Changes in membrane proteins associated with inhibition of the general amino acid permease of yeast (*Saccharomyces cerevisiae*)

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The general amino acid permease ('Gap') system of the wild-type yeast (*Saccharomyces cerevisiae*) strain Y185 is inhibited by the uptake and accumulation of its substrate amino acids. Surprisingly, this inhibition persists even after 'pools' of amino acids, accumulated initially, have returned to normal sizes. Recovery from this inhibition depends on a supply of energy and involves the synthesis of a membrane protein component of the Gap system.

Yeast cells grown under conditions of nitrogen starvation synthesize a general amino acid permease ('Gap') that effects the uptake of a wide variety of D- and L-amino acids. When cells thus de-repressed for the Gap system are exposed to any of its substrates, they take up these amino acids readily. However, they progressively and rapidly lose that capacity: it appears that the accumulation of amino acids in 'pools' inhibits the function of the Gap system. The term 'trans-inhibition' has been applied to this phenomenon (Bellenger *et al.*, 1968; Crabeel & Grenson, 1970; Ring *et al.*, 1970; Pall, 1971; Hunter & Segal, 1973; Woodward & Cirillo, 1977), although there is evidence that it is not due simply to a feedback effect. It is known (Crabeel & Grenson, 1970) that the 'pools' of accumulated amino acids are not exchangeable with amino acids in the external medium. Moreover, as is confirmed in the present paper, cells whose uptake of amino acids has been inhibited by the prior uptake of valine remain inhibited even after the valine initially taken up has been metabolized (Woodward & Cirillo, 1977). A similar effect has been noted with *Penicillium chrysogenum* (Hunter & Segal, 1973).

In the present paper we report experiments, on the changes in amino acid 'pools' after exposure of yeast cells to histidine and valine, that rule out any direct inhibition of Gap activity by these 'pools'. We show that inhibition is associated with a change in a membrane protein component of the Gap system, and that recovery from the inhibition involves the resynthesis of this protein.

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Experimental

Organisms and growth of cultures

The strains of yeast used, Y185 and the Gap⁻ mutant Y185-His3, and the conditions used for their culture, were described by Woodward & Kornberg (1980).

Amino acid uptake

Cells subjected to 'trans-inhibition' were prepared as a 1% (v/v) suspension in 50 mM-potassium phthalate buffer, pH 5.5, containing 2% (w/v) glucose. The appropriate amino acid substrate of the Gap system was added to a final concentration of 2 mM. Samples were removed during incubation with this amino acid, centrifuged, and the cells were resuspended to the same concentration in fresh buffered glucose. The rates at which such cell suspensions took up 0.02 mM ¹⁴C-labelled amino acids were measured as described by Woodward & Cirillo (1977).

Extraction and analysis of amino acid pools

The total amino acid pools in cells accumulating amino acids were measured in 10 ml samples (at A_{680} 1.0) of cells withdrawn at intervals from cultures undergoing trans-inhibition. These cells were harvested, washed twice with distilled water at room temperature and then cooled to 4°C. They were then extracted twice with 2.5 ml of 5% (w/v) trichloroacetic acid; the solid residue was discarded. The extracts were pooled and the trichloroacetic acid was removed from them by washing twice with 50 ml of diethyl ether (Cook & Anthony, 1978). Extracts were stored at -20°C. 'Pools' of individual amino acids were determined in thawed samples with a

Locarte amino acid analyser, with standard amino acid mixtures as reference material.

Cells were also extracted by boiling them with distilled water (5 ml) for 10 min. The two procedures gave virtually identical results.

Preparation and analysis of cell extracts and membranes of labelled cells

Cells were labelled with ^3H - or ^{14}C -labelled amino acid mixtures, and cell-free extracts and plasma membranes were prepared, as described previously (Woodward & Kornberg, 1980). The double-labelling patterns of proteins in these preparations were analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis as also described in that paper.

Results and discussion

A suspension of yeast cells, that had been de-repressed for general amino acid permease activity by growth on L-proline as sole N source, was incubated at 30°C with 2 mM unlabelled valine in the presence of glucose. Portions of this suspension were removed at zero time and at intervals of 30 min over the subsequent 2 h, centrifuged and resuspended to the same cell concentration in fresh buffer containing glucose; the rates at which they took up 0.02 mM - ^{14}C valine were measured. The initial rates of ^{14}C valine uptake were plotted against the lengths of time for which the cells had been preincubated with unlabelled valine (Fig. 1*a*). It is evident that prior exposure to unlabelled valine rapidly and severely diminished the ability of the yeast subsequently to take up labelled valine. This is

not the result of a possible mass-action effect, whereby a high internal concentration of an amino acid specifically reduces the influx of that amino acid into the cells: as apparent from Fig. 1*a*, prior exposure to unlabelled valine also rapidly and severely inhibited the ability of the cells to take up 0.02 mM - ^{14}C histidine. Similarly, prior incubation of yeast suspensions with unlabelled L-histidine rapidly decreased the ability of cells, harvested after various lengths of time, to take up 0.02 mM - ^{14}C valine; this occurred even when the inhibitor of protein synthesis cycloheximide was present during the period of incubation with the unlabelled amino acid (Fig. 1*b*). This makes it unlikely that the progressive decrease in amino acid uptake observed was due to the synthesis of a polypeptide transport inhibitor.

It is known (Woodward & Cirillo, 1977) that valine is rapidly metabolized by yeast. When cells, inhibited by prior incubation with unlabelled 2 mM-valine for 2 h, were left at 20°C overnight in the absence of valine, they recovered their ability to take up 0.02 mM - ^{14}C valine. In contrast, the inhibition caused by prior exposure to L-histidine (which is known to be poorly metabolized) largely remained, even though the cells had been kept in fresh buffer supplemented with glucose overnight (Table 1). Cells recovered from histidine inhibition only when allowed to grow on the medium used for de-repressing the general amino acid permease.

Although these results suggest a direct relationship between the amounts of amino acids accumulated and the subsequent inhibition of amino acid transport, measurements of the amino acid 'pools' in cells taking up histidine or valine did not support such a simple view. As shown in Fig. 2, cells

Fig. 1. 'Trans-inhibition' of valine and histidine transport in cells de-repressed for the *Gap* system

Cells were allowed to accumulate unlabelled valine or histidine (final concn. 2 mM) for 120 min. At the times indicated, 1 ml samples were removed, harvested and resuspended in 50 mM-phthalate buffer, pH 5.5, containing 2% (w/v) glucose. The uptake of ^{14}C valine or ^{14}C histidine (final concn. 0.02 mM) was then measured for 2.5 min, and the initial rates of transport were calculated. (a) Cells incubated initially with unlabelled valine: rates of uptake of ^{14}C valine (Δ) or ^{14}C histidine (O). (b) Cells incubated initially with unlabelled histidine: rates of uptake of ^{14}C valine, in the absence (●) and in the presence (□) of cycloheximide (100 $\mu\text{g/ml}$).

Table 1. Recovery of cells, inhibited by valine or histidine, through overnight storage at 20°C in the absence of the inhibitory amino acid

Cells were treated as described in the legend to Fig. 1.

Incubation conditions	Initial rate of uptake of [¹⁴ C]valine (nmol/min per mg of protein) by cells incubated with unlabelled amino acid under the conditions indicated
Valine for 0 min	122
Valine for 2 h	5
Valine for 2 h and stored in buffer and glucose for 20 h	127
Histidine for 0 min	121
Histidine for 2 h	5
Histidine for 2 h and stored in buffer and glucose for 20 h	19

Fig. 2. Changes in total amino acid pool sizes during accumulation of histidine and valine

Suspensions of cells ($A_{680} = 1.0$) were incubated with either histidine or valine (final concn. 2.0mM) at 30°C for the times shown. At intervals, portions (10ml) were removed, and the amino acids contained in the cells were extracted and analysed as described in the text. The total amino acid ‘pools’ after incubation with histidine (Δ) and valine (O) are here plotted against the times of incubation.

taking up L-histidine indeed accumulated this amino acid in their trichloroacetic acid-soluble ‘pools’; the histidine content increased approximately 10-fold over the 60 min period of incubation. This behaviour is consistent with the known reluctance of yeast to metabolize histidine: presumably, the appropriate catabolic enzymes were not synthesized although the uptake system had been induced. In contrast, the

initial 8-fold rise in valine content observed 15 min after exposure of the yeast suspension to valine was followed by a rapid fall: the valine ‘pool’ appeared to be no higher after 60 min than before the experiment had begun. Moreover, all other amino acids detectable in the trichloroacetic acid extracts had, by that time, returned to the low ‘pool’ concentrations that were routinely observed in cells before incubation with valine. The addition of the proteinase inhibitor phenylmethanesulphonyl fluoride, to 2mM, did not alter the results obtained with valine; this differs from the findings obtained by Seipel & Reichert (1980), that phenylmethanesulphonyl fluoride inhibited the inactivation of xanthine transport by NH_4^+ ions in *Schizosaccharomyces pombe*.

Some information on the mechanism of this control of amino acid uptake was provided by experiments on the requirements for recovery from this inhibition. Cells de-repressed for the Gap system were permitted to accumulate valine, from a buffered glucose solution containing the amino acid at 2mM but lacking the other components necessary for growth, for 90 min; by that time, the ability of the cells to take up valine had fallen to its customary low value (Fig. 3). The cells were harvested thereafter, washed and resuspended in buffer alone, in buffer supplemented with glucose and in buffer supplemented with glucose but also containing cycloheximide (100 μg/ml). In the absence of glucose, the cells did not recover their ability to take up valine over the next 150 min; in the presence of glucose they did, but their recovery was prevented by cycloheximide (Fig. 3). It thus appeared that polypeptide synthesis was required for recovery from inhibition of valine uptake.

It has been argued (Grenson *et al.*, 1968) that addition of cycloheximide, by inhibiting the synthesis of proteins, causes the rapid build-up of amino acid ‘pools’ in the cells, and that it is this accumulation that inhibits the further uptake of amino acids. The data of Fig. 2 already show that, although an initial build-up of amino acid ‘pools’ is associated

with the subsequent inhibition of transport of Gap substrates, this inhibition persists even when (in the case of valine) the 'pool' sizes have returned to normal. Further evidence to this effect was obtained

Fig. 3. Recovery of cells from 'trans-inhibition' by valine. Cells were allowed to accumulate valine for 1.5 h, and their ability to take up [14 C]valine was measured as described in Fig. 1. The cells were then harvested and resuspended in buffer alone (○), buffer plus glucose (▲) or buffer plus glucose plus cycloheximide (100 μ g/ml) (△). The initial rates of [14 C]valine uptake by portions of these cells were measured over a further period of 2.5 h. A control culture that had not been exposed to unlabelled valine initially was maintained in buffer plus glucose for the duration of the experiment (●).

in an experiment in which cycloheximide was added to cells, recovering from valine inhibition, at various times after the onset of recovery. In this experiment, cells de-repressed for the Gap system were first allowed to accumulate 2 mM-valine for 90 min, and their ability to take up [14 C]valine was measured, as in the experiments illustrated by Figs. 1 and 3. After 90 min, the cells were harvested and resuspended in four flasks containing buffered glucose. Cycloheximide was added to one flask immediately, to a second after a further 2 h and to a third after a further 4 h; cycloheximide was not added to the fourth. Portions of the cell suspensions were taken from each flask, and both their ability to take up [14 C]valine, and the total amino acid 'pools' in them, were measured. The results (Fig. 4) show that, whereas the addition of cycloheximide at the onset of the recovery period (90 min) prevented the cells from regaining the ability to take up [14 C]valine (which thus confirmed the data of Fig. 3), addition of cycloheximide at later times had a progressively diminished effect. Of particular significance to the postulates of Grenson *et al.* (1968) is the finding that the addition of cycloheximide at these later times caused neither a build-up of amino acid 'pools' nor a sudden decrease in the ability of the cells to take up [14 C]valine. Clearly, once the function of the general amino acid permease has been restored, its maintenance does not require any major net synthesis of protein.

Fig. 4. Effect of cycloheximide on amino acid transport when added at various times during the recovery from valine 'trans-inhibition'.

Cells were treated as described in Figs. 1 and 2. During recovery from inhibition by valine the culture was split into four subcultures, to which cycloheximide was added at zero time (□), 2 h later (△) and 4 h later (○), as indicated by the arrows. No cycloheximide was added to the fourth culture (▲). The total amino acid 'pools' of samples of the fourth cell suspension (10 ml), taken at various times, are indicated by ■---■; virtually identical results were obtained with suspensions to which cycloheximide had been added.

What, then, is the protein that is synthesized during the process of recovery from valine inhibition, and the synthesis of which appears to be necessary for recovery? Two main lines of evidence suggest that it is a membrane protein associated with the Gap system.

In one type of experiment, wild-type cells were grown, under conditions de-repressing the general amino acid permease, in a medium containing a mixture of ^3H -labelled amino acids. They were harvested, washed, and incubated in buffered glucose containing 2 mM-valine, to elicit the usual inhibition of valine uptake, for 90 min; thereafter, they were harvested and resuspended for 2–3 h in recovery medium that also contained a mixture of ^{14}C -labelled amino acids. Analysis of the plasma membranes of these cells, by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Woodward & Kornberg, 1980), revealed that a major peak of protein, of M_r approx. 31 000, had been formed during the recovery period; it was preferentially

labelled with ^{14}C and was not present among the ^3H -labelled membrane proteins of the valine-inhibited cells (Fig. 5, continuous trace). Repetition of this experiment but with the labels reversed (i.e. de-repression in the presence of ^{14}C -labelled amino acids and recovery from valine inhibition in the presence of ^3H -labelled amino acids) gave similar results and, particularly, also revealed a protein peak of M_r approx. 31 000. This protein peak was not seen when these experiments were repeated with the Gap⁻ mutant Y185-His3 (Woodward & Kornberg, 1980; Fig. 5, dotted trace), which supports the view that it is a component of the general amino acid permease system.

A second type of experiment provided further support for this interpretation. In this, cells were de-repressed for the general amino acid permease and were grown with either ^3H -labelled amino acids or ^{14}C -labelled amino acids; the tritiated cells were subsequently subjected to inhibition by valine for 90 min, whereas the ^{14}C -labelled cells were not.

Fig. 5. *Synthesis of a plasma-membrane protein during recovery of cells from inhibition by valine*

Cultures of the wild-type strain Y185 and the Gap⁻ mutant Y185-His3 were grown for 4 h on L-proline medium in the presence of an ^3H -labelled amino acid mixture (100 μCi ; The Radiochemical Centre, Amersham, Bucks., U.K.). The cells were harvested, washed and incubated for 1.5 h with valine to elicit 'trans-inhibition'; they were then resuspended in 50 mM-phthalate buffer, pH 5.5, containing 2% (w/v) glucose plus a [^{14}C]protein hydrolysate (50 μCi ; The Radiochemical Centre). After 2–3 h, by which time the ability of the wild-type cells to take up amino acids had fully recovered, plasma membranes were prepared from both cultures and the proteins in them were separated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Gels were sliced and analysed as described by Woodward & Kornberg (1980). The results obtained with membranes from wild-type cells (—) and from the Gap⁻ mutant (····) are plotted as the percentage differences per slice in the radioactivity present as ^3H and as ^{14}C . Values above the line represent an excess of ^{14}C over ^3H ; below the line, an excess of ^3H over ^{14}C .

Fig. 6. Disappearance of a ^3H -labelled protein from double-labelled plasma membranes during valine 'trans-inhibition'. Wild-type cells were grown on L-proline medium in the presence of either a $[^{14}\text{C}]$ protein hydrolysate ($50\ \mu\text{Ci}$) or a ^3H -labelled amino acid mixture ($100\ \mu\text{Ci}$). Only the cells labelled with ^3H were subsequently incubated for 90 min with valine; however, plasma membranes were prepared both from these cells (in which amino acid uptake had been inhibited) and from the ^{14}C -labelled cells (in which amino acid uptake had not been inhibited). The two membrane preparations were mixed, and the proteins in them were separated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and analysed as described by Woodward & Kornberg (1980).

Plasma membranes were prepared from both types of cells, were mixed, and the proteins in them were analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. It is apparent (Fig. 6) that, again, a major protein peak of M_r approx. 31 000 is present in the cells not subjected to valine inhibition but absent from the tritiated culture. Again, similar results were obtained when the experiment was repeated with the labels reversed. It is also noteworthy that a peak of material, of M_r approx. 80 000, is present in the membranes of the valine-inhibited cells but absent from the culture that had been de-repressed but not subjected to inhibition by valine. Since proteins of M_r 53 000 and 30 000 have been shown to be associated with the Gap system (Woodward & Kornberg, 1980), it is tempting to assume that the peak at M_r approx. 80 000 is a complex of these two materials that accumulated in the course of inhibition of the general amino acid permease by valine.

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