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Pressure adaptation of teleost gill Na^+/K^+-adenosine triphosphatase: role of the lipid and protein moieties

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Summary. The effects of temperature and pressure on Na^+/K^+-adenosine triphosphatases (Na^+/K^+-ATPases) from gills of marine teleost fishes were examined over a range of temperatures (10–25 °C) and pressures (1–680 atm). The relationship between gill membrane fluidity and Na^+/K^+-ATPase activity was studied using the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH). The increase in temperature required to offset the membrane ordering effects of high pressure was 0.015–0.025 °C·atm^{-1}, the same coefficient that applied to Na^+/K^+-ATPase activities. Thus, temperature-pressure combinations yielding the same Na^+/K^+-ATPase activity also gave similar estimates of membrane fluidity. Substitution of endogenous lipids with lipids of different composition altered the pressure responses of Na^+/K^+-ATPase. Na^+/K^+-adenosine triphosphatase became more sensitive to pressure in the presence of chicken egg phosphatidylcholine, but phospholipids isolated from fish gills reduced the inhibition by pressure of Na^+/K^+-ATPase. Cholesterol increased enzyme pressure sensitivity. Membrane fluidity and pressure sensitivity of Na^+/K^+-ATPase were correlated, but the effects of pressure also dependent on the source of the enzyme. Our results suggest that pressure adaptation of Na^+/K^+-ATPase is the result of both changes in the primary structure of the protein and homeoviscous adaptation of the lipid environment.

Key words: Homeoviscous adaptation – Lipid substitution – Membrane fluidity – Na^+/K^+-ATPase – Pressure

Introduction

High hydrostatic pressures in the range experienced by deep-sea organisms perturb membrane structure and function. High pressure orders phospholipid bilayers, causing the fatty acyl chains to pack together more tightly. The thickness of the bilayer actually increases (Braganza and Worcester 1986), due to the tighter packing. At high enough pressures, the acyl chains undergo a phase transition, from a liquid crystalline to a gel state. Low temperatures have similar effects, with a decrease of 15–30 °C being equivalent to a pressure increase of 1000 atm. Studies of membrane enzymes have shown that putative Arrhenius "break" temperatures are increased at high pressures (Ceuterick et al. 1978; de Smedt et al. 1979; Heremans and Wuytack 1980), with a coefficient of 0.015–0.030 °C·atm^{-1}. The similar pressure-temperature responses of model membrane systems and membrane enzymes have led many researchers to conclude that membrane physical state, particularly the "fluidity" of the lipid bilayer, is of primary importance in controlling membrane protein function at different temperatures and pressures.

Hydrostatic pressure in the ocean increases by 1 atm for every 10 m increase in depth; thus, the deepest part of the ocean, the Challenger Deep in the Marianas Trench (11 034 m), has pressure of over 1100 atm. In concert with the low temperatures of the deep sea (2–4 °C), this corresponds to an effective temperature of about −20 °C at 1 atm as far as membrane properties are concerned. Homeoviscous theory predicts that adaptation of membrane lipid composition to high pressure should resemble low temperature adaptation (Macdonald and Cossins 1985). The few studies to date support this idea (Avrova 1984; DeLong and Yayanos 1985, 1986; Cossins and Macdonald 1986; Wirsen et al. 1987).

Homeoviscous adaptation is expected to have significant consequences for the function of membrane proteins, but few data are available to test this prediction. Acclimation to low temperatures is associated with increased catalytic activites of several membrane proteins (Smith and Ellory 1971; Wodtke 1981; Raynard 1987) and reduced thermal stability of Na^+/K^+-ATPase (Cos-

Abbreviations: *EDTA*; *DPH* 1,6-diphenyl-1,3,5-hexatriene; *PC* phosphatidylcholine; *PL* phospholipid; *SDH* succinate dehydrogenase

sins et al. 1981). Substrate affinities and apparent Arrhenius activation energies of membrane-associated enzymes may also vary with acclimation temperature (Hazel 1972a; Gladwell 1975). The most direct demonstration that homeoviscous adaptation affects membrane protein properties is Hazel's (1972b) study of succinate dehydrogenase (SDH) from differently acclimated goldfish. Hazel showed that reactivation by lipids of detergent-solubilized SDH dependent only on the acclimation temperature of the goldfish from which the lipids were derived, not on the acclimation temperature of the source of the protein.

Homeoviscous adaptation to pressure should also affect membrane proteins, but no data are available on this point. In a previous study, we demonstrated pressure adaptation of Na^+/K^+-ATPases from gills of deep-sea teleost fishes (Gibbs and Somero 1989). Our findings were consistent with adaptation being a lipid-based phenomenon, but could also have resulted from adaptive differences in the primary sequence of Na^+/K^+-ATPase. The present studies were designed to distinguish between these possibilities. First, the relationship between membrane fluidity and Na^+/K^+-ATPase activity was examined using depolarization of fluorescence of the hydrophobic probe, 1,6-diphenyl-1,3,5-hexatriene (DPH), to estimate membrane fluidity. Secondly, in a more direct approach, a lipid substitution technique was used to modify the lipid environment around Na^+/K^+-ATPase, mimicking homeoviscous adaptation in vitro. This allowed the relative importance of lipid-based adaptations to be estimated. Third, by placing Na^+/K^+-ATPases from fishes from different habitat temperatures and pressures in the same membrane lipid environment, we examined the role of changes in protein primary structure in adaptation of this enzyme.

We conclude that pressure adaptation of Na^+/K^+-ATPase is the result of both changes in the primary structure of the protein and homeoviscous adaptation of its membrane lipid environment.

Materials and methods

Specimen collection. The marine teleost species used in these studies (depth range, habitat temperature) were: *Coryphaenoides armatus* (1885–4815 m, 2–4 °C), the Pacific grenadier *C. acrolepis* (700–1820 m, 4–8 °C), the sablefish *Anoplopoma fimbria* (0–1500 m, 4–8 °C), and the barracuda *Sphyraena barracuda* (0–20 m, 24 °C). Cold-living fishes were captured by otter trawl off the coasts of Oregon and northern California. The gills were immediately dissected out, wrapped in aluminum foil, and stored at −80 °C. Barracudas were purchased fresh in Hawaiian fish markets and frozen whole at −80 °C.

Membrane preparations and enzyme assays. Isolation of gill membranes and assay conditions for Na^+/K^+-ATPase activity were as described in Gibbs and Somero (1989). Briefly, gill filaments were trimmed from thawed gills and homogenized in 5–10 volumes homogenization buffer (50 m*M* imidazole, 250 m*M* sucrose, 1 m*M* EDTA, 5 m*M* 2-mercaptoethanol, pH 7.5 at 20 °C). Homogenates were centrifuged for 15 min at 2000 × g, and the supernatants were spun for 90 min at 19000 × g. The pellets were resuspended in approximately 1 ml homogenization buffer per gram of original tissue. Aliquots were stored in Eppendorf microfuge tubes at −80 °C until needed.

Adenosine triphosphatase activities under saturating substrate conditions were monitored continuously by means of a coupled pyruvate kinase/lactate dehydrogenase assay. The assay medium contained 100 m*M* NaCl, 20 m*M* KCl, 5 m*M* $MgCl_2$, 30 m*M* TRIS-HCl (pH 7.5 at 10 °C), 3 m*M* ATP, 0.5 m*M* phosphoenolpyruvate, 0.05 m*M* fructose-1,6-bisphosphate, 0.2 m*M* β-nicotinamide adenine dinucleotide, reduced (NADH), and excess amounts of the coupling enzymes. For assays at varying temperatures, the pH of the TRIS buffer was adjusted to follow the temperature dependence of intracellular pH ($\Delta pH/\Delta T \approx -0.017 \cdot °C^{-1}$; Reeves 1977). Na^+/K^+-adenosine triphosphatase activities were calculated as the difference between total and ouabain-insensitive ATPase activities, and were 2–10 μmol $ATP \cdot h^{-1} \cdot mg\ protein^{-1}$ at 10 °C.

Fluorescence polarization. Fluorescence polarization of the hydrophobic fluorophore, 1,6-diphenyl-1,3,5-hexatriene (DPH, Aldrich Chemical Co.), was measured as described by Litman and Barenholz (1982) using a Perkin-Elmer model MPF-44A fluorescence spectrophotometer. Alignment of the Polaroid© polarizers (Perkin-Elmer no. 063-0571) was checked using a solution of DPH in glycerol, and by light scattering by glycogen at 500 nm (Paladini 1986). Measured polarizations were within 1% of expected values. Excitation of DPH was at 360 nm, with a 5 nm slit width, and emission was measured at 440 nm (20 nm slit width) through the fluorometer's 390 nm cut-off filter.

Thawed membranes were suspended in ATPase salt solution, containing the same concentrations of inorganic salts and TRIS buffer as the ATPase assay medium, to give an optical density at 500 nm of approximately 0.1. At this concentration, polarization values are independent of the DPH-to-membrane ratio, indicating that light scattering is not a problem (Litman and Barenholz 1982). Diphenylhexatriene was added from a 2 m*M* stock solution in tetrahydrofuran, to give a final concentration of 4 μ*M*, and the solution was incubated at 37 °C for 60 min. Other ATPase assay reagents had no effect on either the total fluorescence of the measured polarization of fluorescence, and were omitted. Fluorescence of blanks was less than 1% of that of samples containing DPH. All polarization measurements on a given sample were made on the same day. Steady-state anisotropies (r_s) of DPH were calculated from polarization values (P) using the equation (Litman and Barenholz 1982): $r_s = 2P/(3-P)$.

Fluorescence measurements at increased pressure. The thermal-jacketed, high-pressure optical cell (Mustafa et al. 1971) was modified to include a third window, at right angles to the other two, that served as the excitation port. Samples were allowed to sit for 3–5 min after increase or release of pressure in order to reach thermal equilibrium. Routinely, pressure was increased in 68-atm (1000 $lb \cdot in^{-2}$) intervals, and released in 136-atm intervals. No differences in fluorescence polarization at a given pressure were detected between measurements as pressure was increased or decreased. "Scrambling" of polarized light by the quartz cuvette windows was corrected using the method described by Paladini (1986) using DPH dissolved in glycerol as a standard.

Lipid purification. Fish gill lipids were isolated from gill membrane preparations by the method of Bligh and Dyer (1959). For every 10 ml of membrane preparation, 12.5 ml chloroform and 25 ml methanol were added, and the mixture was stirred thoroughly for several minutes. 12.5 ml $CHCl_3$ were then added, followed by 12.5 ml water. After vacuum filtration through Whatman No. 4 filter paper, the upper aqueous phase was removed by aspiration. The volume of the lower organic layer was reduced by evaporation on a RotaVapor (Rinco Instrument Co., Inc.; Greenville IL). The solution was transferred to a glass vial and stored under N_2 at −80 °C.

Total lipid extracts were fractionated by the column chromatography method of Nevenzel et al. (1985). Purified lipids were

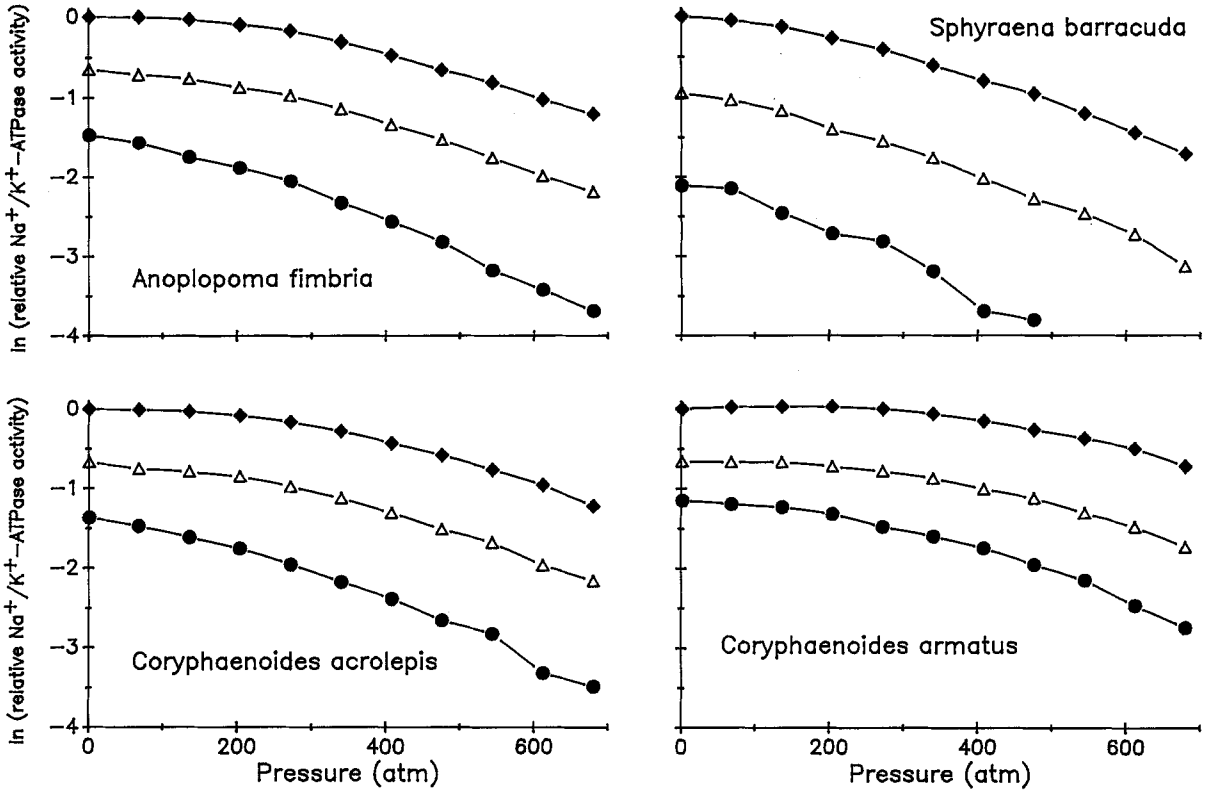

Fig. 1. Effects of pressure and temperature on Na^+/K^+-ATPase activity in gill membrane preparations. Data are means for 2–8 individuals. *Diamonds*: 25 °C; *triangles*: 17.5 °C; *circles*: 10 °C. Data at 10 °C are from Gibbs and Somero (1989)

applied to a BioSilA (BioRad) column equilibrated with $CHCl_3$. The column was washed with 4 column volumes (~40 ml each) of $CHCl_3$, followed by 5 column volumes of acetone, to elute glycolipids and neutral lipids. Phospholipids were collected as the eluate with CH_3OH from 3 column volumes. The volume was reduced on the RotaVapor, and the sample was transferred to a tared glass vial. The yield was ~2 mg phospholipid for each millilitre of membrane preparation. Phospholipids were dried completely under N_2, and resuspended in substitution buffer [50 m*M* imidazole (pH 7.5 at 20 °C), 250 m*M* sucrose, 1 m*M* EDTA, 5.5% sodium cholate], to a concentration of 50 mg phospholipid·ml^{-1}. In other words, the solution contained the same buffer used to prepare membranes, plus detergent and purified phospholipids. Chicken egg phosphatidylcholine (PC) was purchased from Avanti Polar Lipids (Birmingham AL).

Lipid substitution. The protocol used in all lipid substitution experiments was modified from the cholate solubilization/gradient centrifugation method of Warren et al. (1974). 200 µl substitution buffer were added to 1 ml membrane preparation (containing ~5 mg·ml^{-1} each of lipid and protein). The solution was incubated for 60 min at room temperature and centrifuged for 15 min at 10 psi (equivalent to ~50000 × *g*) in a Beckman Airfuge. The pellet (Pellet 1) was resuspended in 1 ml homogenization buffer and 200 µl substitution buffer containing 5% lipids of choice. After incubation at room temperature for 60 min, the sample was centrifuged for 15 min at 15 psi (~85000 × *g*). The pellet (Pellet 2) was again resuspended in 1 ml homogenization buffer and 200 µl substitution buffer containing lipids, incubated for 60 min at room temperature, and spun for 15 min at 15 psi. The pellet (Pellet 3) was resuspended in 0.5 ml homogenization buffer, and ATPase activity was assayed at 17.5 °C. Over 50% of the initial Na^+/K^+-ATPase activity was recovered in the final pellet, and no activity was present in any supernatant fractions.

Results

Temperature and pressure effects on Na^+/K^+-ATPase activity and membrane fluidity

Figure 1 depicts the temperature-pressure relationships of gill Na^+/K^+-ATPase activity for four species of marine teleosts. For all species, increased pressure led to decreased enzymatic activity, but the inhibition by pressure could be offset by increasing the assay temperature. By drawing a horizontal line across one of these graphs, the amount of pressure required to offset the effects of an increase in temperature can be estimated. For Na^+/K^+-ATPase activities of all four species, 400–600 atm counteracted the effects of a temperature increase of 10 °C (equivalent to 0.015–0.025 °C·atm^{-1}).

To examine the relationship between membrane fluidity and Na^+/K^+-ATPase activity, the steady-state anisotropy of DPH (inversely related to membrane fluidity) was measured under the same temperature-pressure regimes as Na^+/K^+-ATPase activity, using membranes prepared in the same manner. Anisotropy increased with pressure and decreased with temperature, with no indication of a sharp break due to a phase transition (Fig. 2). As in Fig. 1, a horizontal line across the figure gives an estimate of the temperature increase required to offset the membrane ordering effects of a given pressure increase. As with Na^+/K^+-ATPase activities, a 15–25 °C increase in temperature offset the effects of a pressure increase of 1000 atm.

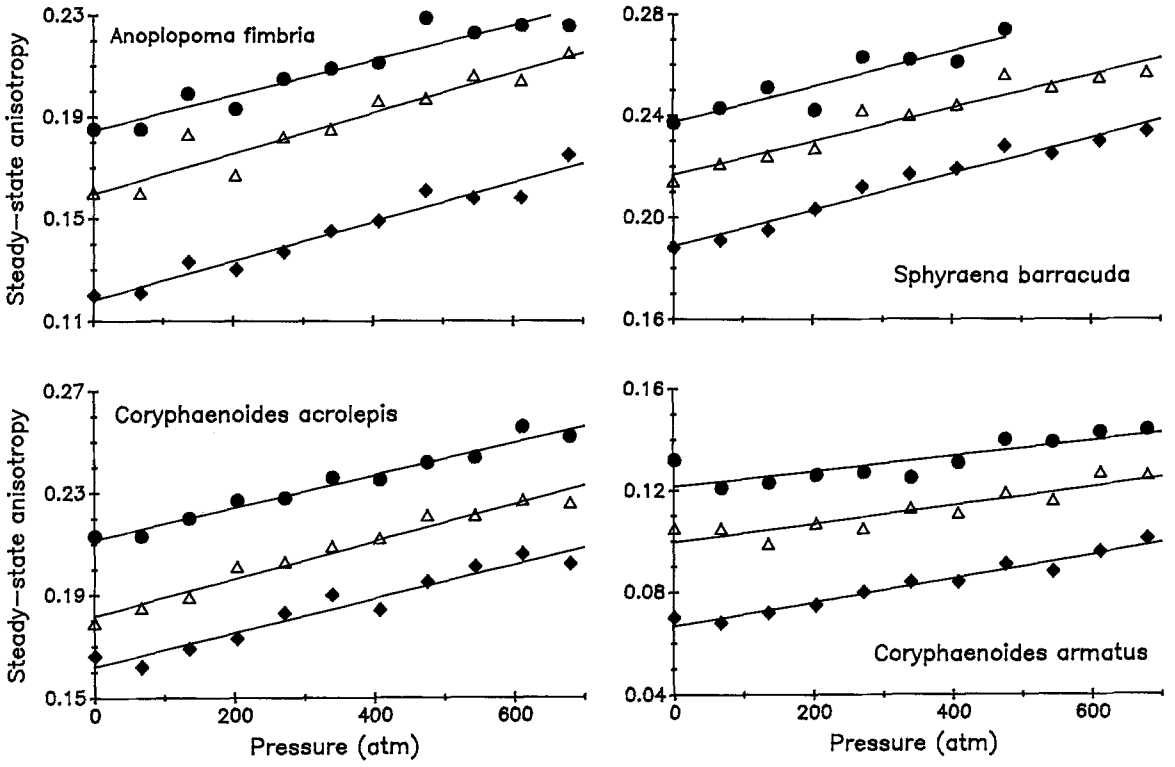

Fig. 2. Effects of pressure and temperature on steady-state anisotropy (r_s) of DPH in gill membrane preparations. Anisotropy is inversely related to membrane fluidity. Data represent means for 2–4 measurements for membranes prepared from one individual of each species. Variability was about ± 0.005. *Diamonds*: 25 °C; *triangles*: 17.5 °C; *circles*: 10 °C

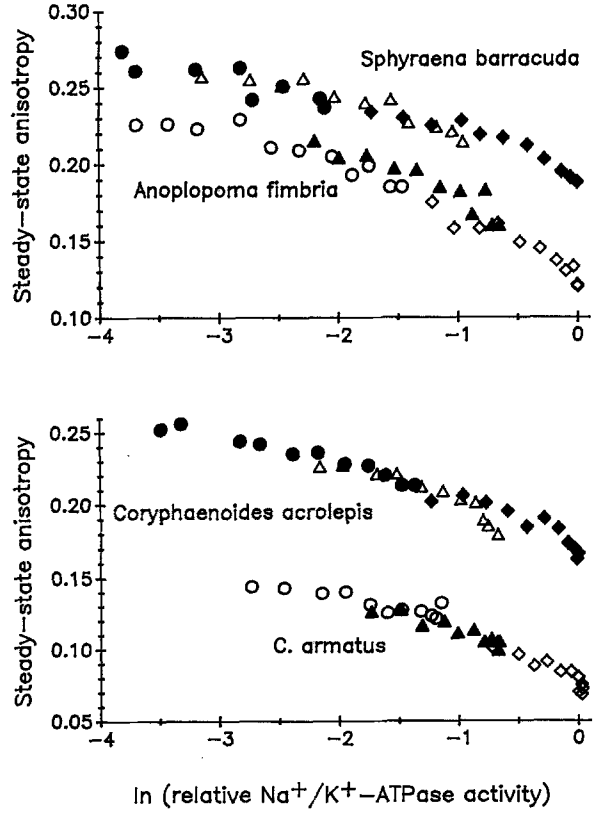

Fig. 3. Relationship between Na^+/K^+-ATPase activity and steady-state anisotropy of DPH at different temperatures and pressures. *Diamonds*: 25 °C; *triangles*: 17.5 °C; *cirlces*: 10 °C

In Fig. 3, the steady-state anisotropy values in Fig. 2 are plotted against Na^+/K^+-ATPase activities measured separately under the same temperature and pressure conditions (data from Fig. 1), to show the close correlation between membrane fluidity and enzymatic activity. Different combinations of temperature and pressure that yielded the same Na^+/K^+-ATPase activities also yielded similar anisotropy values.

Effects of lipid substitution on the pressure responses of Na^+/K^+-ATPase activity

Lipid substitution experiments were performed to determine the extent to which interspecific differences in Na^+/K^+-ATPase were due to the membrane lipid environment. Table 1 shows representative yields of Na^+/K^+- and Mg^{2+}-ATPases and protein at several stages of lipid substitution. Cholate increased Na^+/K^+-ATPase activity by up to 80%, but did not affect the pressure responses of the enzyme (Fig. 4). Cholate levels in the lipid substitution experiments were less than 1%, and most, if not all, cholate should have remained in micelles in the supernatant during centrifugation. A small amount of cholate may have remained associated with Na^+/K^+-ATPase, but should not have affected the pressure dependence of enzymatic activity in these experiments.

Figure 5 depicts the pressure responses of Na^+/K^+-ATPase from *Anoplopoma fimbria* at several stages of substitution with chicken egg phosphatidylcholine (PC). The effects of pressure on Na^+/K^+-ATPase activity of

Table 1. Representative yields of Na^+/K^+-ATPase, ouabain-insensitive Mg^{2+}-ATPase, and protein at various stages of lipid substitution. The Na^+/K^+-ATPase source was *Anoplopoma fimbria* gills, and lipids were egg phosphatidylcholine. All values are relative to 100% of starting gill membrane preparation (1.13 $\mu mol \cdot min^{-1} \cdot ml^{-1}$ Na^+/K^+-ATPase, 0.83 $\mu mol \cdot min^{-1} \cdot ml^{-1}$ Mg^{2+}-ATPase, 6.45 mg protein $\cdot ml^{-1}$. Protein concentrations were measured using the bicinchinonic acid method (Sigma)

Sample	Yields (%)		
	Na^+/K^+-ATPase	Mg^{2+}-ATPase	Protein
Gill membranes	100	100	100
Pellet 1: cholate treated	142	30	43
Pellet 2: 1× substituted	136	23	27
Pellet 3: 2× substituted	80	9	n.d.

n.d. = not determined

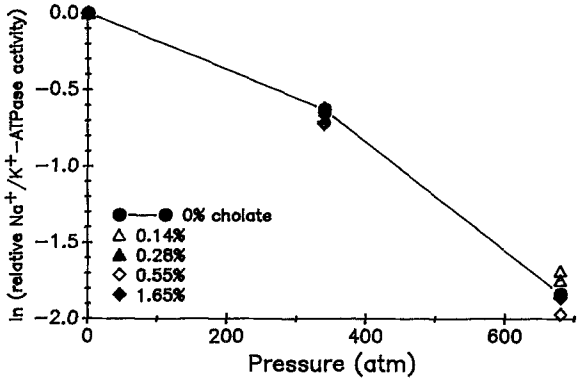

Fig. 4. Effects of cholate on the pressure responses of Na^+/K^+-ATPase activity. 10% Na-cholate was added to gill membranes prepared from *A. fimbria* to give the indicated final concentration, and Na^+/K^+-ATPase activity was assayed at 17.5 °C

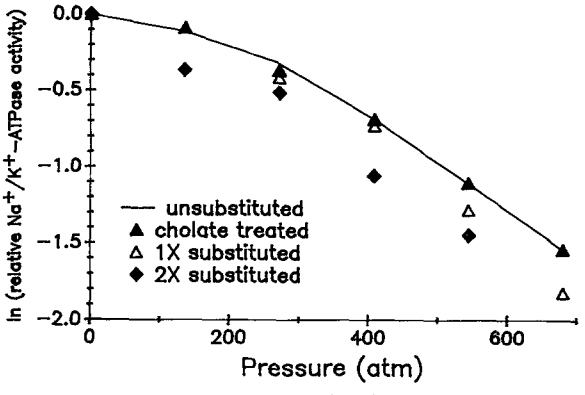

Fig. 5. Effects of pressure on Na^+/K^+-ATPase activity at 17.5 °C at different stages of lipid substitution. Gill membranes were prepared from *A. fimbria*, and the substitution lipids were chicken egg PC. Na^+/K^+-ATPase activity of Pellet 3 (the twice-substituted enzyme) at 680 atm was too low to measure accurately

Pellet 1, the 50000- × *g* pellet of the cholate-treated enzyme, were indistinguishable from the effects on Na^+/K^+-ATPase in its native lipid environment. After one round of lipid substitution, Na^+/K^+-ATPase activity was slightly more pressure-sensitive (Pellet 2), and even more sensitive after two rounds (Pellet 3). No further substitution was attempted, due to limited availability of material. Although we did not analyze lipid composition to attempt to estimate the extent of substitution, Warren et al. (1974) observed greater than 98% replacement of endogenous lipids associated with purified Na^+/K^+-ATPase using similar methods and the same number of substitution cycles.

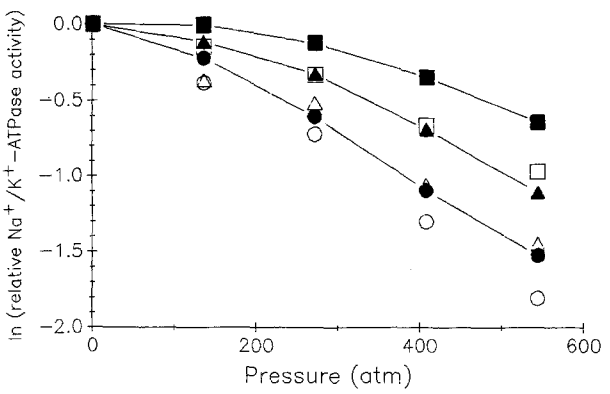

Fig. 6. Effects of lipid substitution with chicken egg PC on the pressure responses of Na^+/K^+-ATPases from three teleost species. *Dark symbols* indicate unsubstituted gill membrane preparations, and *open symbols* indicate samples after two rounds of lipid substitution. *Squares*: *C. armatus*; *triangles*: *A. fimbria*; *circles*: *S. barracuda*. Assay temperature 17.5 °C

The data shown in Fig. 6 for substitution of native lipids with chicken egg PC for Na^+/K^+-ATPases from three species of fish, illustrate two points. The first is that Na^+/K^+-ATPases from different species, in their native lipid environments (bold symbols in Fig. 6), have different pressure responses at 17.5 °C. As a function of habitat conditions, the order of increasing pressure sensitivity is cold deep-sea (*Coryphaenoides armatus*) < cold shallow (*A. fimbria*) < warm shallow (*Sphyraena barracuda*). This is the same pattern seen in Na^+/K^+-ATPases assayed at 10 °C (Gibbs and Somero 1989), although the degree of inhibition by pressure is reduced at the higher assay temperature. Thus, the interspecific pressure-adaptive differences observed at 10 °C remain at 17.5 °C. Second, substitution of native lipids by chicken egg PC markedly increased the pressure sensitivities of Na^+/K^+-ATPases from all three species. Interspecific differences in the pressure responses remained, however, after lipid substitution. These results suggest that there are interspecific differences in the protein component of this membrane enzyme system.

Figure 7 depicts the effects of pressure on Na^+/K^+-ATPase from two species after two substitution cycles with phospholipids purified from two cold-water fishes, the deep-sea rattail *C. armatus* and the shallow-living sablefish *A. fimbria*. In this case, both homologues became less pressure-sensitive, but interspecific differences again remained after substitution with the same phospholipids.

The finding that Na^+/K^+-ATPase from *C. armatus* differs in its pressure responses after substitution with *C. armatus* phospholipids may seem surprising. These results suggest that some other lipid fraction, with signif-

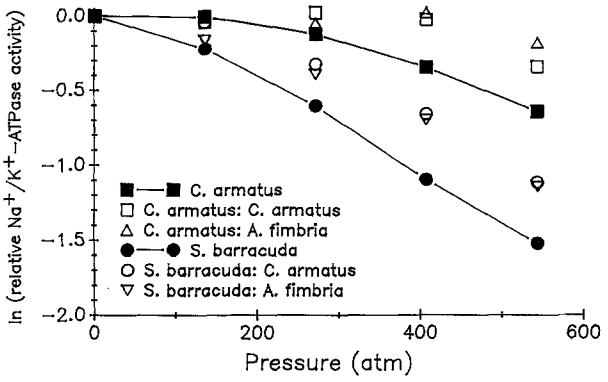

Fig. 7. Effects of lipid substitution with fish gill phospholipids on the pressure responses of Na^+/K^+-ATPases from two teleost species. *Dark symbols* indicate unsubstituted gill membrane preparations from each species. *Open symbols* indicate samples after two rounds of lipid substitution. The first species listed indicates the source of Na^+/K^+-ATPase, the second indicates the source of gill phospholipids. Assay temperature 17.5°C

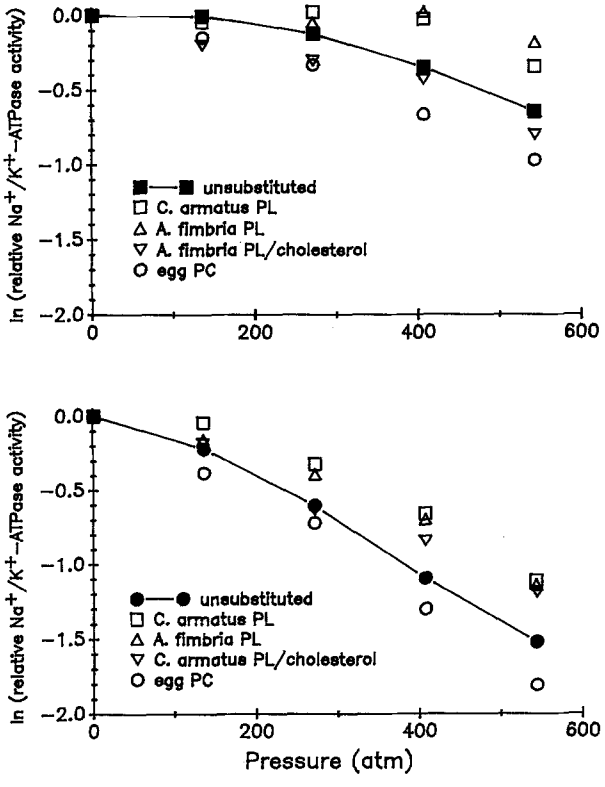

Fig. 8. Effects of lipid substitution on the pressure responses of Na^+/K^+-ATPases from two teleost species. Upper panel: *C. armatus* Na^+/K^+-ATPase; lower panel: *S. barracuda* Na^+/K^+-ATPase. *Dark symbols* indicate unsubstituted gill membrane preparations, and *open symbols* indicate samples after two rounds of lipid substitution. The sources of substitution lipids are indicated. Assay temperature 17.5 °C. PL: phospholipid; PC: phosphatidylcholine

icant effects on the properties of Na^+/K^+-ATPase, was lost during phospholipid purification. An obvious candidate is cholesterol, a major component of animal cell membranes which has significant effects on both membrane physical properties and membrane proteins (Carruthers and Melchior 1986). Substitution of native *C. armatus* lipids with a 1:1 (*w*/*w*) mixture of *A. fimbria* phospholipids and cholesterol resulted in a Na^+/K^+-ATPase that was slightly more inhibited by pressure than the enzyme in its native lipid environment, and much more pressure-sensitive than the phospholipid-substituted enzyme (Fig. 8A). Note that the effects of substitution with phospholipids from *C. armatus* and *A. fimbria* were similar. Results obtained with Na^+/K^+-ATPase from the shallow-living tropical fish, *S. barracuda*, showed the same trends (Fig. 8B), but in every case in which the same lipids were substituted onto different Na^+/K^+-ATPases, the enzyme from *S. barracuda* was more inhibited by pressure than the *C. armatus* homologue.

Discussion

Several authors have varied membrane fluidity and followed the corresponding changes in Na^+/K^+-ATPase activity (Kimelberg and Papahadjopoulos 1974; Kimelberg 1975; Sinensky et al. 1979; Harris 1985). The study most comparable to ours is that of Chong et al. (1985), who also found that fluidity and enzymatic activity correlated as a function of temperature and pressure. Our data extend their results (using dog kidney Na^+/K^+-ATPase) to the homologous enzymes from four marine teleost fishes, inhabiting a wide range of temperatures (2–24 °C) and pressures (1–480 atm). The data also span a 15–45-fold range of enzymatic activities, depending on species (Fig. 3). The close correspondence between membrane fluidity and enzymatic activity over such a wide range suggests that fluidity is a major factor regulating Na^+/K^+-ATPase activity, and that homeoviscous adaptation to pressure will greatly affect enzyme function.

Deep-sea fishes have more fluid myelin membranes (Cossins and Macdonald 1984) and higher amounts of unsaturated fatty acyl chains in liver phospholipids (Cossins and Macdonald 1986) than do shallow-living species. These differences mitigate the lipid-ordering effects of high pressures and low temperatures in the deep sea, and may have concomitant effects on membrane protein function in these tissues. The data in Fig. 3 suggest that the fluidity of gill membrane lipids varied among the fishes studied in a pattern consistent with homeoviscous adaptation. Steady-state anisotropy of DPH, at any combination of measurement temperature and pressure, was consistently highest for the warm-adapted, shallow-living, tropical barracuda, *S. barracuda*, and decreased with increasing depth of occurrence for the cold-adapted species (*C. acrolepis* ≈ *A. fimbria* > *C. armatus*). We observed similar trends in studies of other shallow- and deep-living species (data not shown), but we also found a high level of intraspecific variability which tended to obscure the interspecific differences. We used crude membrane preparations that contained a variety of types of membrane fragments (plasma membranes, mitochondrial membranes, plus other membrane fractions). Fluidities of different membrane fractions from a given tissue may vary (Cossins and Prosser 1982),

and membranes may differ in their efficacies of homeoviscous adaptation (Cossins and Macdonald 1989). Thus anisotropy (fluidity) estimates may differ between membrane preparations containing different relative amounts of different subcellular membrane fractions. Because the relative amounts of different membrane fractions in our preparations are unknown, the interspecific differences in apparent fluidity should be viewed with caution.

The use of crude membrane preparations rather than more purified preparations of Na^+/K^+-ATPase was based on a desire to study homeoviscous adaptation of Na^+/K^+-ATPase in a native lipid environment. Enzyme purification procedures may create artifacts associated with, for example, detergent effects on Na^+/K^+-ATPase activity or differential removal of certain lipid classes. By accepting the uncertainties of working with fairly crude preparations, it is reasonably certain that Na^+/K^+-ATPase was assayed under conditions that were as near to *in situ* conditions as possible. It was also important to measure fluidity using membranes prepared in the same manner as for Na^+/K^+-ATPase assays, since membrane extraction can affect fluidity estimates (Cameron et al. 1983; Kuhry et al. 1985; Mantsch et al. 1988). While our measurements of steady-state anisotropy of DPH cannot quantitatively describe the local membrane environment of Na^+/K^+-ATPase, they do provide a good indication of the relative magnitudes of the effects of temperature and pressure on membrane fluidity.

The effects of pressure on steady-state anisotropy (r_s) at three temperatures are calculated in Table 2. The data are in good agreement with representative values cited by Macdonald and Cossins (see Table 3-2 in Macdonald and Cossins 1985) and data in Cossins and Macdonald (1989). Interestingly, r_s was about half as pressure sensitive in gill membranes from the deepest-living fish, *C. armatus*, as in membranes prepared from shallower species. These results suggest that pressure-adapted membranes may have relatively pressure-insensitive fluidities, as well as being generally more fluid.

Table 2. Pressure and temperature dependence of steady-state anisotropy (r_s) of DPH in fish gill membranes

	Δr_s 1000 atm^{-1} (SE)			$\Delta r_s \cdot °C^{-1}$
	10 °C	17.5 °C	25 °C	
Sphyraena barracuda	0.071 (0.013)	0.066 (0.006)	0.072 (0.005)	−0.0032
Anoplopoma fimbria	0.070 (0.007)	0.080 (0.008)	0.076 (0.006)	−0.0044
Coryphaenoides acrolepis	0.064 (0.004)	0.074 (0.004)	0.067 (0.005)	−0.0033
C. armatus	0.031 (0.007)	0.037 (0.006)	0.046 (0.003)	−0.0037

Steady-state anisotropies were calculated from measurements of fluorescence polarization of DPH, using the equation: $r_s = 2P/(3-P)$. At a given temperature, the effect of pressure on r_s was determined by linear regression. The temperature dependence of r_s was estimated from three measurements at 1 atm

Fluidities of gill membranes from each species were equally temperature-dependent ($\Delta r_s \cdot °C^{-1} \approx -0.0035$). These data are in good agreement with those cited by Macdonald and Cossins (1985).

It must be noted that, although membrane fluidity and Na^+/K^+-ATPase activity correlate closely, this is still only a correlation. The relationship between fluidity and Na^+/K^+-ATPase activity could simply represent the consequences of the effects of temperature and pressure on some other membrane property which regulates enzymatic activity, rather than a cause-and-effect relationship. The coefficient of 0.015–0.030 $°C \cdot atm^{-1}$ holds for properties other than fluidity or enzymatic activity, e.g., phase transitions and bilayer thickness, which have been suggested to control activity of a related cation pump, Ca^{2+}-ATPase (Heremans and Wuytack 1980; Caffrey and Feigenson 1981; but see Squier et al. 1988). It is clear that the membrane lipid environment is critical in modulating the activities of membrane proteins, although exactly which properties of the lipid environment are most important is still open to dispute. Our lipid substitution experiments provide a more direct means of probing the effects of membrane lipids on enzymatic activity and the role of homeoviscous adaptation in pressure adaptation of Na^+/K^+-ATPase.

Several methods have been used to alter the lipid environment of Na^+/K^+-ATPase. Addition of more saturated or longer chain phospholipids (Kimelberg and Papahadjopoulos 1974), or supplementation with cholesterol (Kimelberg 1975; Sinensky et al. 1979) decreases specific enzymatic activity, apparently correlating with reduced membrane fluidity. A drawback of these experiments is that experimental procedures may partially inactivate Na^+/K^+-ATPase. Comparison of the effects of thermodynamic parameters, such as temperature and pressure, allows examination of the effects of the lipid environment on Na^+/K^+-ATPase, without needing to worry about inactivation of an unknown fraction of the enzyme. Higher apparent Arrhenius activation energies of several membrane proteins are correlated with decreased membrane fluidity (Kimelberg and Papahadjopoulos 1974; Kimelberg 1975; Hesketh et al. 1976; Carruthers and Melchior 1984). Similar lipid-based differences in activation energy have been observed for succinate dehydrogenase in thermally acclimated goldfish (Hazel 1972a). Presumably, free-energy barriers associated with protein conformational changes during catalysis are increased in a more viscous membrane.

According to our earlier hypothetical model (Gibbs and Somero 1989), Na^+/K^+-ATPases in more fluid membranes will be less inhibited by pressure than those in more viscous membranes. Homeoviscous theory predicts that more fluid membranes will occur in organisms from deeper, colder habitats. For the lipids in this study, the predicted order of decreasing fluidity is: *C. armatus* phospholipids > *A. fimbria* phospholipids > chicken egg phosphatidylcholine. As the lipids around a given Na^+/K^+-ATPase are replaced by progressively less fluid lipids, Na^+/K^+-ATPase activity should become increasingly pressure-sensitive. The results depicted in Fig. 8 con-

firm these predictions. Na^+/K^+-adenosine triphosphatases from both *C. armatus* and *S. barracuda* became progressively more pressure sensitive as the fluidity of the substituting lipids decreased.

These results provide direct evidence that the lipid environment affects the pressure responses of Na^+/K^+-ATPase, but the question remains whether or not the changes in membrane composition associated with homeoviscous adaptation to pressure are sufficient to adapt Na^+/K^+-ATPase to pressure. The lipids used in these experiments were isolated from animals living at different temperatures as well as pressures, so the lipid effects described here may only reflect thermal adaptation. Comparison of the effects of lipids derived from two cold-water fishes living at different depths, *A. fimbria* and *C. armatus*, should have resolved this question, but the effects of lipids from these two species on Na^+/K^+-ATPases from *C. armatus* (a cold-living, deep-sea fish) and the tropical shallow-living *S. barracuda* were indistinguishable from each other (Fig. 8). Several factors could account for these results.

Incomplete substitution of lipids around Na^+/K^+-ATPase could hide the full effect of lipid-based differences, although Warren et al. (1974) demonstrated that similar methods achieved >98% replacement of endogenous lipids associated with purified Na^+/K^+-ATPase after two substitution cycles. In the absence of lipid analyses, we cannot be certain that we achieved the same degree of substitution as Warren et al. (1974), but the increasing pressure-sensitivity of Na^+/K^+-ATPase as lipid substitution progressed (Fig. 5) may indicate that we did not achieve complete lipid replacement. The extent of substitution, however, was probably substantial given the large effects observed.

Lipids from the sablefish, *A. fimbria*, were isolated from specimens captured near the maximal depth at which this species occurs (1500 m). If intraspecific, depth-related homeoviscous adaptation occurs in this species (as has been demonstrated in deep-sea bacteria; DeLong and Yayanos 1985, 1986; Wirsen et al. 1987), then these lipids may more closely resemble those of deep-sea fishes than those of other shallow-living species. Specimens of *C. armatus* were captured at a depth of 2850 m. As far as membrane properties are concerned, this is equivalent to a decrease in temperature (at 1 atm) of only about 4–8 °C. Differences in the composition and properties of lipids from these two cold-water species are likely to be much smaller than differences between lipids from *A. fimbria* and the barracuda (habitat temperature equals 24 °C) or chicken. If there are differences between the lipids of *A. fimbria* and those of *C. armatus*, they may not be great enough to have measurably different effects on the pressure responses of Na^+/K^+-ATPase activity. Low temperature in the deep sea is certainly a major focus of homeoviscous adaptation, and may make it difficult to detect pressure-related lipid adaptations. We predict that similar studies, using fishes or other organisms from hydrothermal vents (a warm deep-sea environment), or living at depths of greater than 3000 m, will allow the importance of lipid-based adaptations to pressure of membrane proteins to be understood.

Our results do not unequivocally demonstrate a role for lipid variation in pressure adaptation of Na^+/K^+-ATPase, but they do show that the membrane environment influences the pressure responses of this enzyme. In particular, membrane fluidity correlates very closely with Na^+/K^+-ATPase activity. When the lipid environment of Na^+/K^+-ATPase is altered, the effects of pressure on homologous Na^+/K^+-ATPases depend on the source of the enzyme, even when the homologues are in similar lipid environments. Therefore, although membrane lipid composition has significant effects on the properties of Na^+/K^+-ATPase, homeoviscous adaptation alone is insufficient for pressure adaptation of this enzyme. Changes in the primary structure of Na^+/K^+-ATPase are also necessary for function under the environmental conditions of the deep sea.

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