Dynamic Compartmentation of Vacuolar Amino Acids in *Penicillium cyclopium*

CYTOSOLIC ADENYLATEDS ACT AS A CONTROL SIGNAL FOR EFFLUX INTO THE CYTOSOL

Werner Roos‡, Rico Schulze, and Jörg Steighardt

From the Martin-Luther-University Halle, College of Pharmacy, Department of Cell Physiology, 06120 Halle, Federal Republic of Germany

The regulation of amino acid transport from the vacuolar reservoir into the cytoplasm has been studied in hyphal cells of *Penicillium cyclopium*. To avoid artifacts caused by the isolation of vacuoles, efflux was examined "in situ," i.e. in cells whose plasma membranes were permeabilized for micromolecules by a treatment with nystatin. The ATP-dependent proton gradient and amino acid transport activities at the vacuolar membrane remained intact under these conditions. Accumulation of amino acids in the vacuole proved to be the result of a dynamic equilibrium of active, ATP-dependent uptake and energy-independent efflux. The latter was strongly accelerated after the vacuolar amino acid content had surpassed a threshold level.

Efflux of vacuolar amino acids was specifically controlled by extracellular adenylates: ATP, 5'-adenylylimidodiphosphate (an ATPase-resistant ATP-analogue), ADP, or AMP caused a strong inhibition in the concentration range around 200 μmol/liter, whereas both lower and higher concentrations allowed significant efflux rates. Estimates of the cytosolic adenylates (which consisted mainly of ATP) were close to 2 mmol/liter in glucose-metabolizing cells, which concentration allowed maximum rates of both vacuolar uptake and efflux. During 24 h of carbon and nitrogen starvation, the adenylate level decreased toward the efflux-inhibiting region around 200 μmol/liter, whereas 3–4 d of carbon and nitrogen starvation caused a further decline of the adenylate content, leading again to efflux-permitting concentrations. Thus, the cytosolic adenylate pool appears to effectively control the availability of vacuolar amino acids for the cellular metabolism.

The metabolism of amino acids in fungal and plant cells is strongly influenced by transport activities of the vacuole. The majority of free amino acids is usually concentrated in this organelle, the relation between vacuolar and cytosolic pools being different for individual amino acid species (1–5). Vacuolar amino acids are used as a reservoir to maintain the concentrations of cytosolic amino acids within distinct limits, thus compensating for changes of the external or biosynthetic supply and metabolic needs. As amino acid precursors are required for many essential anabolic and catabolic activities, the control over their vacuolar pool is assumed to have a high significance for metabolic regulation. Classical examples are the controlled exchange of vacuolar and cytoplasmic arginine and ornithine during the transition between anabolic and catabolic steady states in *Neurospora crassa* (6–8) or the response of the vacuolar amino acid pool to nutrient-induced changes of the rate of protein synthesis in leaves of barley or spinach (9).

In eukaryotic microorganisms various tonoplast transporters have been identified that catalyze the uptake of amino acids into the vacuole at the expense of proton-motive force generated by the tonoplast H⁺-ATPase and tonoplast H⁺-pyrophosphatase (10–15). In plant cells similar evidence is less abundant (16, 17), and proton-motive force-independent transport (stimulated by ATP and its ATPase-resistant analogue) has been reported as well (4, 9, 18).

The cellular control over the vacuolar amino acid pools suggests that tonoplast transport systems are involved in regulatory circuits that sense cytosolic conditions and respond by switching between accumulation into the organelle and efflux into the cytosol. Whereas some information about the regulation of vacuolar uptake can be deduced from kinetic and regulatory properties of amino acid transport systems at the tonoplast (3, 4, 10, 15, 17, 18) and the energizing proton pumps (19–21), the mechanism(s) governing the efflux of accumulated amino acids into the cytosol is largely unknown. Such knowledge would promote the understanding of the regulatory properties of the vacuolar transporters and of their impact on enzymic processes in the cytoplasm, whose conversion rates might be controlled by the efflux of precursors from their vacuolar pools (e.g. see Ref. 22).

In *N. crassa*, Weiss and co-workers (8, 23) described a stimulating effect of glutamine limitation on the energy-requiring efflux of vacuolar arginine. In vacuoles isolated from barley mesophyll protoplasts Dietz et al. (9) found the efflux of amino acids to be stimulated by ATP and its ATPase-resistant analogue AMP-PNP1 and to be inhibited by neutral cytosolic amino acids. In the present report we describe two novel control mechanisms of vacuolar efflux that became apparent in fungal cells whose plasma membrane was selectively permeabilized for micromolecules: (a) stimulating as well as inhibitory effects exerted by distinct concentrations of extravacuolar adenylates and (b) a sigmoidal concentration dependence of amino acid efflux.

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Dedicated to Prof. Dr. Benno Parthier on the occasion of his 65th birthday.

‡ To whom correspondence should be addressed: Martin-Luther-University, College of Pharmacy, Institute of Pharmaceutical Biology, Dept. of Cell Physiology, Kurt Mothes Str. 3, 06120 Halle (Saale), FRG. Tel.: 0345 5525010; Fax: 0345 5527006; E-mail: roos@pharmazie.uni-halle.de.

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1 The abbreviations used are: AMP-PNP, 5'-adenylyl imidodiphosphate; 4-MUP, 4-methylumbelliferyl phosphate; G6PDH, glucose-6-phosphate dehydrogenase; CCCP, carbonyl cyanide m-chlorophenylhydrazone; fwf, fresh weight; MES, 4-morpholineethanesulfonic acid; CTAB, cetyltrimethylammonium bromide.
**EXPERIMENTAL PROCEDURES**

**Organism, Culture Conditions, and Preparation of Cells for Transport Studies**—Submerged batch cultures of *Penicillium cyclopium*, strain SM 72a, were cultivated from conidiospores as described elsewhere (24). The culture liquid contained (mmol/liter): sucrose, 117; glucose, 55; ammonium tartrate, 35; MgSO₄ 4·0; KH₂PO₄, 2.75; FeSO₄ 0.18; ZnSO₄ 0.17; trace elements (mmol/liter): MnCl₂, 14; CoCl₂, 1.7; CuCl₂, 0.52; MoO₃ 0.41; Triton X-100, 0.003%. 100 ml cultures were grown in 300-ml Erlenmeyer flasks on rotary shakers (250 rpm) at 24 °C.

After 2 d of growth, a 100-ml submerged culture was diluted with 500 ml of maleate buffer, pH 4.5, 50 mmol/liter, and filtered through a nylon mesh (pore diameter ~500 μm) to remove hyphal pellets. The fine, hairy grown mycelium was washed twice with 250 ml of the same buffer by suction filtration. The cells were then resuspended (4 mg wet wt/ml) in half-concentrated culture medium without ammonium tartrate and shaken for another 12 h in order to allow the derepression of nitrogen-regulated transport activities (25).

**Permeabilization of the Plasma Membrane of Hyphal Cells**—After 2 d of growth, subdued hyphal tips (approximately 100 mg wet wt) were filtered and washed with suction filtration with 20 ml of sodium citrate buffer, 5 mmol/liter, pH 4.5, containing 1 mol/liter sorbitol, resuspended in 10 ml of the same buffer, and gently shaken for 6 min at 24 °C. Then 5 μl of a nystatin solution (10 mg/ml in Me₂SO) were added, and the cells were incubated with shaking for another 6 min (or times indicated otherwise). The effect of nystatin was stopped by adding 20 ml of HEPES buffer, pH 7.0, 50 mmol/liter containing 150 mmol/liter KCl, 50 mmol/liter NaCl, 600 mmol/liter sorbitol, 5 mmol/liter CaCl₂, and 5 mmol/liter MgCl₂. Finally, the cells were washed by suction filtration and resuspended in the buffer containing 10 mmol/liter ATP.

The success of permeabilization and the intactness of the vacuolar accumulation capacity was checked prior to each transport experiment by following the vacuolar accumulation of neutral red from 2 mmol/liter solutions in the presence and absence of 1 mmol/liter ATP (see Fig. 2). This was done both microscopically and by following the decrease of $A_{	ext{405}}$ of the supernatant. The test is based on the observation that ATP does not support accumulation in the presence of halichondrin B, so that intact cells stain much slower and do not react to ATP, which is analogous to the uptake of Phe (see Table I).

**Transport Assays**—Hyphal cells were resuspended (4 mg wet wt/ml) in 60 mmol/liter sodium maleate buffer, pH 3.5 (intact cells), or in 60 mmol/liter sodium MES buffer, pH 7.0, with 0.4 mol/liter sorbitol, 0.15 mol/liter KCl, and 1 mmol/liter MgATP (permeabilized cells). These suspensions were gently aerated through a glass sinter funnel. After 1 min the unlabeled amino acid (final concentration 50 μmol/liter), together with the tracer amino acid (14C- or 3H-labeled, final concentration <1 μmol/liter), and effectors in the appropriate buffer were added. Further details for uptake and efflux protocols are given in Figs. 3 and 4. The content of labeled amino acids given in the figures refers to the amount of free amino acids extractable from the cells with 80% ethanol. It was determined by 50-ml samples, which were rapidly injected into 10 ml of the same buffer at 0 °C, sucked onto cellulose nitrate filters (1.2 μm cutoff), and washed with 15 ml of this buffer (intact cells) or with 0.7 mol/liter sorbitol (permeabilized cells) at 0 °C. The cell pellet was then immersed in 2 ml of ethanol 80% (v/v), shaken for 2 h, and centrifuged at 5000 × g for 10 min, and radioactivity in 1 ml of the supernatant was counted by liquid scintillation. A Hewlett Packard LS counter was used in a dual channel mode that allowed the simultaneous detection of 14C and 3H in discrete energy windows by automatically compensating for overlapping radiation via internal calibration curves. The separate detection of the isotopes was confirmed using test mixtures.

Initial rates of uptake and efflux were computed from the experimental curves fitted to a hyperbolic equation (uptake curves) or the equation $y = a + b/(1 + e^{-kx})$ (efflux curves).

At least 88% of the radioactivity extracted from the cells after 80 min of incubation with [14C]Phe was chromatographically identical with phenylalanine as confirmed by reversed phase high performance liquid chromatography amino acid analysis after derivatization with Fmoc-chloride (9-fluorenylmethyl chlorocarbonate) (26). After two extraction steps with ethanol 80% (v/v), the total protein solubilized with 10% SDS (see below) contained 10% of the radioactivity found in the fraction.

**Determination of Nucleotide Content**—Hyphal suspensions were filtered by suction, the cell pellet (approximately 10 mg wet wt) was rapidly immersed in boiling glycerol buffer (100 mmol/liter, pH 11.0) and extracted for 10 min at 100 °C. After cooling to 20 °C, the extract was centrifuged at 6000 × g for 15 min, and the adenylates in the supernatant were assayed luminometrically.

For ATP, 10-μl aliquots were injected into 300 μl of a luciferin/luciferase mixture reconstituted from lyophilized preparations (Boehringer Mannheim) in glycine buffer 100 mmol/liter, pH 7.4, and the luminescence counts integrated over 10 s in a luminometer (Clinilumat, Fa. Berthold). The method was calibrated with ATP solutions in the extraction buffer and validated via internal standards or addition of ATPase. S.D. = 8%, n = 3.

ADP was assayed as the amount of ATP formed from phosphoenolpyruvate via pyruvate kinase as outlined by Hampp (27). AMP was assayed as the amount of ATP used by the adenylate kinase reaction. The reaction mixture contained adenylate kinase (0.9 unit/10 μl) and 10 mmol/liter MgSO₄ in 25 mmol/liter HEPES-K⁺, pH 7.5. ATP present in the native sample was used as the phosphate donor as its concentration always allowed a sufficient excess over AMP (see Fig. 5B). ADP and AMP could be determined in the presence of a ≤2-fold (ADP) or ≥7-fold (AMP) excess of ATP as estimated from calibration experiments at ATP = 10 μmol/liter.

The intracellular water volume was determined as the difference between the total volume of a wet hyphal pellet (dry loss) and the extracellular water. The latter was estimated as the partial volume of the wet cell pellet that was accessible to the non-membrane-penetrating solute bovine serum albumin.

**Indicator Enzyme Activities**—Glucose-6-phosphate dehydrogenase (G6PDH) activities were assayed in hyphal suspensions after exposure to nystatin for various times (see permeabilization procedure above). 2.5 mg of cells (wet wt) in 1 ml were mixed with the same volume of triethanolamine buffer, pH 7.6, containing 0.7 mol/liter sorbitol. After centrifugation at 16,000 × g for 10 min, both the cell pellet (after resuspension in the original volume of the last mentioned buffers) and the supernatant were used to determine conversion rates of glucose 6-phosphate: 800 μl of cell suspension or supernatant were mixed with 150 μl of MgCl₂ solution, 100 mmol/liter, and 60 μl of NADP⁺ solution, 15 mmol/liter. After reading the initial extinction the conversion was started by adding 60 μl of glucose 6-phosphate solution, 30 mmol/liter, and reciprocal shaking at 24 °C. After 30 and 60 s, the reaction was stopped by rapid cooling on ice, and the mixture was centrifuged at 10,000 × g for 10 min at 0 °C. The OD of the supernatants was read at 340 nm after warming to room temperature.

Total cellular protein was extracted from cell pellets by shaking with 10% SDS for 4 h. After centrifugation at 6000 × g for 10 min, the supernatant was assayed by the Lowry et al. (28) procedure.

**RESULTS**

**Accumulation of Amino Acids in Intact Cells**

Hyphal cells of *P. cyclopium* are able to concentrate amino acids to a considerable extent. For example, nitrogen-starved cells accumulate externally added [14C]-labeled amino acids from 5 mmol/liter solutions by the following concentration factors $(C_{\text{int}}/C_{\text{out}})$ calculated on the basis of total intracellular water: Arg, 2600-fold; Glu, 2000-fold; Leu, 1700-fold; Phe, 1300-fold; even from 5 mmol/liter solutions concentration factors of 38 (Arg), 34 (Glu), 32 (Leu), and 24 (Phe) are reached. As expected, the majority of absorbed amino acids accumulated in the vacuolar compartment; after selective lysis of the plasma membrane (see next section) the vacuoles retained 89% of total [14C]Arg, 65% of total [14C]Glu, 93% of total [14C]Leu, and 81% of total [14C]Phe, each preaccumulated from a 5 μmol/liter solution.

Vacuoles liberated from intact hyphal cells via protoplasts contained approximately 65% of the total ninhydrin-positive compounds but showed very low accumulation capacities for added amino acids compared with the protoplasts of their origin (e.g. less than 20% in the case of [14C]Phe or [14C]Arg). This situation is only marginally influenced by the isolation procedure, i.e. lysis with DEAE-dextran (29), EGTA-induced lysis (according to Kringstad et al. (30)), lysis by shearing forces via ultrasonic disintegration (31), all followed by flotation in sorbitol/Ficoll density gradients. Addition to isolated vacuoles of a 20,000 × g supernatant (obtained after disintegration of protoplasts in a glass homogenizer at 0 °C, centrifugation and 3 h flow dialysis against 10 mmol/liter MES, pH 6.5) increased their accumulation capacity for [14C]Phe and [14C]Arg by 30–50% as well as their lifetime detectable by microscopic obser-
Adenylate Control of Vacuolar Efflux of Amino Acids

Selective Permeabilization of the Plasma Membrane for Micromolecules

Selective lysis of the fungal plasmalemma without damaging the tonoplast has been reported hitherto only for yeast cells that had been treated with either DEAE-dextran (33) or CuCl₂ (34). While experiences with the CuCl₂ procedure argue for a damaging side effect on the tonoplast H⁺-ATPase (uptake of arginine or Ca²⁺) was not only reduced compared with intact cells but also insensitive to uncouplers, cf. Ohashi et al. (34), treatment with DEAE-dextran permeabilized hyphal cells of our strain of *P. cyclopium* only partially. In this fungus, treatment with polyene antibiotic nystatin in a buffer of low ionic strength proved to permeabilize the plasma membrane with sufficient efficiency and selectivity. Polyenes, such as nystatin or amphoterin C B, are known for their pore-forming effect, which is based on their binding to membrane sterols of fungal cells (35–37). Selectivity toward the plasma membrane can be expected because the sterol (mainly ergosterol) content of this membrane is more than tenfold higher than that of other cellular membranes. This is not only true for *P. cyclopium* (38), but for other fungal species as well.

The breakdown of the plasma membrane permeability barrier was indicated by the increasing availability of extracellular substrates for intracellular ("latent") enzymes. As seen in Fig. 1, a cytosolically localized indicator enzyme, glucose-6-phosphate dehydrogenase (G6PDH) reports an increasing availability of its external substrates NADP⁺ and glucose-6-phosphate during a 6-min incubation with nystatin. Similar findings were obtained with the conversion of 4-methylumbelliferone phosphate (4-MUP) by cellular phosphatases (data not shown).

The leakage of bulk protein, measured as A₂₈₀ in the supernatant, remained very low for up to 8 min of nystatin treatment (less than 2% compared with detergent treated cells) and correlated with the appearance of marker enzyme activities in the outer medium.

Thus it appears very likely that a 6-min treatment with 5 μg/ml nystatin permeabilizes the plasma membrane for micromolecules as NADP⁺, glucose 6-phosphate, and 4-MUP³ with-

³The indicator reactions reported the permeabilization process with different sensitivity: the dephosphorylation of 4-MUP, a smaller and less hydrophilic substrate than NADP⁺, required shorter times (4 min) of nystatin treatment to accelerate compared with the G6PDH reaction.

Both reactions, after their initial rates had reached a plateau (after 4 or 6 min of nystatin treatment, respectively) showed the same $K_m$ in permeabilized cells compared with the liberated enzymes. Hence, the permeabilization procedure released the indicator reactions from any limitation caused by the membrane transfer of their substrates.
TABLE I
Comparison of Phe transport by intact and nystatin-treated hyphal cells

|                        | Uptake of \([1^{14}C]\)Phe, \(C_i = 50 \mu\text{mol/liter}\*) |
|------------------------|---------------------------------------------------------------|
|                        | Intact cells Nystatin treated                                 |
| pH optimum             | 3.5 7.0                                                       |
| Accumulation ratio (\(C_{in}/C_{out}\)) in the presence of 1 mmol/liter ATP | 1500 1250                                                    |
| Initial uptake rates (%)\(b\) in the presence of 1 mmol/liter ATP          |                                                      |
| + sorbitol 1 mol/liter   | 94 118                                                       |
| No sorbitol             | 107 3                                                        |
| bafilomycin, 2 \(\mu\text{mol/liter}\) | 96 6                                                         |
| NO\(_3^–\), 100 mmol/liter | 100 32                                                      |
| Vanadate, 1 mmol/liter   | 48 93                                                       |
| Azide, 0.1 mmol/liter   | 15 100                                                      |
| In the absence of ATP   | 100 5                                                        |

* Data are means of four experiments, S.D. = 7–11%.
\(b\) 100% of initial uptake rate represent 7.2 nmol of \([1^{14}C]\)Phe/min/mg of protein, determined with intact cells in the absence of ATP and osmotic stabilizers.

out causing a substantial loss of protein. On the other hand, this treatment did not significantly impair the functional integrity of the vacuoles as indicated by the following data. (a) The vacuoles were able to maintain an ATP-dependent proton gradient. Microscopic observations on thevacuolar trapping of neutral red indicated that this dye accumulated much faster in permeabilized than in intact cells (Fig. 2, a and b). The diagnostic value of neutral red accumulation to probe the intactness and energization of the vacuole is supported by showing that the dye accumulates neither in the absence of ATP nor after prolonged nystatin treatment or addition of the detergent CTAB (Fig. 2, c–e). Furthermore, the nucleic acid-based viability probe Fungo-light® (Molecular Probes) accumulated in vacuoles of permeabilized cells supplied with ATP much like in those of intact cells (data not shown). ATP-dependent accumulation of neutral red was used as a test criterion in routine checks of successful and selective permeabilization. (b) The uptake of \(1^{14}C\)-labeled amino acids meets various criteria for vacuolar transport energized by a \(H^+/\text{ATPase}\) (Table I). The most evident properties diverging from intact cells are a pH optimum near 7.0, strong dependence on ATP supply and osmotic stabilization, sensitivity toward bafilomycin and nitrate (inhibitors of tonoplast \(H^+/\text{ATPase}\)), but not toward vanadate and azide (inhibitors of plasma membrane- and mitochondrial \(H^+/\text{ATPases}\), respectively).

Quantitative data suggest that the transport capacities of the permeabilized cells are close to that of vacuoles in intact cells; the accumulation ratio (\(C_{in}/C_{out}\), at an external concentration of 50 \(\mu\text{mol/liter}\)) reached 80% for Phe or Arg and 75% for Leu of that observed with intact cells. The high degree of inhibition by bafilomycin and the absence of inhibition by azide implies that other organelles of the permeabilized cells (e.g. mitochondria) did not significantly contribute to the uptake of the labeled amino acids.

Summarizing, it seems justified to assume that the established procedure permeabilizes the plasma membrane for micromolecules without significantly impairing the ability of the vacuole to maintain a proton gradient and to accumulate external amino acids. Thus, reliable studies of the vacuolar amino acid transport can be performed in situ, i.e. with the vacuolar transport agency kept in place within its macromolecular environment.

**Dynamic Compartmentation of Amino Acids Probed by Efflux Analysis**

Initial rates of efflux of labeled amino acids were determined after the cells had accumulated these compounds over different periods of time and hence had reached different vacuolar concentrations. Most of the data were obtained by a double tracer technique (“indirect method”) that allowed the simultaneous monitoring of unidirectional uptake and net accumulation. Efflux was then calculated as the difference of these both parameters (Fig. 3A). This method, which does not require the exchange of the external medium, is more sensitive and yields more precise results than the direct approach, i.e. following the
decrease of the cellular content of preaccumulated labeled amino acids after resuspension of the cells in tracer-free medium (Fig. 4). Both methods reported similar efflux properties.

Efflux from Intact Cells—As shown in Fig. 4, A, the amino acids did not significantly leak out of intact cells until their total content had reached a distinct threshold value. Above this level efflux tended to increase with the cellular amino acid content. The threshold content required for efflux was linearly related to the cell’s actual accumulation capacity (Fig. 3B). Under the conditions used this relation was true for the four investigated amino acids and for hyphal cells that express different accumulation capacities for a given amino acid (Phe), e.g. hyphae from nitrogen-sufficient compared with nitrogen-starved cell suspensions (25). No threshold level was required for efflux to occur in the presence of uncouplers or respiratory inhibitors, indicating that the threshold phenomenon involves active transport processes (Fig. 4A).

Efflux from Permeabilized Cells—Vacuoles in permeabilized cells allowed an efflux of preaccumulated amino acids from any initial content, i.e. no detectable threshold concentration had to be surpassed. As an example, Fig. 4B shows efflux of [14C]Phe from in situ vacuoles with Phe contents below 200 nmol/mg protein. In the corresponding experiment with intact cells, similar Phe contents do not allow a measurable efflux. The release of efflux from this restriction after permeabilizing the plasma membrane indicates that the threshold phenomenon requires the plasma membrane to function as a permeability barrier. Efflux from the in situ vacuoles is not an energy-requiring process as it proceeded in the presence of uncouplers (cf. Fig. 4B) or in the absence of ATP. It was not influenced by the actual redox state of the medium as it occurred unchanged in the presence of dithiothreitol (Fig. 4B, efflux after a 10-min accumulation).

The efflux of vacuolar amino acids shows a biphasic concentration dependence (Fig. 4B, inset). The ratio of efflux rate versus actual Phe content (given as the percentage of lost tracer content/min) increased after the Phe content surpassed a critical level around 200 nmol/mg of protein. This content comes very likely that its actual concentration determines the steadiness of the efflux of vacuolar amino acids, it appears very likely that its actual concentration determines distinct relations between amino acid uptake and efflux: (a) at

above the critical level were reached only transiently (e.g. within 20 and 60 min of accumulation), and after the return to lower levels (e.g. after 60 min), the enhanced efflux/content ratio was readjusted to the original values. Thus, allosteric acceleration of vacuolar efflux might also be involved in the regulation of the vacuolar pool size of amino acids in intact cells.

Effects of Adenylates on Initial Efflux Rates

The initial rate of amino acid efflux from in situ vacuoles is controlled by the concentration of ATP and other adenylates in the surrounding solution (Fig. 5A). In the absence of external ATP (its endogenous concentration detectable under these conditions was <1 μmol/liter), a fairly high rate of Phe efflux was measurable, which decreased with increasing concentrations of added ATP. A strong inhibition was observed at ATP levels around 200 μmol/liter. Further increase up to 1 mmol/liter of ATP relieved the efflux from inhibition and led to the same or even higher rates than in cells that were not supplied with ATP. The ATP-dependent release of vacuolar Phe is also demonstrated in Fig. 4A (curves starting at t = 15 min). The ATPase resistant ATP analogue AMP-PNP influenced the efflux of Phe in a similar fashion as did ATP (Fig. 5A). AMP and ADP could replace ATP with similar efficiency and concentration dependence. The same is true for mixtures of the adenylates (Table II). The observed effects of adenylates hold true not only for the efflux of vacuolar Phe but also of Leu, Arg, and Glu (data not shown).

Obviously, the adenylate level exerts regulatory effects on the efflux of vacuolar amino acids which are not mediated via an ATPase dependent energy supply but rather by the carrier(s) responsible for amino acid efflux.

The uptake of [14C]Phe by in situ vacuoles required ATP at a minimum concentration of approximately 100 μmol/liter and increased to maximum rates when ATP was raised to 1 mmol/liter (Fig. 5A). Expectedly, AMP-PNP, ADP, or AMP did not support Phe uptake into in situ vacuoles (data not shown), which reflects the ATPase-dependent energization of this process. ATP is the main component of the cellular adenylate pool (cf. Fig. 5B). As this nucleotide acts both as an energy source for uptake and a regulator of efflux of vacuolar amino acids, it
Adenylate Control of Vacular Efflux of Amino Acids

Fig. 5. Influence of different concentrations of adenylates on the rate of efflux from and uptake into vacuoles in situ. A, left axis, initial rates of efflux from permeabilized cells in the presence of ATP (●), AMP-PNP (○), ADP (□), or AMP (▲). Right axis, (⁎), initial rates of uptake of \[^{14}\text{C} \] Phe by permeabilized cells in the presence of increasing concentrations of ATP. Data are means of five experiments, S.D. = 7–12% for efflux, 9–18% for uptake. B, Horizontal columns, adenylate concentrations of intact hyphal cells from 48-h-old submerged cultures after different periods of carbon and nitrogen starvation: ATP (dark), ADP (hatched), AMP (open). (Read the data at the logarithmic concentration scale used in part A.) Nitrogen-derepressed cells (as used also for transport experiments) were resuspended in half-concentrated culture liquid containing neither nitrogen nor carbon sources, and the adenylate contents were assayed after the indicated starvation periods. For the estimation of cytoplasmic adenylate concentrations, the measured contents were related to 40% of the intracellular water as morphometric measurements indicated that the vacuolar compartment occupies an average of 60% of the cell volume. The sums of adenylate concentrations are as follows (μmol/liter): t = 0, 2500; 6 h, 750; 24 h, 255; 96 h, 37. Data are means of four parallel samples, S.D. = 10–15%.

ATP concentrations around 1 mmol/liter, both uptake and efflux reach maximum velocities, i.e. the exchange of vacuolar and cytosolic amino acids occurs at maximum rates; (b) at ATP concentrations around 0.2 mmol/liter, efflux is reduced to a minimum, and hence unidirectional uptake is the dominating process, even though it occurs with only 40% of its maximum rate; (c) at ATP concentrations below 0.1 mmol/liter, no uptake occurs due to insufficient energy input; unidirectional efflux is activated and lasts up to the depletion of the vacuolar pool.

Concentrations of ATP and other adenylates that exert the observed regulatory effects in permeabilized hyphae can be assumed to exist in intact cells under distinct physiological conditions. Fig. 5B displays adenylate concentrations (calculated on the basis of intracellular water) that arise during 4 days of starvation for carbon and nitrogen sources of submerged cultures. It can be seen that adenylate levels drop from 2 mmol/liter to 30 μmol/liter, which indicates that the cells run through all the above mentioned relations of vacuolar uptake and efflux. At any time ATP was found to be the dominant constituent (around 70%) of the total adenylates. Obviously, within the period of starvation the adenylate pool as a whole was gradually degraded without severely changing the relation defined as the free energy charge (see “Discussion”).

The effects of adenylates on the vacuolar efflux were not substantially modified by extravacular amino acids. External amino acids at concentrations >100 μmol/liter accelerated the efflux of the same, preaccumulated amino acid especially in the presence of millimolar concentrations of ATP. However, efflux was not relieved from the inhibition caused by ~200 μmol/liter ATP or AMP-PNP, indicating that the trans-stimulation of efflux did not interfere with its control by adenylates.

**DISCUSSION**

Dynamics of Vacular Uptake and Efflux—The characteristics of amino acid efflux from in situ vacuoles and the comparison with efflux data from intact cells indicate a dynamic equilibrium between active vacuolar accumulation and passive efflux into the cytosol. Most probably, in nutrient-sufficient cells efflux of vacuolar amino acids is a steady process, which is compensated for by active reuptake, thus keeping their cytosolic concentration low enough to prevent an efflux out of the cell. When the vacuolar concentration approaches a critical level, allosteric acceleration causes the rate of efflux to surpass the capacity of active uptake across the tonoplast. The resulting rise of the cytosolic amino acid concentration may then cause the observed efflux out of the cell. Thus, the threshold phenomenon observed with intact cells mainly reflects the functional balance between different transport steps at the tonoplast and the plasma membrane rather than massive binding of amino acids to protein components. The actual size of the vacuolar pool and its share of the cellular amino acid content is controlled by at least four factors. (a) The accumulation capacity of the tonoplasts active amino acid transport systems, which is supported by the observed linear relation between accumulation capacity and threshold content; (b) the affinity of the efflux carriers at the plasma membrane that prevents significant leakage of amino acids at low cytosolic concentrations, which is supported by our finding that efflux required no threshold content after the plasma membrane had been permeabilized; (c) the actual concentrations of vacuolar amino acids that drive the efflux with a biphasic concentration dependence (the involvement of carriers rather than channels in the efflux process is indicated by the trans-stimulation of efflux exerted by extravacular amino acids); and (d) by the actual ATP and adenylate content of the cytosol.

**The Cellular Adenylate Pool as a Regulatory Signal for Vacular Efflux**—To our knowledge, a direct regulatory effect of ATP and other adenylates on vacuolar efflux has not yet been reported from fungal cells. In part, our results might be related to earlier data of amino acid transport in plant vacuoles. Dietz et al. (9) found the efflux of various amino acids from isolated vacuoles of barley protoplasts was stimulated by 10 mmol/liter ATP or AMP-PNP. This finding is analogous to what occurs in the fungal system in the upper range of ATP concentrations. However, our data reveal an additional band of regulatory
active adenylate levels around 100–200 μmol/liter, which cause a drastic reduction of vacuolar efflux. When comparing barley vacuoles and our object, it must be realized that the uptake of amino acids shows significant differences. Whereas in barley vacuoles the transport of [14C]Ala was independent of proton-motive force and hence not coupled to energization via the tonoplast H⁺-ATPase, in *P. cyclopium* uptake of [14C]Phe requires ATP and is sensitive to uncouplers. It is further possible that the effect of low ATP concentrations on vacuolar transport as observed with *Penicillium vacuoles in situ* cannot be detected by *in vitro* experiments (like that performed with barley vacuoles) as it requires cytosolic components that are present under *in situ* conditions but not in *in vitro*. Extended work with different plant and fungal objects is required to determine whether the observed regulatory mechanism is a common phenomenon.

The biological significance of an ATP-mediated control over the efflux from the vacuole into the cytosol appears obvious if one considers the dynamics of cellular adenylates during the adaptive response to a long term carbon limitation (cf. Fig. 5B). Whereas in nutrient-sufficient cells the ATP and adenylate content is high enough to allow an exchange of vacuolar and cytosolic amino acids at maximum rates, cells respond to a decrease of the ATP and adenylate level in two steps. First, a drop toward ~10% triggers an amino acid- and ATP-saving strategy, *i.e.* stop of vacuolar efflux and reduction of vacuolar uptake. Second, a severe loss of energy (adenylate levels far below 10%) leads to increasing rates of efflux and metabolization of stored amino acids thus supporting the survival of the cell.

The mechanism behind the control of efflux exerted by the adenylate concentration remains to be elucidated. The present data argue for a direct influence of cytoplasmic adenylates on the vacuolar amino acid carriers. Some alternate possibilities appear less or not supported by our data, especially because of the similarity of effects caused by AMP, ADP, ATP, or AMP-PNP, which is also consistent with the passive, uncoupler-insensitive nature of the efflux step. Thus, proton-motive force-mediated effects on the amino acid transporters, ATPase-dependent processes, or regulatory influences via the adenylate energy charge are rendered unlikely. In fact, the adenylate energy charge was maintained in the range between 0.75 and 0.85 (calculated from Fig. 5B) during a 4-day starvation period irrespective of whether the adenylate content reached an efflux-permitting or efflux-inhibiting range. This behavior is in contrast with the decline of adenylate free energy in yeast cells during prolonged starvation periods (39). Thus, it appears that in our object the total adenylate level rather than any relationship between the adenylate compounds exerts regulatory functions. Redox-mediated activities of vacuolar ion channels (as found with cation channels in the yeast vacuole) (40) are not consistent with the observed lack of influence of dithiothreitol on efflux rates. Furthermore, the involvement of cation channels cannot explain the similarities of efflux between the neutral (Phe), acidic (Glu), and basic (Arg) amino acids used in this study as well as the observed stimulation of efflux by extra-vacuolar amino acids. In plant cells, some vacuolar ion channels (SV-type) have been found to be influenced by ATP (41, 42), but in contrast to our object nonhydrolyzable ATP analogues were ineffective. Thus, an adenylate-mediated control of the vacuolar pool might add a new facet to our knowledge of intracellular metabolic control and thus deserves further experimental efforts, *e.g.* to identify the site(s) of efflux control.

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