SHORT COMMUNICATIONS

Inactivation *In vivo* of Glutamine Synthetase and NAD-specific Glutamate Dehydrogenase: Its Role in the Regulation of Glutamine Synthesis in Yeasts

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The food yeast, *Candida utilis*, is a highly adaptable organism possessing efficient and flexible pathways for the assimilation and interconversion of nitrogenous compounds. Earlier studies (Sims, Folkes & Bussey, 1968) indicated some of the ways by which appropriate rates of glutamate and glutamine synthesis could be maintained under different nutritional conditions. We now consider some aspects of the regulation of the enzymes glutamine synthetase (GS) and the deaminating NAD-specific glutamate dehydrogenase (GDH_{NAD}).

Candida utilis is similar to *Saccharomyces cerevisiae* (Kohlhaw, Dräger & Holzer, 1965) in that when it is grown on glutamate or some other amino acids there is extensive derepression of GS. There is similar derepression of GDH_{NAD}, the enzyme that under such conditions probably supplies the ammonia required for amide synthesis (Hierholzer & Holzer, 1963). The cellular concentration of free ammonia is very low, and it is believed that the increased synthesis of the two enzymes occurs directly or indirectly in response to this reduced availability of ammonia. We have found that the addition of either ammonia or glutamine to yeast so adapted can result in the rapid and extensive inactivation of both GS and GDH_{NAD}. Our reasons for believing that inactivation of both enzymes is necessary for the readaptation of these yeasts to increased supplies of ammonia are discussed.

METHODS

Organisms. *Candida utilis* (originally designated *C. utilis* BF60) was obtained from Professor Lillian Hawker, Department of Botany, University of Bristol; *Saccharomyces cerevisiae* x2180 from Dr W. Duntze, Biochemisches Institut, Universität Freiburg im Breisgau; and *Torulopsis candida* NCYC576 from Dr J. A. Barnett, ARC Food Research Institute, Norwich.

Growth conditions. Batch cultures were grown at 27° in a Gallenkamp orbital shaker. The medium used was of the following composition: glucose, 55 mM; NaH₂PO₄, 51 mM; K₂SO₄, 9 mM; source of nitrogen, 6 mM; Na₂HPO₄, 5 mM; MgCl₂, 0.6 mM; CaCl₂, 0.3 mM; H₃BO₃, 1.6×10^{-5} M; MnCl₂, 5.1×10^{-6} M; FeCl₃, 3.7×10^{-6} M; ZnSO₄, 3.5×10^{-6} M; KI, 6.0×10^{-7} M; Na₂MoO₄, 4.1×10^{-7} M; CuSO₄, 4.0×10^{-7} M. Growth was routinely followed by measuring opacity changes at 640 nm., and also by measuring changes in the total protein content of the yeast per unit volume of culture.

Preparation of extracts. Cells were harvested by suction filtration on to Whatman GF/A paper. The filter pad was frozen in liquid nitrogen and normally stored overnight at -20°.

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Cells were broken in a stainless-steel hammer-press chilled in liquid nitrogen, and the debris was taken up into phosphate buffer, 5×10^{-3} M, pH 7.2, 5×10^{-4} M-EDTA, 5×10^{-2} M- K_2SO_4 . After filtration through Whatman GF/A paper, the filtrate, which contained between 2 and 3 mg. protein/ml., was assayed for the required enzymes. Enzyme concentrations were adjusted to ensure zero-order time courses.

Measurement of enzyme activities

Glutamine synthetase (L-glutamate: ammonia ligase (ADP) (EC 6.3.1.2). Enzyme activities were usually measured by transferase activity, although synthetase activities gave identical results. The assay solution consisted of adenosine diphosphate, $1.5 \mu\text{mole}$; glutamine, $70 \mu\text{moles}$; hydroxylamine, $17.5 \mu\text{moles}$; manganese chloride, $4.5 \mu\text{moles}$; sodium arsenate, $35 \mu\text{moles}$; all in tris-acetate buffer, 0.05 M, pH 6.4, 5×10^{-4} M-EDTA, in a final volume of 2.0 ml. The assays were carried out at 30° , and initiated by the addition of enzyme extract. The reaction was stopped by the addition of 1.0 ml. of a mixture of 10 g. trichloroacetic acid and 8 g. ferric chloride in 250 ml. 0.5 N-HCl. The optical density at 500 nm. was read after centrifugation.

NAD-specific glutamate dehydrogenase (L-glutamate: NAD oxidoreductase (deaminating) (EC 1.4.1.2). The enzyme was assayed at 30° by following $NADH_2$ oxidation at 340 nm. The assay solution consisted of ammonia, $25 \mu\text{moles}$; 2-oxoglutarate, $24 \mu\text{moles}$; NADH, $0.25 \mu\text{mole}$; all in phosphate buffer; 0.1 M, pH 7.9, 5×10^{-4} M-EDTA, in a total volume of 1.6 ml. The enzyme extract was incubated for 2 min in the presence of 2-oxoglutarate and ammonia before the assay which was initiated by the addition of NADH.

NADP-specific glutamate dehydrogenase (L-glutamate: NADP oxidoreductase (deaminating) (EC 1.4.1.4). Assays were carried out as above (NADPH, $0.25 \mu\text{mole}$), except that the pH of the assay solutions were adjusted to 7.2.

Protein (1 to 1.5 mg) was precipitated in ethanol and estimated by the method of Lowry, Rosebrough, Farr & Randall (1951). Crystalline bovine serum albumin was used as protein standard. For convenience of comparison, specific activities are given as percentages of those at the beginning of each experiment.

RESULTS AND DISCUSSION

When cultures of *Candida utilis*, growing with glutamate as nitrogen source, were transferred to medium containing glutamine, there was a fall in activity of [GS and GDH_{NAD} (Fig. 1a) at a rate faster than could be accounted for by the immediate cessation of enzyme synthesis followed by dilution of existing enzyme by the other cellular proteins produced during continued growth. On resuspension of the culture into glutamate, the levels of both enzymes rose very rapidly: GS to about 90% of the original steady-state level, and GDH_{NAD} to a level higher than that initially present. NADP-specific glutamate dehydrogenase (GDH_{NADP}) was not subject to rapid changes in activity, although a significant but delayed increase in its level did occur during the experiment.

Inactivation of GS also resulted from the addition of ammonia to yeast with derepressed levels of the enzyme, but the rapidity and extent of such inactivation depended on the level of glutamate in the medium. When glutamate-grown yeast was transferred to medium containing ammonia, either alone, or together with very low levels of glutamate, rapid inactivation of GS occurred (Fig. 1b). If, however, ammonia was added to cultures growing in the presence of high levels of glutamate (Fig. 1c) inactivation of GDH_{NAD} occurred, whereas GS was repressed but not immediately inactivated. Thus although inactivation of

GS and GDH_{NAD} was frequently co-ordinated, there must have been separate mechanisms controlling the inactivation of each enzyme.

This rapid and apparently reversible decrease in GS activity is very similar to that described in *Escherichia coli* (Mecke & Holzer, 1966). In that organism, inactivation is brought about by a specific enzyme which catalyses the adenylation of GS, thereby converting it to a form which is biosynthetically less active (see Holzer & Duntze, 1971). Inactivation of GS is also known to occur in *Pseudomonas putida* (A. Segal, E. R. Stadtman & S. B. Hennig, unpublished observations, cited in Shapiro & Stadtman, 1970) and a number

Fig. 1. Inactivation of glutamine synthetase and NAD-specific glutamate dehydrogenase in yeasts. (a) Exponentially dividing cells of *Candida utilis* growing in culture medium containing glutamate (6 mM) were filtered, washed, and transferred to medium containing glutamine (6 mM). After 2 h. the culture was resuspended in a medium containing glutamate (6 mM). At the times shown, enzyme specific activities were measured and are expressed as a percentage of those at the beginning of the experiment.

Ammonia (final concentration 6 mM) was added at the times shown to: (b) a culture of *C. utilis* previously growing in medium with only low levels of glutamate (0.2 mM at time of addition); (c) a culture of *C. utilis* previously growing with glutamate (1.5 mM at time of addition); (d) a culture of *Torulopsis candida* previously growing with glutamate. ●—●, Glutamine synthetase; ■—■, NAD-specific glutamate dehydrogenase; ○—○, NADP-specific glutamate dehydrogenase;, line of repression, as calculated from protein data, assuming an immediate cessation of enzyme synthesis.

of the Enterobacteriaceae, but was previously believed not to occur in yeasts (Gancedo & Holzer, 1968). We have also observed rapid inactivation of GS in two other yeasts, *Saccharomyces cerevisiae* (although here the response was delayed) and *Torulopsis candida* (Fig. 1*d*). Our results indicate that in yeasts the minimum time required for a 50% decrease in activity is about 20 min., whereas in bacteria, comparable inactivation can occur in less than $\frac{1}{2}$ min. after the addition of ammonia (Mecke & Holzer, 1966). It is this difference in the time required for inactivation that probably accounts for the earlier report that GS in yeast is not subject to enzyme inactivation. Inactivation of GDH_{NAD} does not appear to have been previously described.

We have found inactivation of GS only when changed growth conditions have resulted in marked and rapid changes in the relative concentrations of glutamate and glutamine within the cell. Resuspension of glutamate-grown yeasts in either glutamine or ammonia results in a rapid increase in the pool of glutamine and an associated fall in the pool of glutamate (A. P. Sims & A. R. Ferguson, unpublished). The addition of ammonia to glutamate-grown yeast can lead to a fourfold increase in glutamine, accompanied by a halving of the pool of glutamate; this indicates that on such changes in growth conditions the controls operating on GS and GDH_{NAD} are not able to maintain pool homeostasis.

A comparatively rapid and reversible inactivation of GS mediated by changes in the relative concentrations of glutamate and glutamine would provide a coarse but effective means of controlling glutamine synthesis when nutritional conditions are changed. On the one hand it would be sufficiently rapid to prevent excessive diversion of glutamate and ammonia to glutamine with its consequent effects on transamination reactions, yet on the other hand, it would be slow enough to enable extensive build up of a key intermediate, glutamine, under conditions of increased ammonia availability. Inactivation of GDH_{NAD} is important in safeguarding the glutamate pool against excessive depletion. In other experiments using (^{15}N) NH_3 (A. P. Sims & A. R. Ferguson, unpublished), we have shown that on transfer of glutamate-grown cells to ammonia there is a rapid formation of glutamine, an extensive depletion of the glutamate pool, and an appreciable delay before glutamate is synthesized from ammonia. Under such conditions, continued functioning of the GDH_{NAD} at the rate required when it provided all cellular ammonia could rapidly result in exhaustion of the glutamate pool. Inactivation of this enzyme, as well as of GS, would thus help to maintain, under changing growth conditions, appropriate levels of glutamate and glutamine within the cell.

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