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REVIEW

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Analysis of intracellular pH in the yeast *Saccharomyces cerevisiae* under elevated hydrostatic pressure: a study in baro- (piezo-) physiology

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Abstract Hydrostatic pressure is a distinctive feature of deep-sea environments, and this thermodynamic parameter has potentially inhibitory effects on organisms adapted to living at atmospheric pressure. In the yeast *Saccharomyces cerevisiae*, hydrostatic pressure causes a delay in or cessation of growth. The vacuole is a large acidic organelle involved in degradation of cellular proteins or storage of ions and various metabolites. Vacuolar pH, as determined using the pH-sensitive fluorescent dye 6-carboxyfluorescein, was analyzed in a hydrostatic chamber with transparent windows under elevated hydrostatic pressure conditions. A pressure of 40–60 MPa transiently reduced the vacuolar pH by approximately 0.33. A *vma3* mutant defective in vacuolar acidification showed no reduction of vacuolar pH after application of hydrostatic pressure, indicating that the transient acidification is mediated through the function of vacuolar H^+-ATPase. The vacuolar acidification was observed only in the presence of fermentable sugars, and never observed in the presence of ethanol, glycerol, or 3-*o*-methyl-glucose as the carbon source. Analysis of a glycolysis-defective mutant suggested that glycolysis or CO_2 production is involved in the pressure-induced acidification. Hydration and ionization of CO_2 is facilitated by elevated hydrostatic pressure because a negative volume change ($\Delta V < 0$) accompanies the chemical reaction. Moreover the glucose-induced cytoplasmic alkalization is inhibited by elevated hydrostatic pressure, probably because of inhibition of the plasma membrane H^+-ATPase. Therefore, the cytoplasm tends to become acidic under elevated hydrostatic pressure conditions, and this could be crucial for cell survival. To maintain a favorable cytoplasmic pH, the yeast vacuoles may serve as proton sequestrants under hydrostatic pressure. We are investigating the physiological effects of hydrostatic pressure in the course of research in a new experimental field, baro- (piezo-) physiology.

Key words Hydrostatic pressure · Fluorescene analysis · Yeast vacuole · Vacuolar acidification · Cytoplasmic pH homeostasis · Baro-(piezo-) physiology

Introduction

Hydrostatic pressure is a thermodynamic parameter that has recently received further consideration in various experimental fields. This parameter acts to decrease the total volume of a system at equilibrium in the case of liquids and solutions. The pressure effects in biological systems have been analyzed from two perspectives: (i) the physiology of deep-sea organisms and (ii) biochemical reactions as a function of pressure. Although the physicochemical basis of pressure effects is well established (Heremans 1982; Balny et al. 1989), the pressure-induced phenomena that occur in living microorganisms have not been fully defined. Many bacteria adapted to deep-sea environments, called "barophiles," have been reported, and these organisms can grow under high hydrostatic pressure conditions below 100 MPa, the pressure at the deepest point in the ocean. Gene expression under elevated hydrostatic pressure conditions has been explored extensively in barophilic bacteria in recent studies (Bartlett et al. 1995; Kato and Horikoshi 1995; Kato and Bartlett 1997).

In the yeast *Saccharomyces cerevisiae*, high hydrostatic pressure, above 100 MPa, induces cytoplasmic petite mutation (Rosin and Zimmerman 1977) and tetraploid or homozygous diploid forms (Hamada et al. 1992), or disrupts the ultrastructure of the cells (Kobori et al. 1995). Based on the latter observation, high hydrostatic pressure has been applied to food processing for sterilizing food materials without significant loss of nutrients or flavor. A short duration of heat shock induces barotolerance, allowing cells to survive at 150 MPa (Iwahashi et al. 1991). Intracellular trehalose and Hsp104 have a role in heat-shock-induced

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barotolerance (Fujii et al. 1996; Iwahashi et al. 1997). We have been analyzing the physiological effects of nonlethal levels of hydrostatic pressure below 100 MPa in yeast, focusing on cell growth, cellular metabolism, and the functions of organelles. Our recent results showed that hydrostatic pressure promotes the acidification of vacuoles (Abe and Horikoshi 1995a,b, 1996, 1997a,b) and affects hydration and ionization of carbon dioxide (CO_2) generated through ethanol fermentation, which leads to the accumulation of large numbers of protons in the cytoplasm (Abe and Horikoshi 1997a,b). Considering this background, we discuss the physiological effects of elevated hydrostatic pressure in living yeast cells focusing on intracellular pH regulation, and propose to establish a new research field "baro- (piezo-) physiology" in the area of high-pressure bioscience and biotechnology.

Hydrostatic pressure promotes the acidification of yeast vacuoles

Application of hydrostatic pressure to a logarithmic-phase culture of strain IFO2347 causes a delay in cell growth, and pressures above 40 MPa completely inhibit cell proliferation (Abe and Horikoshi 1995a). However, there is no substantial decrease in cell viability as determined by monitoring colony-forming units (CFU) when hydrostatic pressure is applied at pressures up to 60 MPa for 1 h. The pressure-induced repression of cell growth may be ascribed to inhibition of DNA replication, alteration of RNA transcription or protein synthesis, disorganization of microtubules, or deactivation of several enzymes.

The yeast vacuole is an acidic organelle and plays an important role in (i) degradation of cellular proteins, (ii) storage of amino acids and carbohydrates, and (iii) storage of cytoplasmic calcium. Acidic vacuolar pH (pH $\sim$ 6.0) is maintained through the function of vacuolar H^+-ATPase (V-H^+-ATPase) on the vacuolar membrane (Kakinuma et al. 1981; Nelson and Taiz 1989). The V-H^+-ATPase is a *N*′, *N*′-dicyclohexylcarbodiimide- (DCCD-) sensitive electrogenic proton pump that generates a proton motive force of 180 mV, inside positive and acidic, in vacuolar membrane vesicles. The low vacuolar pH is particularly important for all cellular functions mentioned. A number of biochemical and genetic studies of vacuolar functions have been reported; these have been reviewed by Anraku et al. (1992a,b). Exposure to a pressure of 40–60 MPa for 1 h reduces the vacuolar pH, as determined using the pH-sensitive fluorescent dye 6-carboxyfluorescein (6-CF) (Preston et al. 1989), from 6.05 to 5.88 in strain IFO2347 (Abe and Horikoshi 1995a) and in other *Saccharomyces* strains (Abe and Horikoshi 1995b, 1996). The pressure-induced acidification of vacuoles has been confirmed by measuring the vacuolar accumulation of the weak base quinacrine, which was known to accumulate in acidic organelles by a mechanism dependent on the pH gradient across the membrane. Bafilomycin A_1, a specific inhibitor of V-H^+-ATPase, markedly inhibited the pressure-induced acidification, indicating that the process is mediated by V-H^+-ATPase (Abe and Horikoshi 1995a).

Fluorescence analysis under high hydrostatic pressure reveals a transient acidification of vacuoles

Our next challenge was the analysis of vacuolar pH under elevated hydrostatic pressure conditions, i.e., the vacuolar pH of 6-CF-labeled cells was determined when high hydrostatic pressure was applied to the cells. Two items of equipment required were made: (i) a hydrostatic chamber with transparent windows originally designed by Morita (1957) (Fig. 1A) was slightly modified (Abe and Horikoshi 1997a) by connecting an optical cable to one of the windows to detect the fluorescence emission, and (ii) a hydrostatic chamber with three transparent windows (Fig. 1B) (Abe 1998). Fluorescence was emitted through the windows at a right angle. The early time course of changes in vacuolar pH in response to elevated hydrostatic pressure was examined. The vacuolar pH was transiently reduced by approximately 0.33 within 4 min after a pressure of 40–60 MPa was applied

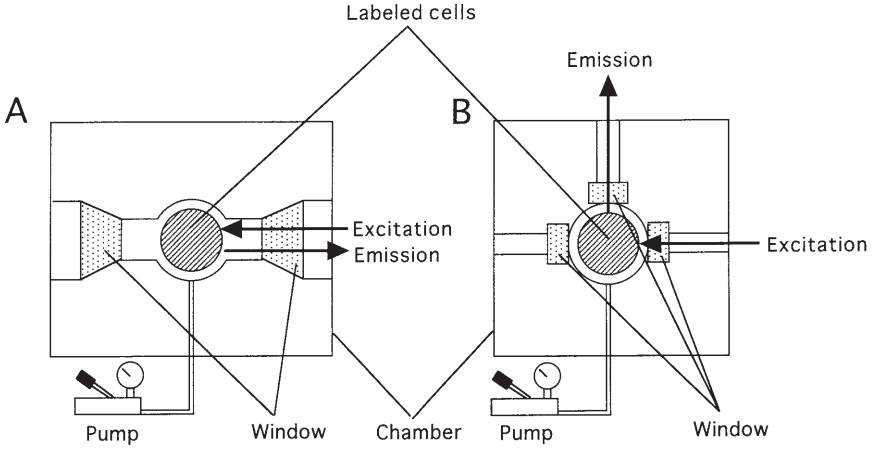

Fig. 1A,B. Diagrams of hydrostatic chambers for fluorescence analysis under elevated hydrostatic pressure conditions. Labeled cells are placed in the hydrostatic chamber, and fluorescence is emitted through the window at the same angle (**A**) or at a right angle (**B**)

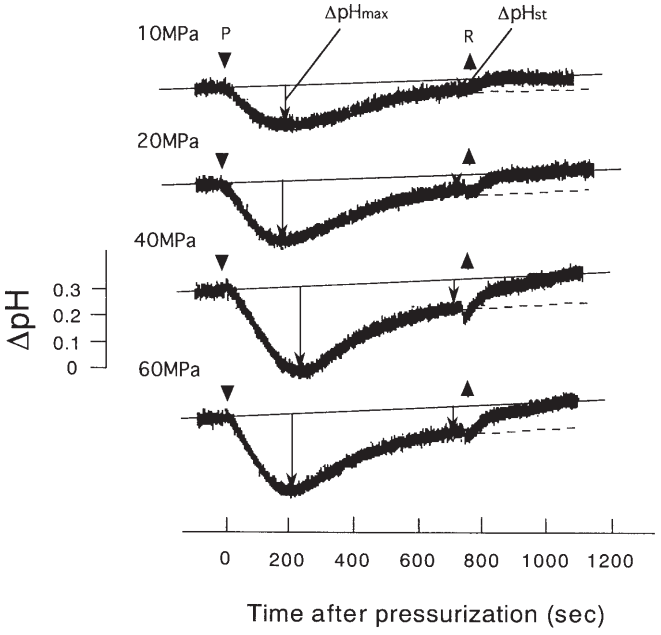

Fig. 2. Time course of vacuolar acidification after the application of hydrostatic pressure. Cells of strain X2180 were exposed to hydrostatic pressures of 10, 20, 40, and 60 MPa at *P* and subsequently removed at *R*. Δ*pHmax*, maximal change in vacuolar acidification; Δ*pHst*, steady level of acidification

(Fig. 2) (Abe and Horikoshi 1997a). After decompression, the pH gradually recovered to its original level. The *vma3* mutant lacks subunit-*c* of the V-H^+-ATPase, and this deficiency results in a vacuolar acidification defect (Umemoto et al. 1990; Tanida et al. 1995). Consequently, the mutant cells never show vacuolar acidification when hydrostatic pressure is applied (Abe and Horikoshi 1997a). This observation indicates that transient vacuolar acidification is mediated through the function of the V-H^+-ATPase and is not caused by simple changes in equilibrium of the vacuolar medium.

What primary factors influence vacuolar acidification induced by elevated hydrostatic pressure?

Transient vacuolar acidification was found to occur in the presence of fermentable sugars such as glucose, fructose, or mannose, but never when ethanol or glycerol was supplied as the carbon source (Fig. 3) (Abe and Horikoshi 1997a). Pressure-induced vacuolar acidification was examined in a glycolysis-defective mutant, ATCC90946 (DFY568; *hxk1, hxk2, GLK*) (Vojtek and Fraenkel 1990), in the presence of different hexoses. Glucose and fructose are known to be

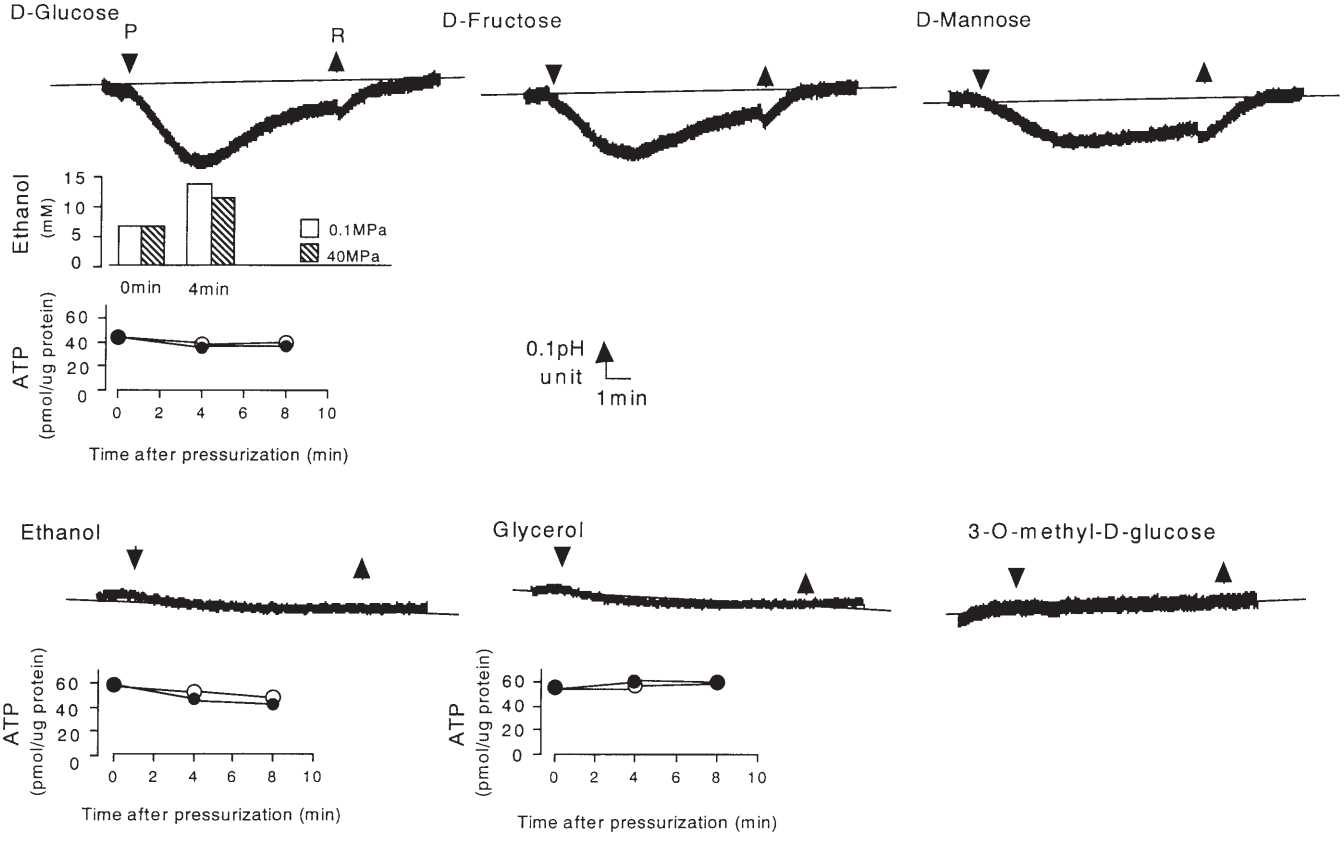

Fig. 3. Time course of vacuolar acidification, the concentration of ethanol, and the amount of cellular ATP in the presence of various carbohydrates (100 mM) after the application of hydrostatic pressure. Cells were exposed to a pressure of 40 MPa at *P* and subsequently removed at *R*. *Open circles*, cellular ATP at atmospheric pressure; *closed circles*, cellular ATP at 40 MPa

transported through the plasma membrane by the same hexose transporter. The first step in metabolism of these hexoses is phosphorylation catalyzed by a hexokinase or glucokinase. Because DFY568 cells completely lack both hexokinases PI and PII, the mutant cannot metabolize fructose. As a result, this mutant displays vacuolar acidification in the presence of glucose, but never in the presence of fructose when hydrostatic pressure is applied (Fig. 4) (Abe and Horikoshi, manuscript in preparation). These results indicate that glycolysis or ethanol fermentation is involved in the process. CO_2 and glycolytic intermediates such as glucose-6-phosphate or fructose-6-phosphate are weak acids, and possibly release protons in solution. Alterations in production or accumulation of these weak acids might have a key role in the mechanism by which hydrostatic pressure induces vacuolar acidification. The cellular content of glycolytic intermediates was determined after exposure to hydrostatic pressure for 1 h. Preliminary results suggested, however, that the content of glucose-6-phosphate, fructose-6-phosphate, or fructose-1,6-bisphosphate in cells cultured at 50 MPa is lower, in each instance, than that of cells cultured at atmospheric pressure (Abe and Horikoshi, manuscript in preparation). However, CO_2 generation in the presence of glucose at 40 MPa was approximately 75% of that produced at atmospheric pressure (see Fig. 3) (Abe and Horikoshi 1997a), which means that a considerable amount of CO_2 would be generated in the cytoplasm at 40 MPa. CO_2 is quite soluble in water; at atmospheric pressure more than 99% of aqueous CO_2 exists as the dissolved gas and less than 1% exists as carbonic acid, H_2CO_3, which partly dissociates to give H^+, HCO_3^-, and CO_3^{2-}. The reaction volume of the reaction $H_2CO_3 \rightarrow H^+ + HCO_3^-$ is negative (−26.0 ml/mol), which means that the dissociation of the weak acid is facilitated by elevated hydrostatic pressure. Therefore, large numbers of protons are likely to accumulate in the cytoplasm under elevated hydrostatic pressure conditions. It is possible that the yeast vacuole serves to take up these protons from the cytoplasm, which results in vacuolar acidification.

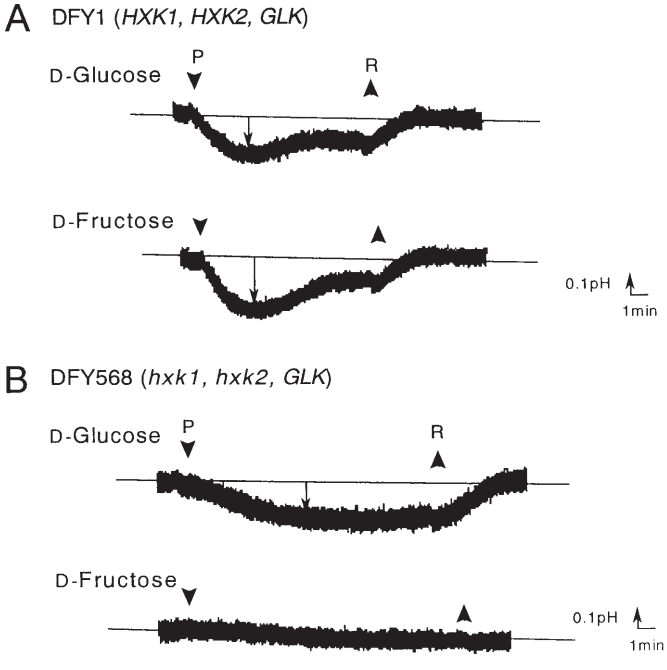

Fig. 4A,B. Time course of vacuolar acidification in a glycolysis-defective mutant after the application of hydrostatic pressure. Cells were exposed to a pressure of 40 MPa at *P* and subsequently removed at *R*. **A** Parental strain DFY1; **B** glycolysis-defective mutant DFY568

Hydrostatic pressure inhibits glucose-induced cytoplasmic alkalization

The plasma membrane ATPase (Pma1) is a P-type ATPase and is a major essential protein required for establishment of cellular membrane potential; it plays a central role in the regulation of cytoplasmic pH (Serrano et al. 1986). This ATPase is essential for cell viability and functions to maintain a suitable cytoplasmic pH (Eraso and Gancedo 1987; Serrano 1993; Van der Rest et al. 1995). When starved cells are challenged with glucose, the external medium is rapidly acidified and simultaneously cytoplasmic alkalization occurs (Haworth et al. 1991; Eraso and Portillo 1994). The efflux of protons is mediated through the direct activation of plasma membrane H^+-ATPase. Hydrostatic pressure reduces the rate of proton extrusion to an extent dependent on the magnitude of applied pressure (Abe and Horikoshi 1995b). The cytoplasmic pH, as determined using the pH-sensitive fluorescent dye carboxy SNARF-1, has been analyzed under elevated hydrostatic pressure conditions. At atmospheric pressure, glucose rapidly induces cytoplasmic alkalization (Fig. 5A). However, a pressure of 25 or 50 MPa considerably slows down the process of cytoplasmic alkalization (Fig. 5B,C) (Abe and Horishoki, manuscript in preparation). This result may be ascribed to inactivation of the plasma membrane H^+-ATPase. Meanwhile, vacuolar acidification is markedly facilitated by elevated hydrostatic pressure up to 50 MPa (Fig. 5A–C). This means that the vacuoles are acidified when cytoplasmic alkalization is inhibited, and the V-H^+-ATPase functions when the plasma membrane H^+-ATPase is inactivated by elevated hydrostatic pressure.

The yeast vacuole may serve as a proton sequestrant under hydrostatic pressure

Cytoplasmic pH tends to decrease in response to elevated hydrostatic pressure for the following reasons: (i) some of the intracellular compounds present in large amounts, such as inorganic phosphates, carbohydrate phosphates, or H_2CO_3 ($CO_2 + H_2O$), may release protons because negative volume changes ($\Delta V < 0$) accompany the ionizing reactions; and (ii) the plasma membrane H^+-ATPase seems be inactivated by elevated hydrostatic pressure. Low cytoplasmic pH could slow down glycolysis, because pH changes

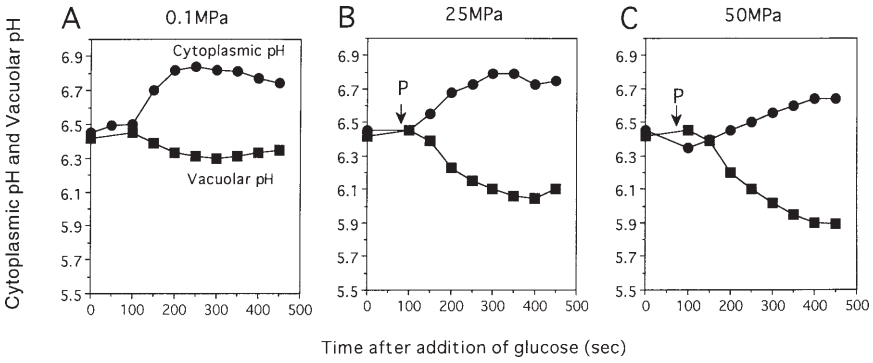

Fig. 5A–C. Time course of cytoplasmic alkalization and vacuolar acidification after addition of glucose at atmospheric pressure (**A**) or under elevated hydrostatic pressure (**B, C**). Cells were exposed to a pressure 25 or 50 MPa at *P*. Changes in cytoplasmic pH (*circles*) and vacuolar pH (*squares*) were analyzed under hydrostatic pressure

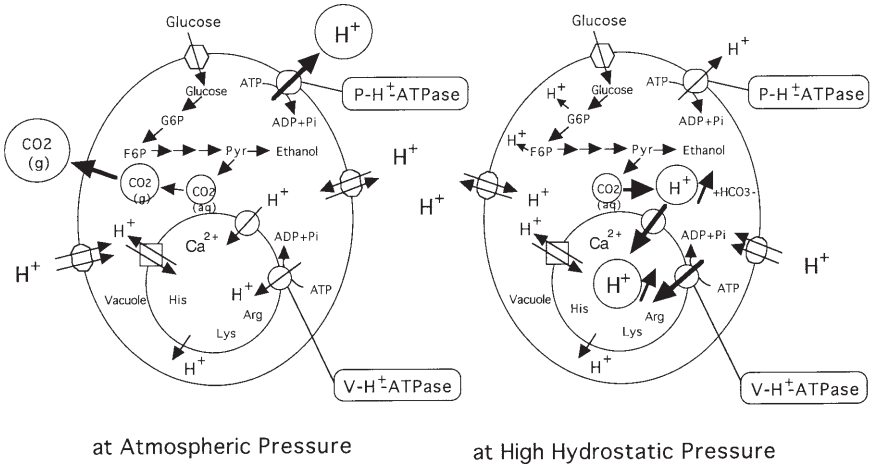

Fig. 6. A model proposed to explain the intracellular events under elevated hydrostatic pressure conditions. Hydration and dissociation of CO_2 are facilitated by elevated hydrostatic pressure, which leads to accumulation of protons in the cytoplasm. To maintain a favorable cytoplasmic pH, the yeast vacuoles may serve as proton sequestrants under elevated hydrostatic pressure conditions

readily affect the activity of a key enzyme of the glycolytic pathway, phosphofructokinase (Hofmann and Kopperschlager 1982). Figure 6 shows a model proposed to explain the intracellular events under elevated hydrostatic pressure conditions. To maintain a favorable cytoplasmic pH, the yeast vacuoles may serve as proton sequestrants under hydrostatic pressure. It is still unclear why the plasma membrane H^+-ATPase seems to be more sensitive than the V-H^+-ATPase to elevated hydrostatic pressure. Hydrostatic pressure is known to affect lipid–protein interactions in P-type ATPases, such as Na^+, K^+-ATPase (De Smedt et al. 1979) or Ca^{2+}-ATPase (Heremans and Wuytack 1980), decreasing the activities. It is worthwhile analyzing the pressure dependence of the transition temperature on the activities of the plasma membrane H^+-ATPase or the V-H^+-ATPase. Although further experimentation is required to fully explain the significance of the vacuolar contribution to regulation of cytoplasmic pH under elevated hydrostatic pressure conditions, concerning cell growth or viability, the findings so far suggest that chemical reactions involving intracellular low molecular weight compounds should be noted to elucidate the physiological responses of living organisms to elevated hydrostatic pressure.

References

Abe F (1998) Hydrostatic pressure enhances vital staining with carboxyfluorescein or carboxydichlorofluorescein in *Saccharomyces cerevisiae*: efficient detection of labeled yeasts by flow cytometry. Appl Environ Microbiol, 64:1139–1142

Abe F, Horikoshi K (1995a) Hydrostatic pressure promotes the acidification of vacuoles in *Saccharomyces cerevisiae*. FEMS Microbiol Lett 130:307–312

Abe F, Horikoshi K (1995b) Effect of hydrostatic pressure on the yeast vacuole. In: Trzeciakowski WA (ed) High pressure science and technology. World Scientific, Singapore, pp 875–877

Abe F, Horikoshi K (1996) Vacuolar acidification under high hydrostatic pressure in *Saccharomyces cerevisiae*. In: Hayashi R, Balny C (eds) High pressure bioscience and biotechnology, vol 13. Elsevier, Amsterdam, pp 53–58

Abe F, Horikoshi K (1997a) Vacuolar acidification in *Saccharomyces cerevisiae* induced by elevated hydrostatic pressure is transient and is mediated by vacuolar H^+-ATPase. Extremophiles 1:89–93

Abe F, Horikoshi K (1997b) Yeast vacuoles may serve as proton sequestrants under high hydrostatic pressure. In: Heremans K (ed) High pressure research in the biosciences and biotechnology. Leuven University Press, Leuven, Belgium, pp 209–212

Anraku Y, Hirata R, Wada Y, Ohya Y (1992a) Molecular genetics of the yeast vacuolar H^+-ATPase. J Exp Biol 172:67–81

Anraku Y, Umemoto N, Hirata R, Ohya Y (1992b) Genetic and cell biological aspects of the yeast vacuolar H^+-ATPase. J Bioenerg Biomembr 24:395–405

Balny C, Masson P, Travers F (1989) Some recent aspects of the use of high pressure for protein investigations in solution. High Press Res 2:1–28

Bartlett DH, Kato C, Horikoshi K (1995) High pressure influences on gene and protein expression. Res Microbiol 146:697–706

De Smedt H, Borghgraef R, Ceuterick F, Heremans K (1979) Pressure effects on lipid-protein interactions in ($Na^+ + K^+$) ATPase. Biochim Biophys Acta 556:479–489

Eraso P, Gancedo C (1987) Activation of yeast plasma membrane ATPase by acidic pH during growth. FEBS Lett 224:187–192

Eraso P, Portillo F (1994) Molecular mechanism of regulation of yeast plasma membrane H (+)-ATPase by glucose. Interaction between domains and identification of new regulatory sites. J Biol Chem 269:10393–10399

Fujii S, Iwahashi H, Obuchi K, Fujii T, Komatsu Y (1996) Characterization of a barotolerant mutant of the yeast *Saccharomyces cerevisiae*: importance of trehalose content and membrane fluidity. FEMS Microbiol Lett 141:97–101

Hamada K, Nakatomi Y, Shimada S (1992) Direct induction of tetraploids or homozygous diploids in the industrial yeast *Saccharomyces cerevisiae* by hydrostatic pressure. Curr Genet 22:371–376

Haworth R, Lemire BD, Crandall D, Cragoe EJ Jr, Fiegel L (1991) Characterization of proton fluxes across the cytoplasmic membrane of the yeast *Saccharomyces cerevisiae*. Biochim Biophys Acta 1098:79–89

Heremans K (1982) High pressure effects on proteins and other biomolecules. Annu Rev Biophys Bioeng 11:1–21

Heremans K, Wuytack F (1980) Pressure effect on the Arrhenius discontinuity in Ca^{2+}-ATPase from sarcoplasmic reticulum: evidence for lipid involvement. FEBS Lett 117:161–163

Hofmann E, Kopperschlager G (1982) Phosphofructokinase from yeast. Methods Enzymol 90:49–60

Iwahashi H, Kaul SC, Obuchi K, Komatsu Y (1991) Induction of barotolerance by heat shock treatment in yeast. FEMS Microbiol Lett 80:325–328

Iwahashi H, Obuchi K, Fujii S, Komatsu Y (1997) Effect of temperature on the role of Hsp104 and trehalose in barotolerance of *Saccharomyces cerevisiae*. FEBS Lett 416:1–5

Kakinuma Y, Ohsumi Y, Anraku Y (1981) Properties of H^+-translocating adenosine triphosphatase in vacuolar membranes of *Saccharomyces cerevisiae*. J Biol Chem 256:10859–10863

Kato C, Bartlett DH (1997) The molecular biology of barophilic bacteria. Extremophiles 1:111–116

Kato C, Horikoshi K (1995) Gene expression under hydrostatic pressure. In: Hayashi R, Balny C (eds) High pressure bioscience and biotechnology, vol 13. Elsevier, Amsterdam, pp 59–66

Kobori H, Sato M, Tameike A, Hamada K, Shimada S, Osumi M (1995) Ultrastructure effects of pressure stress to the nucleus in *Saccharomyces cerevisiae*: a study by immunoelectron microscopy using frozen thin sections. FEMS Microbiol Lett 132:253–258

Morita RY (1957) Effect of hydrostatic pressure on succinic formic, and malic dehydrogenases in *Escherichia coli*. J Bacteriol 74:251–255

Nelson N, Taiz L (1989) The evolution of H^+-ATPases. Trends Biochem Sci 14:113–116

Preston RA, Murphy RF, Jones EW (1989) Assay of vacuolar pH in yeast and identification of acidification-defective mutants. Proc Natl Acad Sci USA 86:7027–7031

Rosin MP, Zimmerman AM (1977) The induction of cytoplasmic petite mutants of *Saccharomyces cerevisiae* by hydrostatic pressure. J Cell Sci 26:373–385

Serrano R (1993) Structure, function and regulation of plasma membrane H(+)-ATPase. FEBS Lett 325:108–111

Serrano R, Kielland-Brandt MC, Fink GR (1986) Yeast plasma membrane ATPase is essential for growth and has homology with ($Na^+ + K^+$), K^+ and Ca^{2+}-ATPase. Nature (Lond) 319:689–693

Tanida I, Hasegawa A, Iida H, Ohya Y, Anraku Y (1995) Cooperation of calcineurin and vacuolar H^+-ATPase in intracellular Ca^{2+} homeostasis of yeast cells. J Biol Chem 270:10113–10119

Umemoto N, Yoshihisa T, Hirata R, Anraku Y (1990) Roles of the *VMA3* gene product, subunit c of the vacuolar membrane H^+-ATPase on vacuolar acidification and protein transport. J Biol Chem 265:18447–18453

Van der Rest ME, Kamminga AH, Nakano A, Anraku Y, Poolman B, Konings WN (1995) The plasma membrane of *Saccharomyces cerevisiae*: structure, function, and biogenesis. Microbiol Rev 59:304–322

Vojtek AB, Fraenkel DG (1990) Phosphorylation of yeast hexokinases. Eur J Biochem 190:371–375