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Different activation energies in glucose uptake in *Saccharomyces cerevisiae* DFY1 suggest two transport systems

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Abstract

The analysis of initial glucose uptake in *Saccharomyces cerevisiae* at 25°, 20°, 15° and 10°C by computer-assisted nonlinear regression analysis predicts two transport systems. The first demonstrates Michaelis–Menten kinetics and the second shows first order behaviour. The activation energies of these two systems were calculated by the Arrhenius equation at four different growth phases, namely early exponential (EE), middle exponential (ME_2), late exponential (LE) and early stationary (ES) with 2% glucose in the batch medium. The activation energies calculated from the V_m values in EE, ME, LE and ES growth phases were 15.8 ± 1.7, 13.5 ± 1.0, 15.1 ± 0.8 and 13.5 ± 0.7 kcal/mol. These values are in agreement with activation energies calculated for the first mechanism, facilitated diffusion, which is the mechanism deduced from countertransport experiments. The activation energies derived for the second transport system from the first order rate constants in cells grown to EE, ME_2, LE and ES were 8.0 ± 2.1, 8.1 ± 1.3, 9.6 ± 3.0 and 7.5 ± 2.6 kcal/mol. These values are still significantly higher than for free diffusion of glucose in water and lower as predicted for passage of glucose through the lipid phase. Therefore, we assume in addition to carrier-mediated facilitated diffusion the entrance of glucose into the cell through a pore.

Keywords: Glucose transport; Kinetics; Temperature dependence; Activation energy; (*S. cerevisiae*)

1. Introduction

In a recent publication Reifenberger et al. [1] described the most relevant HXT transporters from *Saccharomyces cerevisiae*. Kruckeberg [2] reported about the yeast genome project, that the genome contains twenty hexose transporter genes. In protein data banks the notation is commonly made for glucose transporters in *S. cerevisiae* that glucose entrance is thought to be mediated by two kinetically distinct systems, a glucose-repressible 'high affinity system' and a constitutive 'low affinity system'. This assumption was made from glucose uptake experiments in *S. cerevisiae* cells using only 5-s uptake periods at 30°C over a range from 0.2 to 200 mM substrate concentrations. The data were plotted according to the Eadie–Hofstee equation and analysed graphically by drawing two lines through the quasi-linear portions of the curve. In *S. cerevisiae* wild-type strain DFY1 for example [3] two different apparent K_m values of 1.5 ± 0.25 ('high affinity system') and 20 ± 8 mM ('low affinity system') were reported. Despite of publishing the misuse of graphical analysis in nonlinear sugar transport kinetics by Eadie–Hofstee plots [4] there is still a discussion about the significance of these high and low affinity transport systems. Recently Coons et al. [5] have presented data on 10-s uptake periods at 30°C in the *S. cere-*

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visiae strain YPH500 with cells grown glucose-derepressed (0.05% glucose) or glucose-repressed (2% glucose). By using computer-assisted nonlinear regression analysis they offer evidence for a multicomponent transport system with at least two components. One is consistent with a high affinity carrier glucose-derepressed with K_m values of 0.67 ± 0.07 mM or glucose-repressed system with 2.15 ± 0.23 mM. The other is in their hands a low affinity carrier with 27.64 ± 3.55 or 43.4 ± 2.85 mM K_m values, respectively. The mechanisms for the two systems predicted were not investigated. Instead it was claimed [5] that the nonlinear regression analysis under glucose-derepressing conditions results in high and low affinity constants remarkably close to those previously estimated from Eadie–Hofstee plots of similar data (K_m values of 0.67 ± 0.07 correspond to 1.5 ± 0.25 and 27.64 ± 3.55 to 20 ± 8 mM). This remark made by the authors implies that their former incorrect method of graphical analysis of Eadie–Hofstee plots [3] has been confirmed by the new method.

In this study we investigated the mechanism of glucose transport in *S. cerevisia*e strain DFY1. Two kinds of transport experiments were used, namely countertransport of glucose in plasma membrane vesicles and initial glucose uptake in intact cells in 5-s periods at 25°, 20°C and 10-s periods at 15° and 10°C. The plasma membrane vesicles were derived from DFY1 cells grown on 2% glucose (glucose-repressed) or 2% galactose (derepressed) to the middle of the exponential growth phase (ME_1). The presence of countertransport of glucose is a clear-cut diagnostic sign for the presence of facilitated diffusion (Michaelis–Menten kinetics). The studies of initial glucose uptake in intact cells at different temperatures introduce the possibility to calculate activation energies that give information about the resistance encountered as the transported molecule enters and traverses the plasma membrane. The DFY1 cells were grown for this purpose with 2% glucose to the early (EE), middle (ME_2) and late exponential (LE) and finally to the early stationary (ES) growth phase. Our investigations on the activation energies in intact DFY1 cells by initial uptake experiments indicate in addition to facilitated diffusion transport a second transport mechanism which is first order kinetic and by its low activation energy demonstrates the characteristics of a pore.

2. Materials and methods

2.1. Strain and growth conditions

S. cerevisiae strain DFY1 was obtained from Dr. D.G. Fraenkel, Boston, MA, USA. The cells were grown in batch cultures under aeration at 30°C. The composition of the growth medium was 2% peptone, 1% yeast extract and 2% glucose or 2% galactose. Growth phases were determined by counting the cells in a Thoma-counting chamber. Cell growth started from a stationary preculture of about 10^7 cells/ml. At growth phases early exponential (EE) there were approximately $3 \cdot 10^7$ cells/ml, middle exponential (ME_1 to ME_2) 4 to $5 \cdot 10^7$ cells/ml, at late exponential (LE) $8 \cdot 10^7$ cells/ml and early stationary 10^8 cells/ml.

2.2. Vesicle preparation

For vesicle preparation cells were harvested by centrifugation at the middle of the exponential growth phase. Plasma membrane vesicles were prepared as described by Kreutzfeldt and Fuhrmann [6].

2.3. Countertransport experiments

Countertransport with 2% plasma membrane vesicles was carried out under ice bath conditions in 0.4 M KCl solution adjusted to pH 4.5. As shown by Fuhrmann et al. [7] the plasma membrane vesicles are only sealed between pH 4 and 5 and diffusion of glucose is negligibly small in countertransport experiments. The amount of glucose in vesicles was analysed by ^{14}C-labeled glucose. Separation of the vesicles was done by the Millipore filter technique [6,7].

The procedure and mathematics of countertransport are described in detail by Fuhrmann et al. [8,9]. The apparent K_m value was calculated according to Wilbrandt and Rosenberg [10] at the maximal rate of countertransport by:

$$K_m = \left[S_i - (R_i/R_o)S_o\right] / \left[(R_i/R_o) - 1\right]$$

S_i, S_o and R_i, R_o are the concentrations of unlabeled and labeled glucose inside (i) and outside (o) the vesicles.

2.4. Initial glucose uptake experiments

Before measuring glucose uptake the cells were washed three times in ice-cold distilled water and three times in 100 mM sodium phosphate buffer (pH 6.5). The cytocrit was adjusted to 10% cells. Glucose uptake was started by addition of 0.1 ml radioactively labeled glucose solution (0.075 to 6 μCi/μmol D-[U-^{14}C]glucose and unlabeled D-glucose from 0.5 to 150 mM final concentrations) to 0.1 ml of 10% cells at 25°, 20°, 15° and 10°C. Exactly after 5 s at 25° and 20°C or 10 s at 15° and 10°C uptake was stopped by addition of 10 ml ice cold 200 mM sorbitol solution, cells were filtered on a glass fiber filter (GF 92, Schleicher and Schuell, Dassel, Germany) and rinsed with two 10 ml portions of ice cold distilled water. The filters with the cells were immersed into 5 ml scintillation cocktail (Rotiscint eco plus®, Roth, Karlsruhe, Germany) and counted in a Beckman LS 6000IC scintillation counter. Filter control experiments without cells were subtracted.

2.5. Analysis of initial transport kinetics

Experimental data were analysed by computer-assisted nonlinear regression analysis using GraphPAD software [4,11–13]. Two models were used for nonlinear regression analysis:

$$Y = AX/(B + X) + CX/(D + X) \quad (1)$$

$$Y = AX/(B + X) + EX \quad (2)$$

By comparing statistically Eqs. (1) and (2) the fit for the second equation was significantly better than for the first more complex model. Thus only the second model was used for determination of the parameters. For assessing goodness-of-fit the relative distance was used, which is equivalent to dividing each deviation by the value of Y before squaring. This choice is appropriate when the experimental error is expected to be a constant fraction of the Y value. The parameters obtained for the V_m values in (nmol/min)/mg wet weight of cells and the first-order constant in (μl/min)/mg wet weight were plotted according to the Arrhenius equation and analysed for their activation energies (Ea).

2.6. Materials

Peptone from casein and yeast extract were purchased from E. Merck, Darmstadt, Germany. D-[U-^{14}C]Glucose, specific activity 300 mCi/mmol, was obtained from Amersham Buchler, Braunschweig, Germany. All other chemicals were of analytical grade quality.

3. Results

3.1. Glucose countertransport and accelerated efflux in plasma membrane vesicles prepared from repressed and derepressed DFY1 cells

Repressed and derepressed DFY1 cells [11] were grown on 2% glucose and 2% galactose up to the

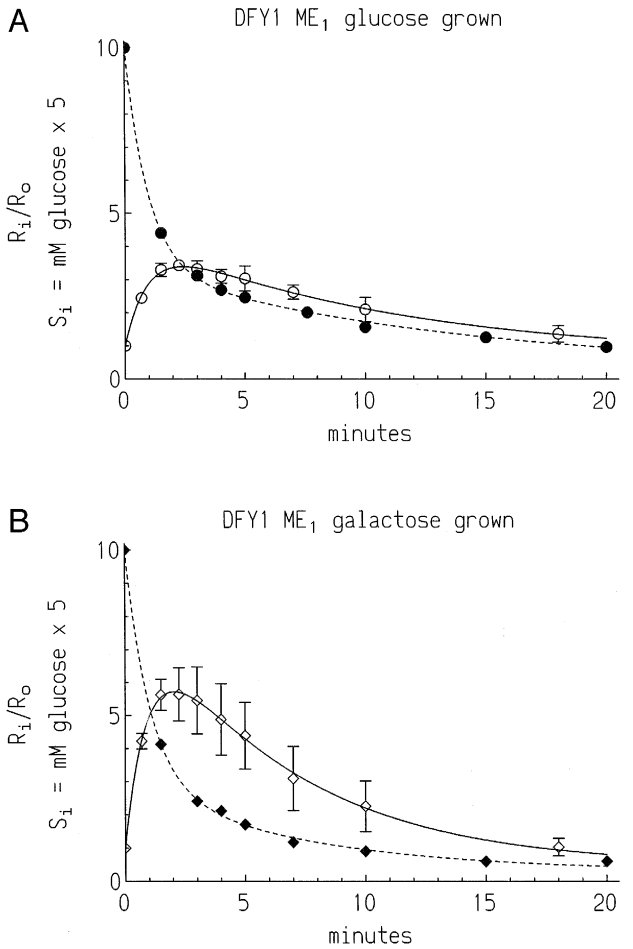

Fig. 1. Glucose countertransport (○,◇) and efflux (●,◆) in plasma membrane vesicles prepared from DFY1 cells. (A) The cells were grown to the middle of the exponential growth phase (ME_1) on 2% glucose and in (B) on 2% galactose in the medium. R_i/R_o is the ratio of ^{14}C-labeled glucose concentration inside to outside; S_i is the inside concentration of unlabeled glucose. Mean values of five (A) and four (B) experiments ± S.D.

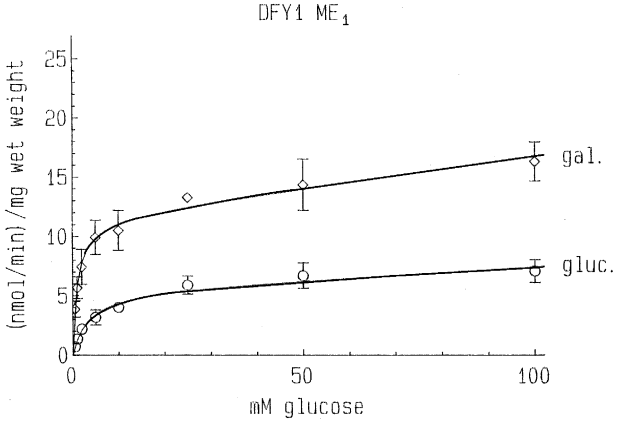

Fig. 2. Initial glucose uptake (5-s) at 25°C in DFY1 cells grown as above on 2% glucose or 2% galactose over a concentration range from 0.5 to 100 mM glucose. Mean of four experiments ± S.D. in glucose and mean of eight experiments ± S.D. in galactose grown cells.

middle of the exponential growth phase (ME_1). The countertransport experiments in vesicles prepared from repressed and derepressed DFY1 cells are depicted in Fig. 1A and B. From the countertransport maximum t_{max} of 3.5 R_i/R_o in vesicles of repressed cells (Fig. 1A) a mean apparent K_m value of 6.0 ± 0.8 mM was calculated from five experiments. A significantly higher affinity was obtained from vesicles derived from derepressed cells (Fig. 1B) with t_{max} values around 6 R_i/R_o and a mean apparent K_m value of 1.7 ± 0.8 mM from four experiments.

3.2. The effect of glucose repression and galactose derepression in initial glucose uptake experiments in DFY1 cells

Fig. 2 shows the corresponding initial glucose uptake experiment to Fig. 1A and B. The DFY1 cells were grown to the middle of the exponential growth phase (ME_1) on 2% glucose or 2% galactose. Experimental data were analysed by computer-assisted non-linear regression analysis. The fit with one Michaelis–Menten and a first order term (Eq. (2)) was statistically favoured over an analysis with two Michaelis–Menten terms (Eq. (1)). The best fit was obtained for the glucose grown cells with a V_m value of 5.4 ± 0.5 (nmol/min)/mg wet weight of cells, an apparent K_m value of 3.5 ± 0.5 mM and a first order rate constant of 0.020 ± 0.007 (μl/min)/mg wet weight. The values are calculated from four experiments and given as mean ± approximated S.E. The corresponding parameters for the galactose initial uptake experiment were 11.5 ± 0.4 (nmol/min)/mg wet weight of cells for the V_m value, 1.0 ± 0.1 mM for the apparent K_m value and 0.053 ± 0.009 (μl/min)/mg wet weight for the first order rate constant (eight single experiments).

Table 1
Kinetic parameters of initial glucose uptake experiments in four growth phases

Growth phase	Temp.(°C)	Time (s)	V_m ± approx. S.E.	K_m ± approx. S.E.	K_d ± approx. S.E.	Number of expts.
EE	25	5	7.9 ± 1.3	5.1 ± 1.2	0.048 ± 0.019	10
EE	20	5	5.6 ± 0.8	3.8 ± 0.9	0.056 ± 0.015	8
EE	15	10	3.5 ± 0.6	3.8 ± 0.9	0.031 ± 0.009	8
EE	10	10	1.8 ± 0.3	3.7 ± 1.0	0.021 ± 0.005	8
ME_2	25	5	14.7 ± 0.6	4.3 ± 0.3	0.019 ± 0.009	10
ME_2	20	5	11.5 ± 0.5	4.3 ± 0.3	0.015 ± 0.007	12
ME_2	15	10	6.9 ± 0.2	3.8 ± 0.2	0.012 ± 0.003	12
ME_2	10	10	4.7 ± 0.2	3.8 ± 0.2	0.010 ± 0.002	12
LE	25	5	14.0 ± 1.2	4.8 ± 0.6	0.033 ± 0.015	6
LE	20	5	10.1 ± 0.7	4.2 ± 0.4	0.040 ± 0.010	8
LE	15	10	5.9 ± 0.5	3.6 ± 0.4	0.018 ± 0.007	10
LE	10	10	3.8 ± 0.3	3.7 ± 0.5	0.013 ± 0.005	10
ES	25	5	19.5 ± 0.7	3.4 ± 0.2	0.042 ± 0.011	10
ES	20	5	13.9 ± 0.4	3.1 ± 0.2	0.048 ± 0.007	10
ES	15	10	9.0 ± 0.3	2.7 ± 0.1	0.027 ± 0.004	10
ES	10	10	5.9 ± 0.2	2.7 ± 0.2	0.022 ± 0.004	10

3.3. The effect of temperature on initial glucose uptake in DFY1 cells in different growth phases

As can be seen from Table 1 the error for the first order term is relatively higher than for the Michaelis–Menten parameters. Therefore, it was necessary to perform sufficient experiments for a successful statistical analysis of temperature dependence. DFY1 cells were grown on 2% glucose to the early exponential (EE), the middle exponential (ME_2), the late exponential (LE) and early stationary growth phase (ES).

Fig. 3A–D show the initial uptake experiments in the different growth phases at the four temperatures investigated. The values are given as mean ± S.D. The curves are calculated by nonlinear regression analysis of the single experiments and depict the best fit of the experimental data. Note that in all graphs the experimental error is not a constant, it increases with increasing glucose concentrations.

3.4. Analysis of the temperature dependence of the V_m values and the first order rate constants of DFY1 cells in different growth phases by the Arrhenius equation

From the difference in V_m values at different temperatures in Table 1 it is already obvious that the rate of maximal velocity for a 10°C difference is more than twice as high. Because of more scattering of the parameter of the first order rate constant no valuable predictions can be made without evaluation of multiple values. In order to use the advantage of statistics we calculated in every single experiment the kinetic parameters by nonlinear regression. In the

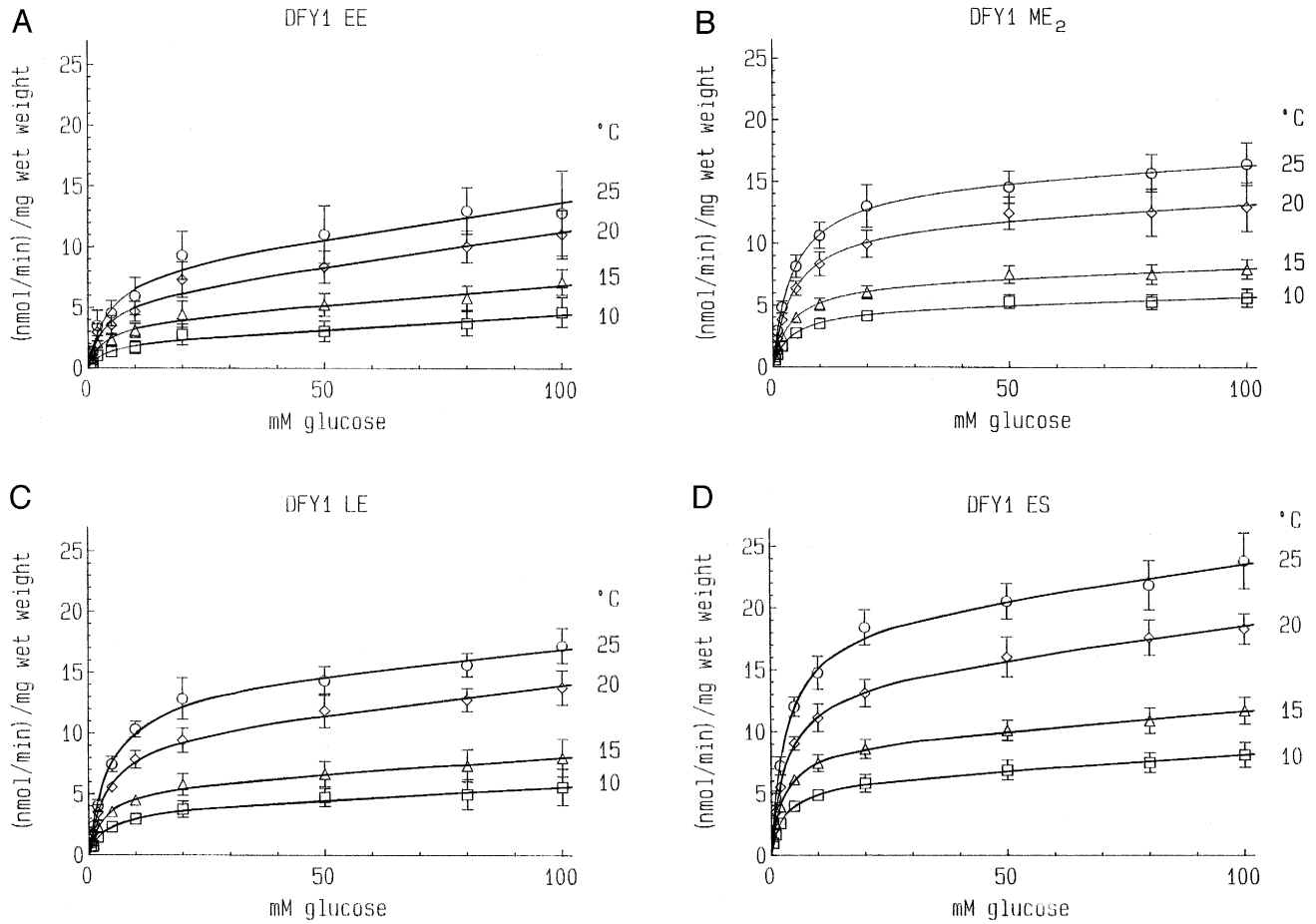

Fig. 3. A–D: Initial glucose uptake at four temperatures in DFY1 cells grown to the early exponential (EE), middle exponential (ME_2), late exponential (LE) and early stationary (ES) growth phase. Mean ± S.D., the number of experiments is given in Table 1.

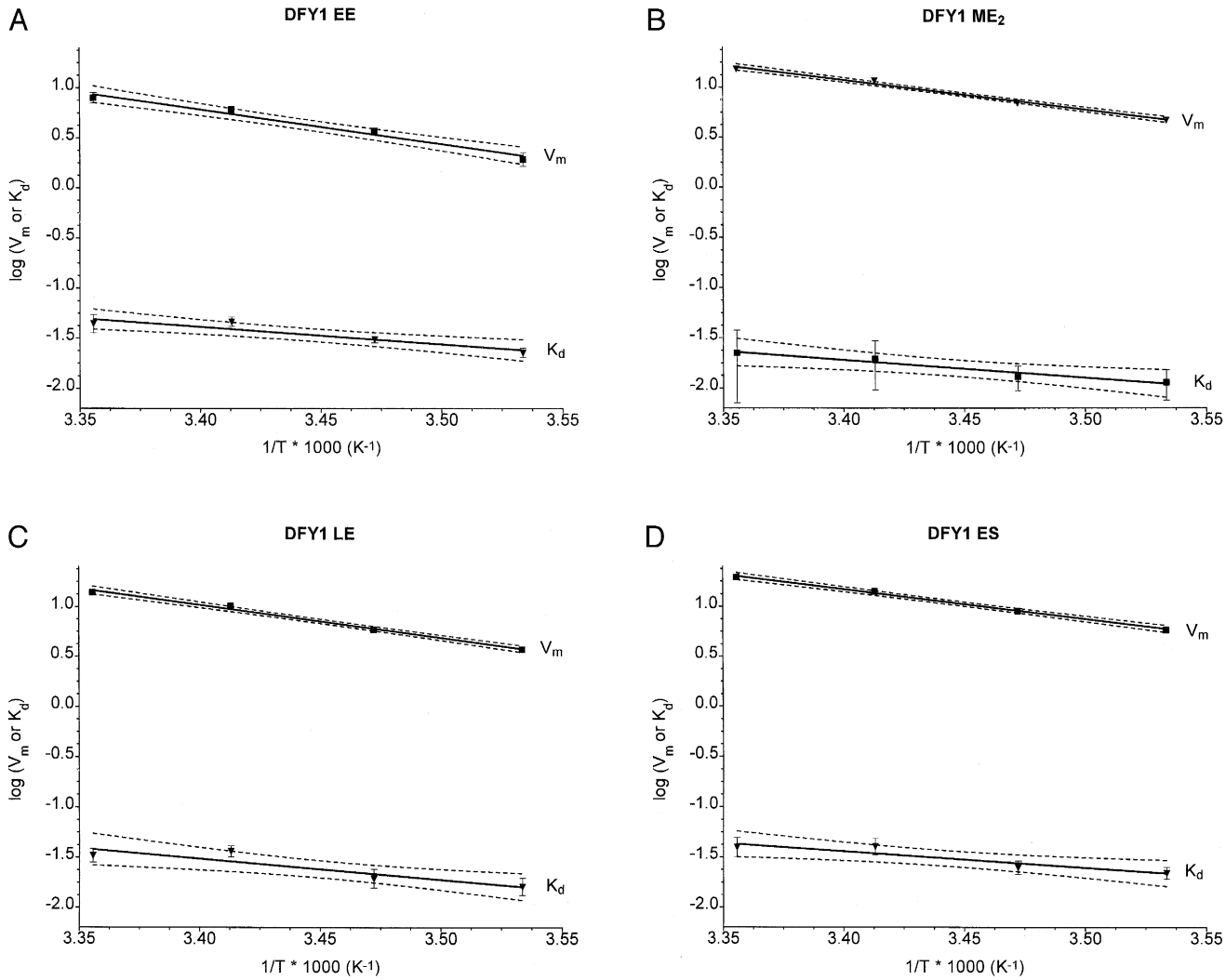

Fig. 4. A–D: Arrhenius plots from glucose uptake experiments in the early exponential (EE), middle exponential (ME_2), late exponential (LE) and early stationary (ES) growth phases. Dotted lines include the 95% confidence intervals; mean values from single experiments ± S.E.

graphs in Fig. 4A–D the log of the reaction rates on the Y-axis was depicted against the reciprocal of the absolute temperature (Arrhenius plot). The figures show the 95% confidence intervals, the mean values of the single experiments and the standard errors. The activation energies (Ea) in kcal/mol were calculated from the slopes of the curves with a standard error resulting from the error of slopes. In all cases the slopes were very significantly different from a zero slope.

The activation energies Ea calculated from the V_m rates were 15.779 ± 1.723, 13.541 ± 0.645, 15.064 ± 0.764 and 13.521 ± 0.725 kcal/mol for the growth phases in EE, ME_2, LE and ES. The corresponding activation energies for the first order rate constant were 7.997 ± 2.096, 8.146 ± 1.315, 9.622 ± 2.992 and 7.527 ± 2.632 kcal/mol.

4. Discussion

Plasma membrane vesicles derived from intact *S. cerevisae* cells are a valuable tool for the investigation of the glucose transport mechanism. By measur-

ing glucose countertransport in plasma membrane vesicles the mechanism is undoubtedly characterized as facilitated diffusion leading to complete equilibrium of the transported sugars. This phenomenon was predicted on theoretical grounds by Widdas 1954 and was demonstrated for the first time experimentally in human red cells by Rosenberg and Wilbrandt in 1957. By using conventional rate equations, as given by Wilbrandt and Rosenberg [10] and introducing osmometer behaviour [8,9], it was possible to simulate the experimentally obtained countertransport data in plasma membrane vesicles by computer-assisted simulation of Michaelis–Menten kinetics. The apparent K_m value calculated from the countertransport maximum in plasma membrane vesicles derived from *S. cerevisiae* cells H 1022 grown on 2% glucose to the middle of the exponential growth phase was 8.6 ± 1.5 mM. This value is comparable with the apparent K_m value of 6.0 ± 0.8 mM obtained in this study by analogous experiments with vesicles prepared from DFY1 cells grown under similar conditions. If the cells were grown to the middle of the exponential growth phase on 2% galactose, which is known to prevent glucose repression of yeast cells [14], the apparent K_m value decreased considerably to 2.9 ± 0.6 mM as measured in plasma membrane vesicles from H 1022 cells [8] and to 1.7 ± 0.8 mM in vesicles of DFY1 cells. Thus, the DFY1 cells used in this study demonstrate regulation of glucose transport very similar to other wild type strains [11,12].

The initial uptake method can only be applied successfully if the inside concentration of glucose is negligibly small during the short uptake period. We have tested this in DFY1 cells grown to the middle of the exponential growth phase on 2% glucose by measuring enzymatically the hexose phosphates appearing during the uptake period of 5-s at 25°C in the cells (results not shown). The sum of glucose 6-phosphate, fructose 6-phosphate and fructose 1,6-bisphosphate was about the same as the amount of transported glucose over a wide concentration range from 0.5 to 50 mM glucose in the uptake experiments.

In contrast to countertransport experiments in plasma membrane vesicles the evaluation of initial glucose uptake experiments in intact cells seems to be more complex which is shown for example, in Eadie–Hofstee plots by the nonlinearity of the slopes [3–5,11]. Instead of using Eadie–Hofstee plots for graphical estimations we used the more advanced computer-assisted nonlinear regression analysis for calculation of the transport parameters [4,11–13]. By statistical analysis the experimental data could be fitted best by a model with one Michaelis–Menten and one first order term; the curves fitted are shown in Figs. 2 and 3. From the figures it is obvious that the experimental error is, on average, not an equal value throughout the experiment but a constant percentage of the Y value. Thus, the points with the large Y values have a larger deviation from the curve than do points with smaller Y values and will be weighted heavily, while points with lower values will be relatively ignored. To get around this problem we always performed goodness-of-fit calculations on the relative deviations of the points by the choice 'relative distance'. In the publication of Coons et al. [5] it was claimed that they used computer-assisted nonlinear regression analysis with the same models we predicted (Eqs. (1) and (2)). However, their method of assessing goodness-of-fit is not the same as we use. These authors did not use our procedure of 'weighting' data points. That this procedure is also necessary for the data of Coons et al. [5] is very clear from their statement: "The data presented contain a margin of error no greater than 10% of the total rate for a given point". Thus, minimizing the sum-of-squares without adequate 'weighting' is not appropriate for initial uptake experiments using labeled sugars.

In initial uptake experiments from DFY1 cells grown to the middle of the exponential growth phase (ME_1) with 2% glucose or 2% galactose in the medium the apparent K_m values of 3.5 ± 0.5 and 1.0 ± 0.1 mM were calculated (Fig. 2). These K_m values are in good agreement with those estimated from countertransport experiments in plasma membrane vesicles with apparent K_m values of 6.0 ± 0.8 and 1.7 ± 0.8 mM (Fig. 1A and B). The slight difference can be explained by the fact that the plasma membrane vesicle population is not uniform in size. Small vesicles should demonstrate higher transport rates because of the greater surface in relation to volume, and the opposite would be true for larger vesicles. The relatively broad countertransport maximum peak, especially in vesicles from glucose-repressed cells, which obviously represents the different vesicle populations, would lead to a slight under-

estimation of the affinities for the glucose transport [8]. In summary there is a good correlation of apparent K_m values calculated by both different methods.

In order to further clarify the mechanism of glucose transport in *S. cerevisiae* the temperature dependence of initial glucose uptake in DFY1 cells in four different growth phases was investigated. The uptake rates measured at 25°, 20°, 15° and 10°C were plotted against the glucose concentrations in Fig. 3. The fitted curves are characterized by the parameters of the Michaelis–Menten kinetics (K_m and V_m) and the parameter of the first order rate constant (K_d). The kinetic parameters calculated by nonlinear regression analysis as outlined under Section 2 are summarized in Table 1. The apparent K_m values demonstrate dependence on the growth conditions as shown in other wild type strains [11,12]. For example, at 25°C the apparent K_m value decreased from 5.1 ± 1.3 to 3.4 ± 0.2 mM from early exponential to early stationary growth phase. In addition there is a consistent temperature dependent slight decrease of the apparent K_m values from 25° to 10°C. This result is similar to the glucose transport in human red cells by efflux experiments where the apparent K_m value decreased, for example, from 37° to 20°C from 5.8 to 2.3 mM [15,16]. As can be seen from Table 1 not only the K_m values but also the V_m values and the first order rate constants K_d changed significantly during cell growth.

Studies of temperature dependence of transport processes introduce the possibility to calculate activation energies that give information about the resistance encountered as the transported molecule enters and traverses the plasma membrane. The parameters obtained for the V_m values in (nmol/min)/mg wet weight of cells and the first order rate constant in (μl/min)/mg wet weight were plotted according to Arrhenius (Fig. 4) and analysed for their activation energies Ea. The activation energies Ea derived from the V_m values and from the first order rate constant are significantly different. The activation energies for the growth phases in EE, ME_2, LE and ES calculated from the V_m values were 15.779 ± 1.723, 13.541 ± 0.645, 15.064 ± 0.764 and 13.521 ± 0.725 kcal/mol and those calculated from the first order rate constant were 7.997 ± 2.096, 8.146 ± 1.315, 9.622 ± 2.992 and 7.527 ± 2.632 kcal/mol. The first values calculated from the V_m values are in the same order as for facilitated diffusion transporters. In general for such processes the activation energy is higher than 12 kcal/mol [17]. For example, the activation energy of the glucose transport in human red cells determined in efflux experiments [16] between 10° and 30°C was especially high with 23 kcal/mol.

The low activation energies calculated from the first order rate constants in the different growth states were between 7.5 and 9.6 kcal/mol and thus significantly higher than for a free diffusion of glucose in water with about 5.0 kcal/mol [18] and significantly higher than expected for diffusion of glucose through the lipid phase of the plasma membrane [17]. Thus, these results exclude a free diffusion of glucose into the cell. However, the activation energy calculated for the first order rate constants are in accordance with activation energies for channels or pores with values of 12 kcal/mol or less [17].

In summary our investigation of the temperature dependence of glucose transport in *S. cerevisiae* is in agreement with two different ways for glucose penetration, the first is facilitated diffusion transport and the second one is entry through a pore. We can not decide yet if these pores are simple glucose transporters which do not oscillate and stay open or if we have special pores for glucose permeation.

Acknowledgements

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