J. Membrane Biol. 138, 29–35 (1994)

The Journal of
Membrane
Biology

Dependence of the Kinetics of Secondary Active Transports in Yeast on H^+-ATPase Acidification

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Received: 13 May 1993/Revised: 31 August 1993

Abstract. Acidification of the external medium of the yeast *Saccharomyces cerevisiae,* mainly caused by proton extrusion by plasma membrane H^+-ATPase, was inhibited to different degrees by D_2O, diethylstilbestrol, suloctidil, vanadate, erythrosin B, cupric sulfate and dicyclohexylcarbodiimide. The same pattern of inhibition was found with the uptake of amino acids, adenine, uracil, and phosphate and sulfate anions. An increase of the acidification rate by dioctanoylglycerol also increased the rates of uptake of adenine and of glutamic acid. In contrast, a decrease of the membrane potential at pH 4.5 from a mean of −40 to −20 mV caused by 20 mM KCl had no effect on the transport rates. The ATPase-deficient mutant *S. cerevisiae pmal*-105 showed a markedly lower uptake of all the above solutes as compared with the wild type, while its membrane potential and ΔpH were unchanged.

Other types of acidification (spontaneous upon suspension; K^+ stimulated) did not affect the secondary uptake systems.

A partially competitive inhibition between some individual transport systems was observed, most pronouncedly with adenine as the most avidly transported solute.

These observations, together with the earlier results that inhibition of H^+-ATPase activity affects more the acidic than the basic amino acids and that it is more pronounced at higher pH values and at greater solute concentrations, support the view that it is the protons in or at the membrane, as they are extruded by the ATPase, that govern the rates of uptake by secondary active transport systems in yeast.

Key words: H^+ symports — Plasma membrane ATPase — Local *vs.* delocalized protons — Yeast

Introduction

Yeast cells, particularly those of *S. cerevisiae,* were among the first experimental objects where the proton-driven secondary active transports were postulated—for a fine review, *see* [7] — and, using heavy water to replace ordinary water, more or less definitively established [22]. (It still remains to be shown whether it is the H^+ or the H_3O^+ ion that binds to the carrier [3]; in the following sections, the word protons will be used for either of the ionic species.) In applying the approach of protonmotive-force-driven transports introduced by Mitchell [27], thermodynamic and kinetic effects have often been confused (as criticized in [14, 43]) and the protonmotive force or rather the "bulk" electrochemical gradient of protons has been introduced into kinetic equations describing the rates of transport [33].

However, when the temperature and pH dependence of some secondary active transports were measured in the yeasts *S. cerevisiae* and *Rhodotorula gracilis* [12, 20], the optimum rate did not coincide with the highest protonmotive force (pH 2.5 and 32°C) but rather with the optimum of H^+-ATPase activity (pH 6.1 and 37°C). This raised some doubt as to the relevance of the "bulk" membrane potential and ΔpH for the secondary active transports, particularly when it was found by using a dual-excitation fluorescence technique [39] that the pH profile across the outer cell envelope is smooth and that, within the resolution limit of 300 nm, there is no steep pH drop across the membrane.

Still, no direct proof exists that local protons in or at the membrane determine the activity of the various proton symports. An attempt to relate the kinetics of uptake of amino acids to the activity of the plasma membrane H^+-ATPase was published recently [23] and in the present paper the suggestion of this relationship is

extended to include a variety of other H^+-associated transports in the yeast *S. cerevisiae*.

Materials and Methods

Cell Cultivation

Baker's yeast collection strain *S. cerevisiae* K (CCY 21-4-60) was mainly used. Other strains were *S. cerevisiae* Y55 (wild type) and its ATPase-deficient mutant *pmal-105*, both a kind gift from Prof. J.E. Haber of the Rosenstiel Basic Sciences Research Center, Waltham, MA. The strains were grown aerobically at 30°C in a yeast extract—glucose medium with mineral salts at pH 5 [18]. After 20 hr (for *S. cerevisiae* K) or 24 hr (for *S. cerevisiae* Y55 and its mutant), when the early stationary phase had been reached, the cells were harvested, washed in distilled water and aerated for 60 min at room temperature to deplete their endogenous reserves.

To achieve maximum transport rates (in fact, the number of active transport protein molecules) the cells were preincubated for 60 min at 30°C with 50 mM D-glucose [19]. For the transport of uracil and of phosphate and sulfate anions, 50 mM D-glucose was also present during the uptake experiment itself.

Uptake Experiments

Suspension of yeast cells was made to a density of 6–8 mg dry wt. per ml of 0.1 M triethanolamine—phthalic acid buffer of pH 4.5. Two ml of this suspension was placed in 25-ml Erlenmeyer flasks and incubated for 10 min at 30°C with the inhibitors or activators in a Dubnoff-type reciprocating water bath. The labeled substance to be transported was then added, generally to a final concentration of 10 μM (distinctly lower than the half-saturation constants of the various transport systems). Samples (0.2 ml) were withdrawn at suitable time intervals, ranging from 30 sec for adenine to 5 min for uracil, filtered through a Synpor 5 membrane filter (Synthesia, Czechoslovakia) with 0.6 μm pores and washed with 2 ml ice-cold distilled water. The filter was then transferred to a toluene-ethanol scintillation cocktail for radioactivity counting. The whole procedure from the beginning of filtration to filter transfer took no more than 10 sec.

Membrane Potential and ΔpH

Membrane potential was estimated from the distribution of ^{14}C-labeled tetraphenylphosphonium ions across the plasma membrane, starting from a 75 nM solution of the chloride.

Extracellular pH was recorded in a constant-temperature vessel (10 ml) using a WTW pH-meter (Germany) with a combination pH-electrode (Radiometer, Denmark) and a conventional flatbed recorder.

Intracellular pH was determined by the dual-excitation fluorescence technique with fluorescein diacetate [38].

ATP hydrolysis in vitro was determined in membrane fragments as described in [34].

Chemicals

The reagents used were uniformly ^{14}C-labeled L-amino acids (Ala, Glu, Lys, Pro) that were obtained from the Institute for Research, Production and Uses of Radioisotopes (Czechoslovakia); uniformly ^{14}C-labeled adenine, uracil and tetraphenylphosphonium chloride were from Amersham International (Great Britain); ^3H-labeled 6-deoxy-D-glucose was prepared in the author's laboratory [16]. Carrier-free [^{32}P]-NaH_2PO_4 and [^{35}S]-Na_2SO_4 were from the Central Institute for Nuclear Research (Germany). Heavy water D_2O was from Merck (Germany) and was 99.9% pure. Diethylstilbestrol, suloctidil, dioctanoylglycerol, erythrosin B and dicyclohexylcarbodiimide were from Sigma (Germany). All other chemicals, as well as growth medium components (with the exception of Difco Yeast Extract) were supplied by Lachema (Czechoslovakia).

Results and Discussion

Inhibitors of the plasma membrane H^+-ATPase, some described by Dufour, Boutry and Goffeau [6] and Serrano [35], others, such as D_2O, described earlier in this laboratory [22], were applied earlier in a study of glucose-induced acidification of the external medium and of the uptake of 13 amino acids [23]. Three of these inhibitors, D_2O, diethylstilbestrol and suloctidil, were used here to examine the uptake of other, presumably H^+-symported, solutes in yeast, *viz.* adenine, uracil and phosphate and sulfate anions (adenine uptake was studied with all the above inhibitors) [5, 10, 31, 32]. Table 1 shows that the inhibitory effects are, at least qualitatively, very much like those on the external medium acidification rate. The only exception is the mediated diffusion of 6-deoxy-D-glucose where no cotransport of H^+ ions exists in this yeast [15, 17]. The relatively lower effect on uracil may be due to its utilizing several transport systems, one of them possibly also thermodynamically passive [26].

It should be noted that, like in the previous publication [23], glutamic acid is much more affected by ATPase inhibitors in its uptake than is lysine. It appears that this is due to glutamic acid requiring two, possibly three, protons to be transported into yeast [7] while lysine could move as such or with a single proton as it is positively charged at the pH used.

To check the hypothesis that the effects observed on the various transports were actually due to inhibition of the ATPase-catalyzed acidification, the ATPase-deficient mutant *pmal-105* was used. Again, the mutant showed substantially lower uptake rates than the parent strain (Table 2). What is striking here is the substantial effect with some substrates whose transport was examined in the presence of glucose. The following explanation is offered.

The H^+-ATPase is activated both by an intracellular acidification [2], such as is brought about, among other things, by proton symport with a suitable substrate, and by the addition of glucose or an analogous fermentative substrate [36, 37]. If the mutant lacks most of the ATPase activity, this will produce a greater effect on the various symports than if only a fractional inhibition takes place in the presence of inhibitors in the wild strain.

Table 1. Effect of ATPase inhibitors on the initial rate of uptake of various compounds in *S. cerevisiae* K, preincubated for 1 hr with 50 mM glucose

Transported solute (10 μM)	Glucose present during assay	Rate of uptake[a]			
		Control	A[b]	B	C
L-Glutamic acid	−	83 ± 18	9–13	16–21	38–52
L-Lysine	−	31 ± 9	70–80	70–89	71–79
Adenine	−	920 ± 78	10–14	32–45	32–40
Uracil	+	18 ± 6	46–54	75–87	73–81
Phosphate anion	+	36 ± 11	9–14	19–29	15–21
Sulfate anion	+	8 ± 3	33–45	71–79	33–40
6-Deoxy-D-glucose	−	180 ± 32	95–103	94–100	95–105
Acidification rate of external medium by ATPase (%)	+	100	20	31	27

[a] The control is shown in nmol solute per min per ml cell water ± SD. The range observed in 5–8 experiments is shown with the inhibitors as a percentage of the control. [b] A: 0.1 mM diethylstilbestrol; B: 97% D_2O; C: 0.05 mM suloctidil.

Table 2. Initial rate of uptake of various compounds in the wild-type and in the ATPase-deficient strain at pH 4.5

Transported[a] solute (10 μM)	Glucose present during assay	Rate of uptake[b]		
		S. cerevisiae Y55	*S. cerevisiae*	*pmal-105* (% of control)
L-Proline	−	17.6	6.4	(36)
L-Alanine	−	23.5	11.9	(51)
L-Lysine	−	19.4	11.7	(60)
Uracil	+	6.4	0.6	(9)
Adenine	+	167	14.1	(8)
Phosphate anion	+	63	1.1	(2)
Sulfate anion	+	1.8	0.1	(6)
Acidification rate of external medium by ATPase (μmol H^+ per min per ml cell water)	+	18.9	10.0	(53)
Membrane potential (in mV)	+	30	30	(100)
ΔpH (in units)	+	1.1	1.2	(110)

[a] No acidic amino acid could be tested with significant results as the uptake of Glu as well as Asp was negligible even in the parent strain. [b] The rate is shown in nmol transported solute per min per ml cell water, as means of at least three experiments.

It should be noted that, in contrast with the experiments reported in [30] where midexponential-phase cells were used, the acidification rate here was lower in the mutant than in the wild-type strain as might be expected. At the same time, the membrane potential at pH 4.5 was virtually the same in both strains.

In contrast to the number of useful inhibitors of the ATPase (even if not necessarily very specific), there are few activators known. Unlike with the analogous H^+-ATPase of plant cells where, in vivo, fusicoccin is a powerful activator of the ATPase—kinase functional complex, the compound is inactive in yeast [16]. However, preliminary data were described in [1, 4] that diacylglycerols activate the plasma membrane ATPase, apparently via the phosphatidylinositol—protein kinase C pathway. Indeed, dioctanoylglycerol (1 mM) caused

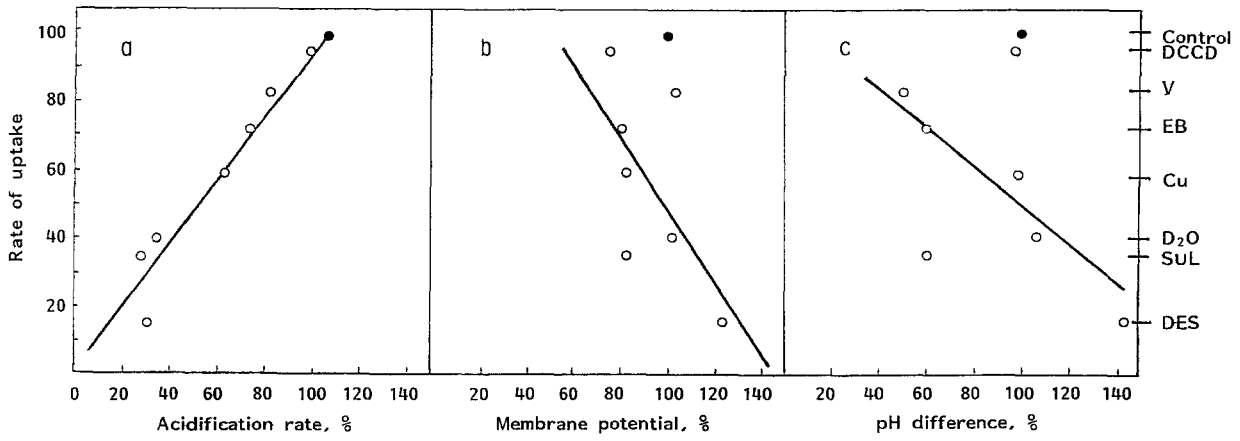

Fig. 1. Rate of uptake of 10 μM adenine by *S. cerevisiae* K in the presence of various inhibitors related (*a*) to the acidification rate between 1 and 3 min after addition of glucose (the control rate was 7.0 nmol H^+ per min per mg dry wt. of yeast), (*b*) to the membrane potential (its value in the absence of inhibitors was here −45 mV) and (*c*) to the pH difference across the membrane (in the control this was 1.1 pH units, corresponding to −66 mV). All values are shown as a percentage of the control without inhibitor. The heavy lines are regression lines for the dependence of the rate of adenine uptake on the rate of glucose-induced acidification (*a*), on the membrane potential (*b*) and the pH difference (*c*). The correlation coefficients were 0.95 in *a*, −0.60 in *b* and −0.56 in *c*. The inhibitors were used in the following concentrations: dicyclohexylcarbodiimide (*DCCD*) 0.1 mM; sodium vanadate (*V*) 50 μM; erythrosin B (*EB*) 50 μM; copper(II) sulfate (*Cu*) 10 μM; suloctidil (*SUL*) 50 μM; diethylstilbestrol (*DES*) 0.1 mM; heavy water (D_2O) 97%.

a drop of pH of a yeast suspension (particularly after the cells had been starved by aeration), amounting to 18–25% of the acidification rate observed after adding 50 mM glucose. Likewise, the uptake of 100 μM adenine was, over the first 16 min, by 12–27% faster with dioctanoylglycerol than without it.

The effects of ATPase inhibitors on some transports have been observed before (e.g., [9, 35] but they were always attributed to the assumed decrease of the membrane potential and/or ΔpH across the membrane. It was shown here that the effects of a total of seven inhibitors are in no way related to either of the above (Fig. 1). Likewise, glucose consumption or endogenous respiration were not affected by any of these inhibitors (*not shown here in detail*). The inhibitory effects, however, are in a highly satisfactory agreement with the rate of ATPase-catalyzed acidification, the correlation coefficient being 0.95 for the uptake of adenine, while for the relationship between the rate of uptake and either the membrane potential or the pH difference, the correlation coefficient was in fact negative. A similar relationship between the uptake of glutamic acid and the rate of acidification, on the one hand, and its lack with the electrochemical potential gradient, on the other, was published before [23].

In agreement with the suggestion made above that the membrane potential has little to do with the initial rates of transport of various solutes, it was shown here that 20 mM KCl, which collapses the membrane potential (from −100 to −50 mV at pH 6.5, and from −40 to −20 mV at pH 4.5) had no effect on the uptake of glutamic acid or adenine. The initial rates at pH 4.5 were 89–106% of the control for Glu and 91–102% for Ade. Addition of 50 mM glucose at this pH had never more than a ±10% effect on the potential.

The correlation found to exist between the rate of acidification upon addition of glucose to a suspension and the various symports should not be taken as to absolutely exclude other H^+-generating processes of the yeast plasma membrane, such as the "spontaneous" acidification observed on suspending cells in water [29] or opening of alkaline ion channels that, under some conditions, substantially enhances the outflow of H^+ ions (*cf.* [24]). However, some inhibitors of H^+-ATPase, such as D_2O and, to a degree, diethylstilbestrol, have a positive effect on the alkaline ion channels [16]. Moreover, activation of the H^+ extrusion in the presence of glucose by K^+ (and Rb^+, Cs^+ and Tl^+) does not enhance the uptake of either glutamic acid or adenine [25]. All this suggests that it is primarily the ATPase that is responsible for the acidification which bears on the secondary active transports.

It is suggested on the basis of the above results, plus the fact that at higher concentrations of the transported solute and at higher pH values the inhibition was more pronounced [23], that the H^+-ATPase is the immediate source of protons used for the various secondary transports, in addition to its functioning as a generator of the membrane potential and of the ΔpH.

One might argue that there should be no preference by the secondary transport systems for the ATPase-generated protons over those present in the bulk aqueous buffer washing the cell surface. Indeed, the rate of movement of protons in water has been shown to be far

greater than predicted by simple diffusion [28] and is ascribed to proton jumps from a H_3O^+ hydronium ion to a neighboring H_2O, plus a rotational motion which carries the protons in one direction. In the relatively concentrated buffer used here, the so-called "buffer shuttle" mechanism [8] should ensure a relatively rapid equilibration between the perimembrane region and the bulk solution. However, the results obtained here show that the perimembrane protons produced by the ATPase are distinctly preferred over those provided from the bulk. Several considerations should be brought forth in this context.

(i) The equilibration with the bulk may be hindered by the mesh of polysaccharides in the thick cell wall.

(ii) The lateral movement of protons along the lipid-water interface is faster than that from the bulk [40].

(iii) The H^+-ATPase may be localized in close vicinity of the transport systems in question, something in line with the views of Kell [11] and Westerhoff et al. [42] about microdomains or coupling units in the membrane where the local concentrations of protons (as well as other solutes) may substantially differ from those in the surrounding solution.

This last view appears to tally with the results since it is the H^+-ATPase-mediated acidification that affects the secondary transports, not the "spontaneous" acidification [29] or the K^+-stimulated H^+ extrusion [25]. Indeed, the greater dependence of some of the transports on H^+-ATPase activity than of others might suggest (although this is purely speculative at this stage of research) that these systems lie closer to the ATPase than the others.

If the supply of H^+ ions to the secondary transporters is limited by the momentary capacity of the ATPase, one could expect some competition for the H^+ ions even by independent transporters using H^+ as a "second substrate," in analogy with the bi-bi systems of enzyme kinetics.

A somewhat laborious derivation of the rate equations, starting from the premise that the concentration of the carriers in the membrane is similar to that of the H^+ ions available to them, leads to the conclusion that a partially competitive inhibition between individual transports should be observed [13]. All the combinations of L-glutamic acid, L-lysine, uracil, adenine, sulfate and phosphate were tested in this respect, but clearly the most pronounced inhibition was that of sulfate uptake by adenine, apparently because adenine is the most avidly transported substance of all those examined while the sulfate anion is transported slowly (*see* Table 1).

The half-saturation constants of uptake, determined in independent experiments, were 30–50 μM for the sulfate anion and 15–20 μM for adenine. Figure 2 shows (*a*) the Lineweaver-Burk plot of sulfate uptake as inhibited by adenine, indicating competitive inhibition; (*b*) a Dixon plot of reciprocal rate of sulfate uptake against

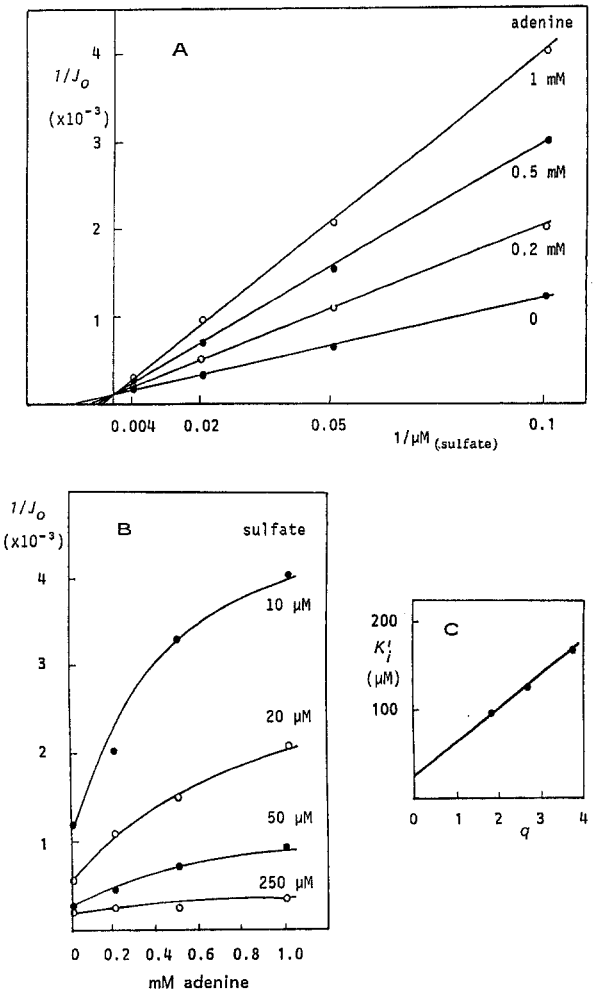

Fig. 2. Partially competitive inhibition of sulfate uptake in the yeast *S. cerevisiae* K by adenine. (*A*) Lineweaver-Burk plot of sulfate uptake at different concentrations of adenine; (*B*) Dixon plot of sulfate uptake at different concentrations of adenine; (*C*) auxiliary plot to determine the inhibitor constant of adenine toward sulfate (intercept with the ordinate). For details, *see text.*

adenine concentration, suggesting a partial inhibition, and (*c*) a plot of apparent inhibitor constants at different concentrations of adenine (K_i's from the Lineweaver-Burk plot) against a value q, representing the ratio of slopes of Lineweaver-Burk lines at these inhibitor concentrations to the slope of the control without inhibitor [41]. This is seen to yield an inhibitor constant of almost exactly 25 μM. However, agreement of this constant with the half-saturation constant for adenine uptake is apparently fortuitous because the inhibitor constant of adenine reflects the affinity of the carrier — adenine complex for H^+.

This is, to my knowledge, the first case where this type of interaction, *viz.* competition for a source of energy, has been quantitatively evaluated in the transport field.

On the whole, the results support the view advocated by Williams [43, 44], Kell [11], Westerhoff et al. [42], and others, that in the *kinetics* of H^+ symports what counts is the actual concentration of protons at the site they are utilized, whether it is provided by diffusion from the bulk or by a membrane-localized proton generator. The overall *thermodynamic* quantities, the membrane potential and the pH difference, are only applicable if equilibrium distribution of the transported solute is considered, a situation that rarely occurs in living systems [21].

I would like to thank those who assisted with the experiments, Dr. M. Dvořáková and G. Georghiou, MSc., and one of the referees for valuable suggestions for improving the manuscript. The work was supported by grant no. 51127 of the Czechoslovak Academy of Sciences.

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