Competitive intra- and extracellular nutrient sensing by the transporter homologue Ssy1p

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Recent studies of *Saccharomyces cerevisiae* revealed sensors that detect extracellular amino acids (Ssy1p) or glucose (Snf3p and Rgt2p) and are evolutionarily related to the transporters of these nutrients. An intriguing question is whether the evolutionary transformation of transporters into nontransporting sensors reflects a homeostatic capability of transporter-like sensors that could not be easily attained by other types of sensors. We previously found *SSY1* mutants with an increased basal level of signaling and increased apparent affinity to sensed extracellular amino acids. On this basis, we propose and test a general model for transporter-like sensors in which occupation of a single, central ligand binding site increases the activation energy needed for the conformational shift between an outward-facing, signaling conformation and an inward-facing, nonsignaling conformation. As predicted, intracellular leucine accumulation competitively inhibits sensing of extracellular amino acids. Thus, a single sensor allows the cell to respond to changes in nutrient availability through detection of the relative concentrations of intra- and extracellular ligand.

Introduction

Many cells adapt to changes in extracellular nutrient levels by altering the expression of genes encoding transporters for these molecules. This is accomplished through signal transduction pathways that respond to the binding of extracellular nutrients to plasma membrane–sensor proteins. In some cases (for review see Boles and André, 2004), including plants and animals (for reviews see Forsberg and Ljungdahl, 2001; Hyde et al., 2003), the sensors share significant amino acid sequence similarity with transporters of the nutrient in question. The plasma membrane of *Saccharomyces cerevisiae* contains transporter-like sensors for amino acids (Ssy1p) and glucose (Snf3p and Rgt2p), but these proteins apparently do not transport their respective nutrients (Liang and Gaber, 1996; Didion et al., 1998; Özcan et al., 1998; Iraqui et al., 1999). Sensing of extracellular amino acids through Ssy1p leads to transcriptional induction of genes encoding amino acid transporters (Didion et al., 1996, 1998; Jørgensen et al., 1998; Iraqui et al., 1999; Klasson et al., 1999) and some other genes through activation of the transcription factors Stp1p and Stp2p (for review see Boles and André, 2004).

How nutrient sensors convert changes in extracellular nutrient concentrations into appropriate signals is key to the understanding of nutrient homeostasis. Kinetic and biochemical studies (for reviews see Wheeler and Hinkle, 1985; Abramson et al., 2003a) and recent structural data (Abramson et al., 2003b; Huang et al., 2003) indicate that carrier-type transporters have a single substrate binding site (for symporters, a single site for each ligand molecule transported in a cycle) that is exposed to either side of the membrane, depending on the conformational state of the transporter. Observations that mutations in *SSY1* can increase the apparent affinity for sensing of amino acids and concomitantly confer an increased basal level of signaling (Gaber et al., 2003; Poulsen et al., 2005a) suggests that these mutations alter an equilibrium between a signaling and a nonsignaling conformation of Ssy1p in the absence of ligand. In fact, ligand-independent occurrence of a signaling conformation has previously been found among the very different 7TM-type receptors (Rosenkilde and Schwartz, 2000). We propose, then, that extracellular amino acids are sensed because of their ability to bind to and stabilize a signaling conformation. Given the structural similarity of Ssy1p to transporters, existence of states such as I (inward facing), O (outward facing), and O·L (outward facing, ligand bound; Fig. 1), interconverting through reactions 1 and 2, would provide a formal model for the initial

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Abbreviation used in this paper: DW, dry weight.

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step of sensing and would, at the same time, interpret hyper-responsive and constitutive *SSY1* mutants in a simple way, namely, as being affected in the equilibrium constant for reaction 1. The additional idea that a cytoplasmic ligand might bind to state I (i.e., existence of reaction 3 and state I·L [inward facing, ligand bound]) represents an extended model for transporter-like sensors, which has the salient feature that ligand binding inhibits the conformational shift (i.e., reaction 4 is not efficient). Reaction barriers for conformational changes are actually common in transporters. For example, in the case of the anion carrier in erythrocytes, reaction 1 is at least 10,000-fold less efficient than reaction 4 (Hunter, 1977; Knauf et al., 1977), a fact that explains the strong antiport function of the carrier for chloride and bicarbonate. We decided to test our model by investigating whether the signaling potency of extracellular ligand is influenced by the cytoplasmic ligand concentration.

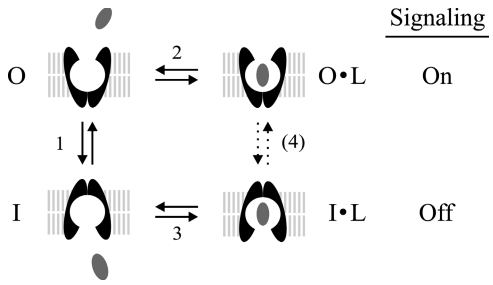

Figure 1. **Model for transporter-like sensors.** States corresponding to those of a canonical transporter are presented. The elliptic object symbolizes the ligand (i.e., amino acid in the case of Ssy1p), and the vertical shaded bars indicate the membrane lipids. The dotted arrows for reaction 4 indicate that for a sensor working as proposed for Ssy1p, states O·L and I·L cannot be directly turned into one another. For a real transporter, on the other hand, reaction 4 must be efficient. The outward-facing conformation of the sensor (states O and O·L) is hypothesized to be signaling, whereas states I·L and I are nonsignaling. O, outward facing, O·L, outward facing, ligand bound; I·L, inward facing, ligand bound; I, inward facing.

Results and discussion

Manipulating cytoplasmic leucine levels by extracellular supplementation during growth

To study the effect of intracellular amino acids on signaling by Ssy1p, we chose to manipulate the cytoplasmic concentration of leucine, as extracellular leucine is the most potent known elicitor of signaling through Ssy1p (Didion et al., 1996; Gaber et al., 2003). To increase the cytoplasmic level of leucine, cells were grown in minimal medium with increasing levels of extracellular leucine. This resulted in an increase in the cytoplasmic concentration of leucine from 0.5 μmol/g dry weight (DW) without addition of leucine to 26 μmol/g DW for cells grown in medium with 1 mM leucine (Table I, experiments 1 and 2), allowing us to measure the effect of intracellular leucine levels on Ssy1p signaling. After removal of leucine from the growth medium by washing the cells, signaling was induced by addition of extracellular amino acid at various concentrations and measured as cleavage of the transcription factor Stp1p. The dose–response relationship (Fig. 2) allowed determination of the median effective concentration (EC_{50}, apparent dissociation constant). In agreement with the model (Fig. 1), loading of cells with leucine by previous growth in presence of 1 mM leucine produced an EC_{50} that was four times higher than that of cells grown without leucine (Fig. 2 B and Table I, experiments 1 and 2). The value of 13 μM for cells grown without leucine agreed with data previously determined (Poulsen et al., 2005a,b). Thus, increasing the cytoplasmic leucine concentration led to an increase in the apparent dissociation constant in the sensing of extracellular leucine. Consistent with the model, the increased cytoplasmic leucine also produced a similar increase in EC_{50} for the sensing of phenylalanine (Table I, experiments 10 and 11).

EC_{50} in cells with different growth history

We tested in several ways the possibility that changes in the apparent affinity of extracellular ligand might be a consequence of the history of signaling, rather than a direct consequence of the cytoplasmic leucine concentration. In one experiment, cells were grown in media containing 50 or 125 μM leucine, amounts that are sufficient to almost fully induce signaling but which did not strongly increase the cytoplasmic leucine concentration. These cells exhibited an EC_{50} close to that obtained with cells grown without leucine (Table I, compare experiments 3 and 4 with 1), suggesting that the signaling history was irrelevant. Next, we tested a leucine uptake-defective strain (M5568), which lacked the broad-spectrum amino acid transporter Agp1p (Iraqui et al., 1999) and the leucine transporters Bap2p, Tat1p, and Bap3p (Didion et al., 1998). In the control experiment, i.e., after growth in medium without leucine, this strain exhibited a normal EC_{50} for leucine (Table I, compare experiment 5 with 1 and 7). After growth for 2 h in medium containing 500 μM

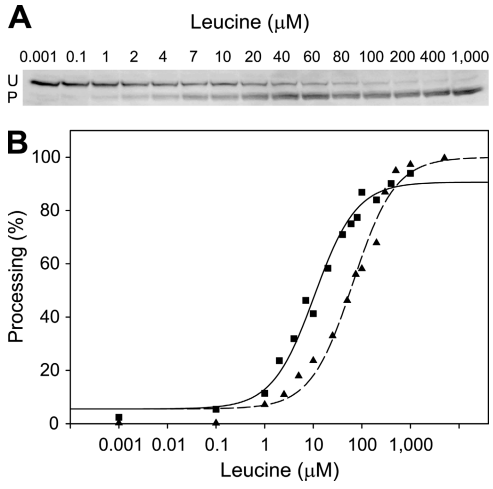

Figure 2. **Cytoplasmic leucine inhibits extracellular amino acid signaling by influencing the median effective concentration of extracellular amino acid.** A representative experiment is shown. (A) Western blot of protein extracts of yeast cells (strain M5447) grown in minimal medium (SD) and exposed to leucine, showing processed (P) and unprocessed (U) forms of the transcription factor Stp1p. (B) Signaling measured as Stp1p processing shown in A was fitted as described (see Material and methods). Squares indicate data from A (solid curve is best fit, giving EC_{50} = 12 μM), whereas triangles and broken curve indicate data and fit obtained with cells grown in SD medium with 1 mM leucine and subsequently washed (EC_{50} = 66 μM).

Table I. **Cytoplasmic leucine (Leu_c) and EC_{50} for sensing of extracellular amino acid**

Experiment	Strain[a]	Growth medium	Leu_c	Extracellular sensed amino acid	EC_{50}
			μmol/g DW		*μM*
1	M5447 (*ura3 gap1Δ*)	SD[b]	0.5	Leu	13
2	M5447 (*ura3 gap1Δ*)	SD + 1 mM Leu	26	Leu	66
3	M5447 (*ura3 gap1Δ*)	SD + 50 μM Leu	1	Leu	18
4	M5447 (*ura3 gap1Δ*)	SD + 125 μM Leu	3	Leu	23
5	M5568 (*ura3 gap1Δ bap2Δ tat1Δ bap3Δ agp1::URA3*)	SD	1	Leu	15
6	M5568 (*ura3 gap1Δ bap2Δ tat1Δ bap3Δ agp1::URA3*)	SD + 500 μM Leu[c]	10	Leu	27
7	M5593 (*gap1Δ*)	SD	0.4	Leu	11
8	M5593 (*gap1Δ*)	SD + 500 μM Leu[c]	20	Leu	53
9	M5446 (*ura3 gap1Δ LEU4-fbr*)	SD	12	Leu	30
10	M5447 (*ura3 gap1Δ*)	SD	1[d]	Phe	253
11	M5447 (*ura3 gap1Δ*)	SD + 1 mM Leu	29[d]	Phe	1,025

The values are single determinations except for 1 (mean of triplicate, including Fig. 2) and 2 (mean of duplicate, including Fig. 2 B). The SDs were estimated to be 24% for Leu_c and 10% for EC_{50}. The SDs were determined in separate experiments by repeated cultivations. Different strains were grown at different conditions giving at least six separate sets, and the mean SDs were estimated using all data. For EC_{50}, the variance obtained in the regression was added to the one of repeated cultures to get the SD presented.
[a]All four strains are isogenic to strain S288C except for the genes indicated.
[b]SD is a standard minimal medium (see Materials and methods).
[c]Cells were grown overnight without leucine, leucine was added to the indicated concentration, and growth was continued for 2 h before washing and determination of Leu_c and EC_{50}.
[d]Determined in the same batch of cells as EC_{50}.

leucine, cytoplasmic leucine in this strain only increased to 10 μmol/g DW (Table I, experiment 6) as compared with 20 μmol/g DW in the uptake-proficient strain (M5593; Table I, experiment 8), and the EC_{50} of leucine only increased to 27 μM (Table I, experiment 6), as compared with 53 μM for the uptake-proficient strain (Table I, experiment 8). Thus, the shift in EC_{50} follows intracellular leucine rather than history of exposure, and it can occur independently of whether the cells were treated with leucine for many hours or 2 h.

Increasing cytoplasmic leucine level by overproduction

In a third experiment, the cytoplasmic leucine concentration was perturbed in a manner that did not involve feeding of leucine from the outside. Organisms that synthesize leucine do so by a series of four reactions, using 2-oxoisovalerate as a precursor. In *S. cerevisiae*, *LEU4* encodes the major isoform of the enzyme catalyzing the first step (Beltzer et al., 1988), which is subject to feedback inhibition by leucine (Ulm et al., 1972). The *LEU4-fbr* mutation (Baichwal et al., 1983) confers reduced or eliminated sensitivity to leucine, leading to an increased intracellular concentration of leucine. Introduction of the *LEU4-fbr* mutation caused an increase of the cytoplasmic leucine pool from 0.5 to 12 μmol/g DW (Table I, compare experiment 9 with 1). The EC_{50} toward extracellular leucine increased from 13 to 30 μM. Thus, this independent method of increasing cytoplasmic leucine produced the same effect on signaling as growth in medium with a high leucine concentration.

We also considered the possibility that the catabolic product of leucine, isoamyl alcohol, might influence EC_{50} by dissolving into the lipid bilayer of the plasma membrane and changing its characteristics. However, there was no significant effect of adding isoamyl alcohol at relevant concentrations (unpublished data).

Quantitative aspects of the model

We have considered equilibrium equations for reactions 3 ($K_3 = [I][L_c]/[I{\cdot}L]$), 1 ($K_1 = [O]/[I]$), and 2 $\{K_2 = [O{\cdot}L]/([L_e][O])\}$ in Fig. 1, where [O], [O·L], [I·L], and [I] are the concentrations (amounts) of the four depicted forms of the sensor. The cytoplasm may contain several compounds that can appreciably bind to I but, for a start, we consider a single compound, L_c, which may or may not be identical to the offered extracellular ligand, L_e. The concentrations of the ligand-free forms of the sensor, [I] and [O], can be eliminated from the three equations to yield a single equation:

$$\frac{[L_c]}{K} = [L_e]\frac{[I \cdot L]}{[O \cdot L]}$$

where [I·L] and [O·L] are the concentrations of the ligand-containing forms of the sensor and $K = K_1 \cdot K_2 \cdot K_3$ (Fig. 1). If ligand bound forms of both conformations are strongly predominant, [I·L] will be the concentration of nonsignaling Ssy1p and [O·L] will be that of signaling Ssy1p. Then, the ratio of signaling to nonsignaling Ssy1p is proportional to the concentration ratio across the plasma membrane; i.e., the sensor output is determined by the ligand concentration ratio. If a measured response is linear with the amount of signaling form of Ssy1p, the right part of the equation becomes the apparent dissociation constant, EC_{50}, of an extracellular ligand to Ssy1p in a dose–response analysis. Thus, EC_{50} will be approximately proportional to $[L_c]$. If several cytoplasmic ligands with different affinities are considered, EC_{50} becomes approximately proportional to $\Sigma\,[L_{ci}]/K_{3i}$, a weighted sum of cytoplasmic ligand concentrations, where each ligand is referred to by an index integer, i, and K_{3i} is the equilibrium constant of reaction 3 (Fig. 1) characteristic for the cytoplasmic ligand in question.

Relationship between EC_{50} and cytoplasmic leucine

If the concentration of a single cytoplasmic ligand, [Leu_c], is varied and the concentrations of the other cytoplasmic ligands are kept constant, the model, even when taking multiple ligands into account, predicts that the EC_{50} of an extracellular ligand will vary linearly with [Leu_c]. Indeed, our data were consistent with linearity (Fig. 3). Best fit ($R^2 = 0.80$) to $EC_{50} = M\,[Leu_c] + N$ yields $M = 1.57$ μM/(μmol/g DW). An analogous relation can be made in which the abscissa is the cytoplasmic leucine concentration, rather than amount per DW. With an approximate value of 2 ml/g DW for the specific cytoplasmic volume (Larsson et al., 2000), this relation has a slope of 0.003 (analogous to M, but dimensionless).

At sufficient concentrations of cytoplasmic and extracellular leucine, this relationship means that the leucine concentration ratio across the plasma membrane is sensed, rather than the absolute extracellular leucine concentration. This reflects a sensing principle that would not be straightforward to obtain with nontransporter-like sensors. We interpret the intercept with the ordinate in Fig. 3 to reflect binding to the inward-facing form of Ssy1p of cytoplasmic ligands other than leucine, presumably other L-α-amino acids. It is rather modest (fourfold lower than EC_{50} of the highest point in Fig. 3), considering the relatively high amounts of some cytoplasmic amino acids measured; e.g., we see rather constant levels of ∼120 μmol/g DW (i.e., ∼60 mM) of glutamate, which is fourfold higher than the leucine concentration at the highest point in Fig. 3. However, this is consistent with the possibility that the relative affinities of the various amino acids for binding to outward-facing Ssy1p (Gaber et al., 2003) are fully or partially conserved when inward-facing Ssy1p is considered, i.e., that leucine is the strongest binding amino acid, also from the inside.

Concluding remarks

We present a model in which binding of amino acids to transporter-like sensors from outside or inside stabilize signaling and nonsignaling conformations, respectively. We tested the model by looking for an influence of the concentration of a cytoplasmic amino acid on Ssy1p-mediated sensing. Consistent with the model, we found the median effective concentration, EC_{50}, of signaling to correlate linearly with the concentration of cytoplasmic leucine. It will be of interest to determine whether our model can account for sensing by other transporter-like sensors, including the *S. cerevisiae* glucose sensors Snf3p and Rgt2p (Özcan et al., 1996). The model makes obvious sense in terms of intracellular nutrient homeostasis. It can also account for the function of sensors that can transport their respective solutes, such as the *S. cerevisiae* general amino acid permease (Gap1p; Donaton et al., 2003) if the rate constants for reaction 4 are different from those for reaction 1.

We have not shown that the interaction between intracellular ligand and the sensor is direct, but our results are readily explained by the structural similarity of the sensor with transporters and suggest a mechanism by which cells can monitor the relative concentrations of a nutrient across the plasma membrane.

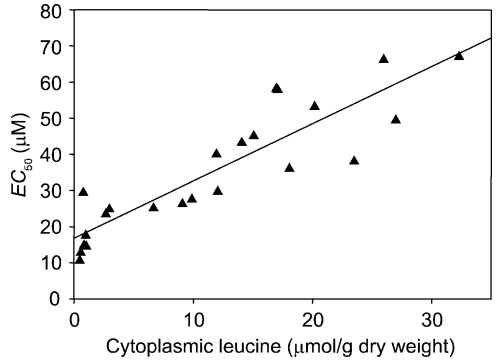

Figure 3. **Relationship between amount of cytoplasmic leucine and EC_{50} for sensing of extracellular leucine.** The 23 pairs of data include experiments 1–9 presented in Table I and were fitted to the linear relationship $EC_{50} = M[Leu_c] + N$. Each point represents a separate condition or strain.

Materials and methods

Yeast strains

Yeast strains were derived from strain M4054, originating (Grauslund et al., 1995) from S288C via X2180-1A by a spontaneous, low-reverting *ura3* mutation and a deletion of most of *GAP1*. Genetic fusion of the ZZ tag to Stp1p was as described previously (Poulsen et al., 2005a), and deletions were introduced (Didion et al., 1998) by standard techniques. Strain M5446 was made by integrating a PCR fragment with *LEU4-fbr* from strain XK14-15D, provided by G.B. Kohlhaw (Purdue University, West Lafayette, IN), into strain M4054, followed by introduction of the ZZ construct.

Cultivation and media

Yeast cells were grown aerobically batch-wise in shake flasks overnight to a turbidity (OD_{600}) of 0.25–0.6, corresponding to 3–6 × 10^6 cells/ml, in glucose- and ammonium-based minimal medium supplemented with uracil (Sherman, 1991) and buffered with 85 mM succinic acid + 150 mM NaOH (SD medium). As indicated, some cultures were inoculated in medium with additional leucine, and others contained additional leucine during the last 2 h of cultivation.

Determination of cytosolic amino acids

To determine the cytosolic pool of amino acids, 200 ml of culture with a known DW of cells were mixed with crushed ice (100 g), harvested by centrifugation, and washed twice with ice-cold water. For specific release of cytosolic amino acids, the plasma membrane was selectively permeabilized with the cupric ion method as described previously (Ohsumi et al., 1988), using the following specific protocol: cells were resuspended in 1.5 ml of permeabilization buffer (5 mM MES, pH 6.0, and 0.4 mM $CuCl_2$), incubated at 30°C for 10 min, and centrifuged. The supernatant was pooled with the supernatant obtained after a step of washing (0.75 ml 5 mM MES buffer, pH 6.0) and dried in a SpeedVac. For some experiments, the aforementioned volumes were scaled a few times up or down. The sample was dissolved in 100 μl of water, acidified with 10 μl of 3% sulfosalicylic acid, and centrifuged at 20,000 *g* for 20 min at 4°C. The supernatant was neutralized with 10 μl of 1 M NaOH. After evaporation, the volume was adjusted, and part of the sample was applied to the amino acid analyzer (Biochrom 20; GE Healthcare), using ninhydrin for detection.

Quantification of signaling and determination of EC_{50}

The quantification of signaling by Ssy1p is based on the findings that the transcription factor Stp1p is proteolytically activated by removal of a 10-kD NH_2-terminal inhibitory part and that it has such a short half-life that monitoring of signaling is possible irrespective of the physiological history of the cells (Andréasson and Ljungdahl, 2002).

Stp1p was expressed as a fusion protein (Stp1-ZZ) containing a bacterial IgG binding domain, allowing monitoring of proteolytic processing by Western blotting as described previously (Poulsen et al., 2005a,b). Leucine or phenylalanine at appropriate concentrations was

added to aliquots of the culture to induce signaling. After 10 min, proteins were extracted and separated by electrophoresis, and Stp1-ZZ in processed and unprocessed form was quantified.

The median effective concentration (EC_{50}, apparent dissociation constant) was determined by measurement of Stp1p processing ($S = P/[U + P]$, where P is the amount of processed form and U is the amount of unprocessed form) at 0.001–1,000 μM of leucine or 0.01–10,000 μM of phenylalanine. The data were fitted to a hyperbolic relationship ($S = S_1\{(EC_{50}/[L_e]) + 1\}^{-1} + S_0$, sigmoid in the semilog plot) to extracellular ligand concentration ($[L_e]$), using the SigmaPlot software with the constraints that S_0 cannot be negative and $S_1 + S_0$ cannot be >1.

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