Water channels are important for osmotic adjustments of yeast cells at low temperature

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The importance of aquaporin expression in water permeability in *Saccharomyces cerevisiae* was assessed by measuring the osmotic water permeability coefficient (P_f) and the activation energies (E_a) from both hypo- and hypertonic experiments performed with whole protoplasts from four strains differing in aquaporin level of expression: parental, double-deleted and overexpressing AQY1 or AQY2. Double-deleted (lower P_f) and AQY1-overexpressing strains (higher P_f) presented linear Arrhenius plots with E_a consistent with fluxes mainly through the lipids [$16.3 \text{ kcal mol}^{-1}$ (68.2 kJ mol^{-1})] and with a strong contribution of channels [$9.6 \text{ kcal mol}^{-1}$ (40.2 kJ mol^{-1})], respectively. The Arrhenius plots for the parental (swelling experiments) and overexpressing AQY2 strains (swelling and shrinking experiments) were not linear, presenting a break point with a change in slope around 23°C . The E_a values for these strains, calculated for temperatures ranging from 7 to 23°C , were lower [$9.5 \text{ kcal mol}^{-1}$ (39.7 kJ mol^{-1})] than the values obtained from 23 to 38°C [17 kcal mol^{-1} (71.1 kJ mol^{-1})]. This behaviour indicates that only in the lower temperature range did the water fluxes occur predominantly via the water channels. The permeabilities for each strain relative to the deletion strain show that an increase in permeability due to the presence of aquaporins was more relevant at low temperatures. Following our results, we propose that water channels play an important role for osmotic adjustment of yeast cells at low temperature.

Received 9 November 2005

Revised 20 January 2006

Accepted 3 February 2006

INTRODUCTION

The first experimental evidence for the existence of water channels in cell membranes was obtained for red blood cells (Farmer & Macey, 1970; Macey *et al.*, 1972; Paganelli & Solomon, 1957; Vieira *et al.*, 1970) and renal epithelia (Verkman, 1992), and was based mainly on measures of high osmotic water permeability (inhibited by mercurial reagents) and of low activation energy for water transport. In 1992 Agre and co-workers revealed that a 28 kDa integral membrane protein was the erythrocyte water channel and that the same protein was also present in renal epithelia (Preston *et al.*, 1992). This protein is now known as aquaporin 1 (Aqp1) and it belongs to a large family of proteins that are widely distributed in nature. In mammals and plants they have important functions in water homeostasis and

osmoregulation of individual cells and whole organisms. In micro-organisms, aquaporins were also recognized but their physiological role is yet being explored.

Analysis of the *Saccharomyces cerevisiae* genome revealed four ORFs encoding members of the major intrinsic protein (MIP) family (Bonhivers *et al.*, 1998): *FPS1* and *YFL054c* related to glycerol facilitator homologues (genetically close to the aquaglyceroporins), and *AQY1* and *AQY2*, more closely related to aquaporin water channels. The functionality of aquaporins in *S. cerevisiae* has been debated in the literature. To study the polymorphism of aquaporins in yeast, a survey on 52 strains was performed, revealing that all strains carry an allele encoding a functional Aqp1p, but in none of these strains did the Aqp2p seem to be functional, with the exception of strain $\Sigma 1278$ (Laizé *et al.*, 2000). The authors considered the hypothesis that selective pressure kept an active Aqp1p, but not Aqp2p, in yeast.

Abbreviation: CFDA, carboxyfluorescein diacetate.

Expression of *AQY1* in *Xenopus* oocytes showed an active Aqp1p, mediating water transport (Bonhivers *et al.*, 1998), while the expression of *AQY2* from strain $\Sigma 1278$ was not detectable. Nevertheless, evidence that Aqp2p was expressed and functional in vesicles derived from strain $\Sigma 1278$ cells has been presented, while the expression of Aqp1p was not detectable (Meyrial *et al.*, 2001). By immunocytochemistry and biochemical subcellular fractionation it was found that Aqp2p was located in the endoplasmic reticulum and in the plasma membrane. Under the experimental conditions tested, *AQY2* was expressed only in rich medium, during exponential growth, when glucose was the energy source. The expression of *AQY2* was repressed by ethanol or galactose in rich medium, by minimal medium, hyperosmolar medium and by sporulating conditions.

Recently, it was shown that Aqp1p was involved in sporulation (Sidoux-Walter *et al.*, 2004) and that this could be its only function. The authors claimed that Aqp1p was detectable exclusively in spore membranes under ambient conditions and that the lack of Aqp1p reduced spore fitness.

An important role for aquaporins was found in the increase of freeze tolerance in baker's yeast (Tanghe *et al.*, 2002, 2004). Deletion of the genes *AQY1* and *AQY2* rendered a laboratory strain more sensitive to freezing, while overexpression of these genes improved freeze tolerance. In these transformants growth and fermentation characteristics were not affected as compared with the parental strains, but the improvement of freeze-thaw resistance was so notorious that a transformant was selected based on this characteristic (Tanghe *et al.*, 2002). This phenotype was observed only when freezing occurred rapidly (Tanghe *et al.*, 2004).

Surprisingly, it was found that when spores are rapidly frozen, those that lack Aqp1p survive better, providing for a functional test of active spore water channels (Sidoux-Walter *et al.*, 2004). Comparing all these recent works (Sidoux-Walter *et al.*, 2004; Tanghe *et al.*, 2002, 2004), the amazing conclusion is that water channels improve freeze tolerance in vegetative cells and reduce freeze tolerance in spores.

The purpose of our work was to evaluate the role of aquaporins in plasma membrane water permeability of yeast strains for which the effect of aquaporins on freeze tolerance has been documented. We approached this problem by measuring activation energies of osmotic water transport in protoplasts prepared from *S. cerevisiae* $\Sigma 1278$ b-derived parental strain 10560-6B, and from three derivatives of this strain, one where *AQY1* and *AQY2* were deleted and two others where either *AQY1* or *AQY2* was overexpressed. These protoplast populations were submitted to hypo- and hyperosmotic shocks with an impermeant solute at different temperatures; the time dependence for volume equilibration was followed and water permeability coefficients evaluated.

METHODS

Yeast strains and plasmids used in this study. Strains used in this work were 10560-6B *MAT α leu2::hisG trp1::hisG his3::hisG ura3-52*; 10560-6B/pYX012 *KanMX* (=strain ANT29, indicated as parental); 10560-6B/pYX012 *KanMX AQY1-1* (=strain ANT27, indicated as overexpressing *AQY1*); 10560-6B/pYX012 *KanMX AQY2-1* (=strain ANT26, indicated as overexpressing *AQY2*); 10560-6B *aqy1::KanMX4 aqy2::HIS3* (=strain YSH 1172, indicated as double deleted).

Integrative plasmid pYX012 (Novagen) was modified with a dominant marker gene by cloning the *EcoRV*–*PvuII* fragment containing the *loxP*–*KanMX4*–*loxP* cassette from pUG6 (Güldener *et al.*, 1996) in the *URA3* marker (*StuI* restriction site), resulting in plasmid pYX012 *KanMX*. The aquaporin ORF *AQY1-1* was PCR-amplified using primers *AQY1*-FP (5'-GCGAATTCCTTAACATAACATGTCTTCGAACG-3'; *EcoRI* site underlined) and *AQY1*-RP (5'-CACCCGGGAA-AAACACTAATTACCTCAGTAG-3'; *XmaI* site underlined) on genomic DNA of strain 10560-6B. The resulting fragments were cloned into pYX012 *KanMX* downstream of the constitutive *TPH1* promoter using *EcoRI* and *XmaI* restriction sites. The aquaporin ORF *AQY2-1* was subcloned from pYX242/*AQY2-1* (Meyrial *et al.*, 2001) in pYX012 *KanMX* using *EcoRI* and *BamHI* restriction sites. Integration of *SphI*-linearized pYX012 *KanMX/AQY1-1* or *AQY2-1* at the *TPH1* locus resulted in geneticin-resistant strains of 10560-6B, overexpressing *AQY1-1* and *AQY2-1* respectively.

Preparation of yeast protoplasts. Cells were grown in YPD medium [1% (w/v) peptone, 0.5% (w/v) yeast extract, 2% (w/v) glucose], with orbital shaking, at 28 °C. Protoplasts were prepared essentially as described by Faye *et al.* (1974). Cells were harvested in the exponential phase ($OD_{640} \approx 1$) by centrifugation (18 000 g) for 5 min at 4 °C, and washed once with 1.2 M sorbitol, after which 10 g (wet wt) was resuspended in 30 ml digestion buffer and incubated in an orbital shaking water bath at 30 °C. To prepare the digestion buffer, 30 ml 2 M sorbitol was mixed with 3 ml 1 M potassium phosphate buffer, pH 7.5; 0.1 ml 0.5 M EDTA; 1.25 ml 0.25 mM dithiothreitol; 15.25 ml water; and 2 units zymolase 100T ml⁻¹. When the digestion was complete, 150 ml of a 1.2 M sorbitol solution was added to the suspension, which was centrifuged at 3000 g for 10 min (4 °C). The pellet was then washed twice with 150 ml 1.2 M sorbitol. The protoplasts were resuspended in 1.2 M sorbitol. The concentration of 1.2 M sorbitol was chosen throughout the preparation of protoplasts, because disruption of the protoplasts was observed when they were suspended in lower concentrations of sorbitol. In 0.8 M sorbitol many disrupted membranes were observed under the microscope and only at 1 M did most protoplasts remain intact. The concentration of 1.2 M guarantees that protoplasts will not burst during swelling experiments.

For fluorescence experiments, protoplasts were pre-loaded for 10 min at 30 °C with the non-fluorescent precursor 5-(and 6)-carboxyfluorescein diacetate (CFDA, 1 mM in iso-osmotic solution) prior to the osmotic challenges in the stopped-flow apparatus. To avoid pH interference in fluorescence, cell suspensions and osmotic solutions were prepared in 50 mM K⁺-citrate buffer, pH 5.

Protoplast size determination. Protoplast size (initial equilibrium volume) of all the preparations tested was determined by quasi-elastic light scatter (QELS) using a particle sizer (BI-90 Brookhaven Instruments). An application of this technique in the determination of vesicular sizes has been published (Perevucnik *et al.*, 1985).

The protoplast preparations were homogeneous and protoplasts were spherical in shape, as observed under light microscopy. The QELS technique for measuring the initial diameter has shown unimodal populations for all the preparations with a mean value of 1.9 ± 0.2 μ m.

Stopped-flow experiments

Experiments were performed on a HI-TECH Scientific PQ/SF-53 stopped-flow apparatus, which has a 2 ms dead time and is temperature controlled, interfaced with an IBM PC/AT compatible 80386 microcomputer. Experiments were performed at temperatures ranging from 7 to 38 °C. Three runs were usually stored and analysed in each experimental condition. In each run 0.1 ml protoplast suspension [1:20 dilution in preparation buffer, initial osmolarity (osm_{out}^0) = 1.2 M] was mixed with an equal amount of iso-, hypo- or hyper-osmotic sorbitol solutions to reach different out or inwardly directed gradients of solute. The time-course of volume change was followed by light scatter and/or fluorescence of a trapped fluorophore.

Light-scatter experiments. The time-course of 90° scattered light intensity at 400 nm was followed until a stable light-scattered signal was obtained (1024 data points were acquired for periods ranging from 15 to 90 s).

Fluorescence experiments. A concentration-dependent self-quenching fluorophore was used to monitor cell volume changes. Carboxyfluorescein (CF) was excited using a 470 nm interference filter and detected using a >530 nm cut-on filter. Cells were subjected to hyper- and hypo-osmotic shocks following the same protocol applied for the light-scatter experiments, and changes in CF fluorescence due to fluorescence quenching were followed until a stable signal was obtained.

Signals were recorded on a computer interfaced directly with the Hi-Tech SF-53 using dedicated software (Hi-Tech Scientific) and were fitted to a single exponential from where the total signal amplitude and the rate constant K were evaluated.

P_f evaluation from the rate constant of volume equilibration.

The osmotic water permeability coefficient P_f can be estimated from the linear relationship between P_f and K (van Heeswijk & van Os, 1986), $P_f = K(V_0/A)(1/V_w(osm_{out}^\infty))$, where V_w is the molar volume of water, V_0/A is the initial volume to area ratio of the protoplast population, and (osm_{out}^∞) is the final medium osmolarity after the osmotic shock.

The activation energy (E_a) of water transport was evaluated from the slope of the Arrhenius plot ($\ln P_f$ as a function of $1/T$).

RESULTS

To determine the importance of aquaporins in water permeability, we measured the osmotic water coefficient (P_f) in four *S. cerevisiae* strains: parental, double-deleted and overexpressing AQY1 or AQY2. The experiments were performed under hypo- or hypertonic conditions at different temperatures using the stopped-flow technique.

Water permeability measurements

Fig. 1 shows records of a typical stopped-flow experiment (obtained with AQY2 overexpressing strain) where the light-scatter intensity from a protoplast suspension suddenly exposed to three different solutions (iso-, hyper- and hypo-osmotic) was followed for 20 s. The three curves are identified by the tonicity of the shock (Λ), defined as the ratio of the final to initial osmolarity of the outside medium, i.e. $\Lambda = (osm_{out}^\infty)/(osm_{out}^0)$. Fig. 1 insert shows the linear dependence of the signal amplitude on $1/\Lambda$, obtained from these stopped-flow traces. For all protoplast preparations,

Fig. 1. Record of a typical stopped-flow experiment where the light scatter intensity from a protoplast suspension (AQY2-over-expressing strain) equilibrated in 1.2 M sorbitol solution was suddenly exposed to different osmotic shocks ($\Lambda = 0.75$, 1 and 1.25). Data acquisition was followed for 20 s at 23 °C. Insert: signal amplitudes as a function of the $1/\Lambda$, obtained from the previous traces.

similar traces were obtained and linear relations between the total amplitude and $1/\Lambda$ were also obtained for all the temperatures tested. The time-dependent volume changes at 23 °C for the different strains subjected to the same osmotic shocks ($\Lambda = 0.75$, or $\Lambda = 1$ for the baseline) are shown in Fig. 2. These traces show similar time constants for the deletion and parental strains, while strains harbouring overexpression constructs for AQY1 or AQY2 show higher rate constants. Equivalent symmetrical traces were obtained for hypertonic shocks of $\Lambda = 1.25$ (data not shown). Each strain presented equivalent P_f values for both hypo- and hypertonic shocks at all temperatures tested. The mean P_f ($\times 10^4$ cm s⁻¹) values ($\pm$ SD) at 23 °C were 7.28 ± 1.07 and 5.13 ± 0.59 for the strains overexpressing AQY1 and AQY2, respectively, and 1.88 ± 0.25 and 2.02 ± 0.20 for the parental and deletion strain, respectively.

To assure that other light-scattering artefacts not related to volume changes did not contribute to the light-scattering signal, we validated this methodology on yeast protoplasts with fluorescence measurements applied to the AQY1 strain. The fluorophore used is an ester form of CFDA, which is membrane permeable but of low fluorescence until cleaved intracellularly by non-specific esterases to give the impermeable fluorescent form. As the cells shrink or swell in response

Fig. 2. Light-scatter signals obtained for the four yeast strains subjected to the same osmotic shock at 23 °C. $\Lambda = 1$ (baseline) and $\Lambda = 0.75$.

to osmotic changes, the concentration of the entrapped fluorophore will rise or fall with a consequent change in fluorescence output (Chen & Knutson, 1988); the concentration-dependent self-quenching properties of the fluorophore enable cell volume changes to be recorded as changes in fluorescence.

Applying in these fluorescence experiments the same osmotic shocks used in the light-scatter experiments ($\Lambda = 0.75$, 1 and 1.25), it was observed that, at all the temperatures tested, the rate constants for volume equilibration obtained by this approach were similar to the rate constants obtained by light scatter, giving similar P_f values. Table 1 compares the P_f values obtained at 23 °C by the two different methods and for the lower and higher temperature tested. These results demonstrate that for small perturbations, the light scatter is an adequate methodology to follow protoplast volume changes and perform measurements of osmotic water permeability in yeasts.

Activation energies

The activation energy (E_a) for water transport was estimated from Arrhenius plots for the four populations tested. Fig. 3 shows the plots obtained for the four strains for swelling and shrinking respectively when P_f was evaluated using the light-scatter technique. The deletion strain and the strain with overexpression of AQY1 show a linear plot with a lower E_a value for the overexpressing AQY1 strain (confirmed in fluorescence experiments, data not shown). The shrinking and swelling experiments for the parental strain showed different behaviours. A single straight line could only be fitted to the shrinking data points with E_a value equivalent to the deletion strain (Fig. 3).

Table 1. P_f values (cm s^{-1}) obtained by light scattering and fluorescence for the AQY1-overexpressing strain

Temperature (°C)	$10^{-4} \times P_f$ light scatter (cm s^{-1})	$10^{-4} \times P_f$ fluorescence (cm s^{-1})
7	2.99 ± 0.26	3.11 ± 0.39
23	6.32 ± 0.15	6.42 ± 0.74
38	15.60 ± 2.03	17.11 ± 1.50

The protoplasts derived from the parental (swelling experiments) and AQY2 overexpression strain (swelling and shrinking experiments) showed a different behaviour for

Fig. 3. Arrhenius plots for the four strains tested: Δ , AQY1; $\bullet$, AQY2; $\circ$, parental; $\blacksquare$, deletion. (a) Hypotonic conditions, swelling experiments; (b) hypertonic conditions, shrinking experiments. All the experimental points are presented as mean values $\pm$ SD of triplicates.

Table 2. Activation energies for swelling experiments

The strains used were 10560-6B/pYX012 *KanMX* (parental); 10560-6B/pYX012 *KanMX AQY1-1* (*AQY1*); 10560-6B/pYX012 *KanMX AQY2-1* (*AQY2*); 10560-6B *aqy1::KanMX4 aqy2::HIS3* (deletion).

Strain	Activation energy [kcal mol ⁻¹ (kJ mol ⁻¹)]	
	7–23 °C	23–38 °C
Parental	9.5 (39.7)	17.8 (74.5)
<i>AQY1</i>	9.6 (40.2)	9.6 (40.2)
<i>AQY2</i>	9.5 (39.7)	17.1 (71.5)
Deletion	16.3 (68.2)	16.3 (68.2)

different temperature spans. The E_a values calculated for temperatures ranging from 7 to 23 °C were lower than the values obtained from 23 to 38 °C. The E_a values obtained for the lower temperature range were similar to the values obtained for the *AQY1* strain, while the values obtained for the higher temperature range were equivalent to those for the deletion strain (Table 2).

Table 3 presents the P_f values for all the strains obtained for the lowest and highest temperatures tested (7 and 38 °C). For both temperatures the lowest value was obtained for the deletion strain. The table also shows the permeabilities for each strain relative to the deletion strain, calculated as the ratio $P_f/P_f^{\text{deletion}}$. This ratio shows that the increase in permeability due to the presence of aquaporins becomes more relevant at low temperatures.

DISCUSSION

Water may cross cell membranes by two different parallel pathways with distinct mechanisms for permeation: partition/diffusion of water molecules across the hydrophobic bilayer (high activation energy for transport) and single file diffusion of water molecules through the channels (low activation energy). The flux of water across the membrane is the sum of the fluxes through these two pathways and the

resulting activation energy depends on the relative contribution of each pathway to the total flux. In order to establish which pathway is dominant in a particular cellular system, measures of the activation energy for osmotic water transport are usually performed. High values suggest that the lipid pathway predominates while lower values indicate the presence of functional aquaporins in these cells.

All our experiments were performed in protoplasts derived from strains with different levels of expression of aquaporins and they responded osmotically to the different tonicity shocks (hypo- and hypertonic, Fig. 1 and Fig. 2), rendering these protoplasts a useful model to study water transport in yeast. For small volume perturbations, the light-scatter technique has proved to be an adequate method to evaluate the osmotic permeability coefficient as the use of an independent technique (concentration-dependent quenching fluorescence) applied to the same *AQY1* strain gave equivalent values of P_f for all the temperatures tested and, consequently, an equivalent E_a value.

A similar methodology was previously used (Laizé *et al.*, 1999) when working with strain $\Sigma 1278b$ presenting P_f values much higher than those we measured. We speculate that the differences observed may be explained by different initial concentrations of sorbitol used. In the work by Laizé *et al.* (1999), the initial concentration was 0.5 M, but in our experiments we observed that an important fraction of the protoplasts was disrupted when suspended in 0.8 M sorbitol and only at 1 M did most protoplasts remain intact. We chose 1.2 M to guarantee that disruption would not occur during swelling experiments.

Our results from both hypo- and hypertonic experiments (protoplast swelling and shrinking) show that, in general, the P_f values for all the temperatures tested were higher for the *AQY1* followed by the *AQY2* overexpressing strains. Since both genes are expressed behind the same promoter, this result suggests that *Aqy1p* may be slightly more active than *Aqy2p*, although the hypothesis that this difference is due to different levels of expression cannot be discarded.

Table 3. Osmotic permeability coefficients for the lower (7 °C) and higher (38 °C) temperature limits

For each strain the P_f value is compared with the value obtained for the deletion strain at the same temperature ($P_f/P_f^{\text{deletion}}$).

Strain	7 °C		38 °C	
	$10^{-4} \times P_f$ (cm s ⁻¹)	$P_f/P_f^{\text{deletion}}$	$10^{-4} \times P_f$ (cm s ⁻¹)	$P_f/P_f^{\text{deletion}}$
Parental	0.64 ± 0.14	1.9	7.91 ± 0.43	1.1
<i>AQY1</i>	2.99 ± 0.26	8.8	15.60 ± 2.03	2.1
<i>AQY2</i>	1.74 ± 0.03	5.1	21.50 ± 2.61	2.9
Deletion	0.34 ± 0.12	1.0	7.52 ± 1.27	1.0

For the lower temperature range (7–23 °C) the parental strain showed higher P_f values than the deletion strain, representing clear evidence that at least one of the aquaporins was expressed in the parental strain under the growth conditions used for the experiments (Table 3). Nevertheless, the values for both these strains were similar for the higher temperature range tested (23–38 °C). This may indicate that the presence of aquaporins is of major importance at lower temperatures and is in agreement with the aquaporin-mediated improvement of freeze tolerance as suggested (Tanghe *et al.*, 2002). Although the concept of cold-induced improvement of freeze tolerance is highly controversial, the most recent studies on this topic are all indicative of an adaptation of yeast cells to cold stress (Alves-Araujo *et al.*, 2004; Rodriguez-Vargas *et al.*, 2002; Sahara *et al.*, 2002).

The activation energy values obtained for the deletion strain were high and compatible with water flowing mainly through the lipid bilayers as expected from the genotype of this strain. For the strain overexpressing AQY1, much lower values were found for the E_a , indicating the presence of active water channels, under all the temperatures tested (Table 2).

Interestingly, however, the Arrhenius plots for the parental (swelling experiments) and overexpressing AQY2 strains (swelling and shrinking experiments) were not linear, presenting a break point with a change in slope around 23 °C. The calculated E_a for the higher temperature range (23–38 °C) was high and only for temperatures below 23 °C was the value low (Table 2, Fig. 3). This behaviour indicates that below this temperature the water fluxes through the water channels become important.

The question is, why were the water fluxes across the channels not visible at higher temperatures? Were the water channels less active at higher temperatures or was their activity masked by the flux across the bilayer at higher temperatures? If this latter explanation is true then at the very low temperatures the contribution of the lipid pathway may even become insignificant and the activation energy could become much lower. On the other hand, and according to this hypothesis, if we could increase the temperature range to much higher values (above 40 °C), we should probably also detect a break in the AQY1 plot.

A numerical simulation of this hypothesis is shown in the Arrhenius plot of Fig. 4. The two dashed straight lines represent the typical behaviour for the lipid pathway with an activation energy of 18 kcal mol⁻¹ (75.3 kJ mol⁻¹; line with greater slope) and the channel pathway with an E_a of 4 kcal mol⁻¹ (16.7 kJ mol⁻¹; line with lesser slope). These lines will cross at a certain point corresponding to the temperature below which the channel pathway overcomes the lipid pathway (right side of the graph). This temperature can vary depending on the number of active channels present. The sum of these two contributions for the total permeability will give rise to a non-linear Arrhenius plot (solid curve). In this example, if the temperature is

Fig. 4. Numerical simulation of an Arrhenius plot. The two dashed straight lines represent the typical behaviour for the lipid pathway with an E_a of 18 kcal mol⁻¹ (75.3 kJ mol⁻¹; line with greater slope) and for the channel pathway with an E_a of 4 kcal mol⁻¹ (16.7 kJ mol⁻¹; line with lesser slope) respectively. The solid line represents the sum of both pathways for the total permeability and simulates the behaviour of all the four strains shown in Fig. 3. The experimental temperature range for each particular strain is differently positioned in the x -axis.

sufficiently high (left side of the graph) the total permeability will equal the lipid permeability with an E_a of 18 kcal mol⁻¹ (75.3 kJ mol⁻¹) while, if the temperature is sufficiently low (right side of the graph), the total permeability will be equivalent to the channel permeability with an E_a of 4 kcal mol⁻¹ (16.7 kJ mol⁻¹). In the middle temperature range, the slope varies from 18 to 4 kcal mol⁻¹ (75.3 to 16.7 kJ mol⁻¹). Within our experimental temperatures the four strains behaved differently, showing a diverse contribution of both pathways. The deletion strain had no contribution of the channel pathway, as expected. The overexpressing AQY1 was the strain with the highest contribution of the channel pathway at all temperatures tested. The overexpressing AQY2 and parental strains showed a mixed behaviour, since at high temperatures they behaved like lipid membranes but as the temperature decreased the channel pathway became visible and the activation energy decreased accordingly.

We would point out that the results were obtained with protoplasts and this delicate balance in water transport through the lipid bilayer and channels may be disturbed as compared with intact cells. Nevertheless, it is very probable that, in any case, aquaporins become more relevant for yeast behaviour at low temperatures. We should not forget that the only visible phenotypes for genotypes associated with aquaporins were found in freeze tolerance (Sidoux-Walter *et al.*, 2004; Tanghe *et al.*, 2002, 2004). If the lipid membrane

is more rigid and less permeable it makes sense that water channels become important during water volume increase and crystal formation at low temperature, preventing cell bursting (Tanghe *et al.*, 2003).

Cold stress responsiveness in plants has also been associated with aquaporins. It was shown that cold promotes the expression of the water channel γ TIP in *Tulipa gesneriana* associated with the stalk elongation (Balk & de Boer, 1999). In maize, a contribution of aquaporins to the recovery of root hydraulic conductance after chill injury was reported (Aroca *et al.*, 2005). The hypothesis that aquaporins may be involved in the cryopreservation of animal cells was also considered. Data indicate that the regulation of aquaporins can attenuate cold-induced osmotic damage in rat kidney cells (Wang & Ben, 2004).

In summary, and following our experimental results, we propose that yeast aquaporins may have a relevant function restricted to low temperature.

ACKNOWLEDGEMENTS

Strains 10560-6B and YSH1172 were kindly provided by Gerald Fink, Whitehead Institute, Cambridge, USA and Stefan Hohmann, Göteborg University, Sweden, respectively. Plasmid pYX242/AQY2-1 was kindly provided by Vincent Laizé, Göteborg University, Sweden. This work was supported by Fundação para a Ciência e a Tecnologia, POCTI/AGR/57403/2004. A. V. received a fellowship (SFRH/BPD/8548/2002) from Fundação para a Ciência e a Tecnologia. A. T. received a fellowship from the Institute for Scientific and Technological research (IWT) and the Katholieke Universiteit Leuven (PDM/02/114). We thank V. Neves and A. Madeira for technical support.

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