, raft-depleted plasma membrane.

(P<0.05, N=8) than that for the raft by 59%, while these fractions did not differ in the warm-acclimation group.

Discussion

Plasma membranes (PMs) were isolated from trout using a combination of differential and density gradient centrifugation. The fraction obtained by this method has been previously characterized. It comprised 1.2–1.6% of the total cell protein, regardless of acclimation group. The apical domain marker 5′ nucleotidase was enriched by a factor of 11.14±2.03 (20°C acclimated trout) and 10.96±1.51 (5°C trout) while the basolateral domain marker Na^+/K^+-ATPase was enriched by a factor of 38.64±9.26 (20°C trout) and 52.53±6.72 (5°C trout) (Hazel et al., 1992). Thus, both major domains of the PM are present and the preparation is reasonably representative of the cell membrane.

We used sonication and density gradient centrifugation to separate the PM into low and high buoyant density fractions. Liver from rainbow trout does not express the protein caveolin (Fig. 2A). Nevertheless, the low density fraction was enriched in molecules characteristic of lipid microdomains, including cholesterol (Fig. 3A; Table 1) and both the β_2 adrenergic receptor and adenylyl cyclase (Fig. 2B,C). These three molecules have been reported to be lipid microdomain enriched in other systems (Ostrom et al., 2001; Rybin et al., 2000; Xiang et al., 2002). Furthermore, the low density fraction was substantially depleted in the β subunit of the insulin receptor, which is also consistent with previous observations (Mastick et al., 1995; Ohira et al., 2000). The even distribution of the protein clathrin among PM, RDPM and raft in our system is surprising since it is not associated with raft or caveolae microdomains (Razani and Lisanti, 2001). However, unlike all other proteins examined in this study, clathrin is not an integral membrane protein and can be separated from membranes by gentle dissociation (Keen et al., 1979). Therefore, it is possible that the sonication step used in separating the fractions acted to redistribute the protein among all membrane particles present in the preparation. Regardless, the weight of evidence suggests that this method separates trout liver PM into biochemically distinct fractions, including a low buoyant density fraction consistent with lipid microdomains. As this membrane fraction lacks caveolae, we will refer to it operationally as raft-enriched PM (raft) and the high buoyant fraction as raft-depleted PM (RDPM).

The discovery that the PM of most cells is not homogenous, but contains microdomains with distinct compositions, presents an opportunity for the re-examination of membrane thermal acclimation. In response to changing environmental temperatures, poikilotherms substantially alter the lipid composition of the PM (Hazel, 1997). Many studies have sought to rationalize these compositional changes as mechanisms of homeostasis for specific physical properties, notably hydrophobic region fluidity/order (homeoviscous adaptation), with the implication that critical functions are sensitive to these properties (Hazel, 1997). However, these studies have examined the PM as a whole. It is possible that the compositions of different regions of the PM are altered in different ways during thermal acclimation. Furthermore, microdomains form as a result of a separation of liquid-ordered (L_o; raft) and liquid-disordered (L_d; RDPM) phases within the membrane (Ahmed et al., 1997). Since lipid phase behavior is temperature sensitive, it is reasonable to hypothesize that acute changes in temperature could disrupt raft structure and the function of raft-targeted proteins. Consequently, acclimatory compositional changes of the PM may be aimed at stabilizing raft structure against thermal perturbation. Therefore, we

sought to determine what general compositional changes occur in raft and RDPM portions of the PM during thermal acclimation. We measured the cholesterol, phospholipid and total protein content of raft, RDPM and total PM of livers from cold- and warm-acclimated trout.

Rafts from both acclimation groups are substantially depleted in total protein compared with PM or RDPM (Table 1). The protein is primarily replaced by an enrichment in phospholipids in the cold-acclimated raft (although cholesterol is also enriched). By contrast, cholesterol accounts for most of the lipid enrichment in warm-acclimated rafts (Fig. 3A,B; Table 1). As a result, the cholesterol/phospholipid (Ch/PL) ratio is highly elevated in warm-acclimated raft while the Ch/PL is not changed in cold-acclimated raft compared with its PM source (Fig. 3B). The Ch/PL ratio is probably the most important measure for raft stabilization since it is an interaction between cholesterol and specific lipids, and not cholesterol concentration *per se*, that favors raft formation. This suggests that there are important differences in how rafts are stabilized in cold- and warm-acclimated animals. The data also demonstrate that the increase in cholesterol concentration is larger in raft than in RDPM with acclimation to 20°C (Table 1). Collectively, these data suggest that the compositional changes associated with thermal acclimation are at least partially related to properties specific to raft membrane.

If thermal acclimatory compositional adjustments can be explained in terms of raft stabilization, we would expect that molecular interaction strength (and thus the tendency to form the L_o phase) would be regulated in rafts to offset thermal perturbation. Membrane solubilization by detergent has previously been used to measure this property (Ahmed et al., 1997), but most analyses have failed to appreciate or account for the different stages of detergent–membrane interaction. Detergent molecules added to a membrane suspension are initially incorporated into the bilayer structure, suppressing the free detergent monomer concentration below the critical micelle concentration (CMC; Fig. 1A, plates 1, 2). As the membrane saturates, detergent monomer concentration in solution exceeds the CMC, mixed micelles form and the membrane begins to be solubilized (Fig. 1A, plates 3, 4) (Lichtenberg et al., 1983). The capacity of a membrane to incorporate detergent monomers is inversely proportional to the lipid–lipid interaction strength in the membrane (Lichtenberg et al., 1979). For example, a Langmuir trough study demonstrated that small increases in lateral pressure, with concomitant increases in lipid cohesion forces, resulted in large decreases in the incorporation of Triton X-100 (Nyholm and Slotte, 2001).

We found this interpretation to be well supported in model vesicle studies. The membrane condensing effect of an addition of 30 mol% cholesterol to palmitoyloleoyl phosphatidylcholine (POPC) vesicles was easily detected as a dramatic reduction in the SP (Fig. 4B). Furthermore, myristoylpalmitoyl phosphatidylcholine (MPPC) vesicles saturated with less detergent in the tightly packed gel phase than in the fluid phase (Fig. 4A). Similar results have previously been reported for sphingomyelin vesicles (Patra et al., 1999). A reduction in SP was observed with an increase in temperature up to, but remaining below, the acyl chain melting temperature in MPPC vesicles (Fig. 4A), sphingomyelin vesicles (Patra et al., 1999) and biological membranes (Fig. 7B). Prior to the dramatic decreases in packing density associated with a phase transition, increases in temperature within a phase cause increased membrane lateral pressure (de Kruijff, 1997). This may explain the lowered SP observed in these cases.

Membrane molecular interaction strength also influences the detergent/lipid values necessary to disrupt bilayer morphology and incorporate membrane molecules into mixed micelles (solubilization slopes). For example, a relatively high concentration of detergent was required to fully dissolve the vesicles made of POPC and 30 mol% cholesterol, producing a shallow solubilization slope (Fig. 5B). As predicted, the solubilization slope produced during dissolution of the more loosely packed POPC vesicles was comparatively steep. However, this measure tends to be less informative than SPs since this process is much more sensitive to variables other than molecular interaction strength. For example, the CMC of Triton X-100 is temperature sensitive (Elworthy et al., 1968a) and, contrary to prediction, the solubilization slopes for MPPC vesicles are much steeper in the gel phase compared with the fluid phase (Fig. 5A). Furthermore, the ability of detergent micelles to incorporate lipids, a property known as maximum additive concentration, is strongly dependent on the molecular species to be solubilized (Elworthy et al., 1968b). This complicates the interpretation of these data collected on biochemically distinct fractions.

Nevertheless, differences in solubilization slope between raft and RDPM are probably responsible for the detergent resistance of rafts, which is widely observed and commonly used in their isolation. The most commonly used detergent-based raft isolation method exposes isolated PM to Triton X-100 at 4°C and floats the remaining undissolved raft-enriched membrane on a sucrose density gradient (Brown and Rose, 1992). In the current study, raft membranes produced lower normalized optical densities than either PM or RDPM at most detergent concentrations (Fig. 6). This reflects the large differences in the SPs of these membranes but seems contradictory to the use of detergent as a means of raft isolation since it suggests that raft would dissolve more readily than RDPM. However, this is easily resolved when considering the solubilization process of PM as a mixture of raft and RDPM membranes. The PM sample as a whole would become saturated with detergent before the process of solubilization became dominant. This would effectively remove the differences in SPs between raft and RDPM membranes, and the solubilization process would be primarily defined by the process measured by the solubilization slopes of the RDPM and raft. The RDPM fractions had steeper solubilization slopes than the raft or PM in both acclimation groups (Fig. 10). Therefore, RDPM membrane would be more rapidly solubilized, leaving a raft-enriched fraction behind.

The difference in packing strength between raft and RDPM indicated by the solubilization slopes is also evident in the SP data (Fig. 7A). The more shallow solubilization slope and lower SP of raft, compared wiht RDPM, probably reflects the L_o phase of this microdomain. Proteins can be targeted to the L_o phase rafts by modification with saturated lipids that are more soluble in the tightly packed L_o than in the L_d phase (Melkonian et al., 1999). Since the SP (and packing strength) of membrane in either phase is acutely temperature sensitive (Fig. 7B), it is possible that perturbations of this property could influence the targeting of proteins to rafts. Furthermore, differences in packing strength may have functional consequences for the proteins embedded in the membrane. Integral membrane proteins may require a specific range of lateral pressure to allow appropriate flexing without allowing the protein to relax into non-functional conformations (de Kruijff, 1997). Finally, a sufficiently large increase in temperature could drive a phase transition, effectively melting L_o phase raft and resulting in mixing with the RDPM membrane. Conversely, a large decrease in temperature could result in accretion of lipids and proteins normally excluded from rafts.

Therefore, we examined the fractions from both acclimation groups at both assay temperatures to determine if membrane packing strength was conserved during thermal acclimation (Fig. 8). In both the PM and RDPM, samples assayed at a common temperature gave similar SPs. As a result, the warm-acclimated samples assayed at 20°C were different from the cold-acclimated samples assayed at 5°C (physiological comparisons; Fig. 8, dots). However, this perturbation was not seen in the raft membranes. The SPs of the warm-acclimated rafts were substantially up-shifted with respect to the cold-acclimated rafts such that there was little difference in this property when examined at physiological temperatures. To our knowledge, this is the first evidence of an apparent regulation of a physical property of a subdomain of the PM. Such regulation would be expected to maintain raft structural integrity and packing properties despite differing thermal environments experienced by the organism.

An increase of assay temperature above the gel–fluid phase transition was detected as a dramatic increase in SP for the MPPC vesicles (Fig. 4). Our observation that an increase in assay temperature from 5°C to 20°C resulted in an *increase* in SP in one raft sample from cold-acclimated fish suggested that rafts from these animals were close to their L_o–L_d melting temperature at 20°C. To test this hypothesis, all samples were assayed at 28°C (Fig. 9). Since RDPM is in the L_d phase, an increase in temperature should not cause an increase in SP. Indeed, there were only small and non-significant SP increases in RDPM from both acclimation groups. By contrast, the SP of the raft samples assayed at 28°C was significantly greater than those measured at 20°C in both acclimation groups. The dramatic increase in SP at 28°C suggests that the raft membranes from both acclimation groups were above their L_o–L_d melting temperature at this temperature. In the plasma membrane, clustered raft proteins and lipids would be expected to mix randomly with RDPM molecules under these conditions.

In conclusion, there is apparent spatial heterogeneity in the response of the PM to thermal change. Compositional changes associated with thermal acclimation are different in the raft and RDPM portions of the PM. Specifically, the ratio of cholesterol to phospholipid is elevated in rafts from warm-acclimated, but not from cold-acclimated, animals compared with their PM sources. Furthermore, there is a conservation of membrane packing strength in the raft, but not the raft-depleted, regions of the PM. This is consistent with a regulation of this physical property, which may have functional consequences for phase-dependent protein targeting. Furthermore, the detergent data suggest that elevated temperature can abolish the L_o/L_d phase separation responsible for raft structure. The raft-associated compositional changes observed may also function to stabilize these microdomains.

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