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The range of amino acids whose limitation activates general amino-acid control in *Neurospora crassa*

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Summary

Several amino-acid synthetic enzymes, belonging to arginie, glutamine, leucine, lysine and phenylalanine biosynthesis, respectively, were investigated under conditions of reduced availability of any one of 16 out of the 20 amino acids represented in proteins. The enzymes showed simultaneous derepression under each condition, albeit to different degrees. Derepression was abolished and the remaining basal enzyme levels reduced by mutations at the *cpc-1* locus which governs general amino-acid control in *Neurospora*. Glutamine synthetase was shown to be under *cpc-1* and additional controls. The evidence emphasizes the global nature of general amino-acid control.

1. Introduction

In *Saccharomyces cerevisiae* (see review by Hinnebusch, 1988), *Neurospora crassa* (Carsiotis *et al.* 1970; Carsiotis & Jones, 1974) and other fungi the enzymes of many amino-acid biosyntheses are controlled by a complex regulatory system. This 'general amino-acid control' (formerly called 'cross-pathway control' in *Neurospora*) causes derepression of the enzymes when the supply of any one of several amino acids is limited. Molecular studies in *Saccharomyces cerevisiae* have made this system one of the best characterized examples in eukaryotic organisms of the coregulated expression of a large number of unlinked genes.

In *Neurospora* derepression of amino-acid synthetic enzymes is specifically abolished by mutations at a locus designated *cpc-1* (cross-pathway control, Barthelmess, 1982). Control is exerted at the level of transcription (Flint & Wilkening, 1986). Molecular studies by Paluh *et al.* (1988) indicate that *cpc-1* is the locus that specifies the major positive *trans*-acting transcription factor that regulates expression of amino-acid synthetic genes subject to general control in *Neurospora*.

The full range of amino acids whose limitation would lead to enzyme derepression is not known in *Neurospora* or in any other organism. Here we take advantage of the wealth of mutants affecting amino-acid biosynthesis available in *Neurospora crassa*, to investigate this problem in *cpc-1*$^+$ or *cpc-1* mutant strains. Individual limitation for 16 out of the 20 amino acids represented in proteins was achieved by either introducing auxotrophic mutations into these strains and growing them on a low level of the required amino acid or by growing wild-type or *cpc-1* mutant mycelia in the presence of an inhibitor that selectively reduces the endogenously synthesized level of one amino acid. Under such conditions the specific activities of five enzymes belonging to a variety of amino-acid pathways were studied.

2. Materials and methods

(i) *Strains*

Table 1 lists the mutant strains employed. The wild type used was St Lawrence 74A (D. Newmeyer). For construction of double-mutant strains the *cpc-1* alleles *j-5* (Barthelmess 1984) or *CD-86* were used. *cpc-1* (*CD-86*) and *arg-6* (*CD-25, R1*) strains were obtained from R. H. Davis (Davis 1979); other amino-acid auxotrophs came from the Fungal Genetics Stock Center, Kansas City. *cys-4, ilv-2, leu-1, leu-2, lys-5, met-5, thr-3* and *trp-2* were used as obtained, all other strains were backcrossed once into St Lawrence background.

Table 1 indicates whether the strains were found to be complete or leaky auxotrophs, behaving as non-growers or slow growers, respectively, on unsupplemented minimal medium. Isolation of certain double mutant strains carrying a lesion at the *cpc-1* locus together with an auxotrophic mutation succeeded only on very low supplement concentrations (Barthelmess, 1986). *gln-1* segregants and *gln-1, cpc-1* double mutants could only be isolated on media containing glutamine as the sole nitrogen source.

Table 1. *Description of mutant strains and growth conditions*

Locus	Allele	Phenotype: no (a) or slow growth (b) on minimal medium	L-amino acid supplement	Final concentration (%)	*cpc-1* allele[a]
arg-6	*CD-25,R1*	a	Arginine	0·002	*j-5*
arg-8	*44207*	b	Proline	0·001	—
asn	*S1007*	b	Asparagine	0·001	*CD-86*
cys-4	*K7*	a	Cysteine	0·001	*j-5*
gln-1	*R1015*	b	Glutamine	0·01	*CD-86*
his-2	*Y152M43A*	a	Histidine	0·0003	*j-5*
ile-1	*OY338*	a	Isoleucine	0·0005	—
ilv-2	*T319*	a	Isoleucine	0·002	*j-5*
			+ valine	0·002	
leu-1	*33757*	a	Leucine	0·002	*j-5*
leu-2	*37501*	a	Leucine	0·002	*j-5*
leu-3	*R156*	a	Leucine	0·003	*CD-86*
leu-4	*R108*	a	Leucine	0·003	*CD-86*
lys-4	*STL4*	a	Lysine	0·002	*CD-86*
lys-5	*STL7*	a	Lysine	0·001	—
met-5	*9666*	b	Methionine	0·002	*j-5*
phe-2	*Y16329* and *E5212*	b	Phenylalanine	0·001	*CD-86*
pro-1	*21863*	b	Proline	0·001	*CD-86*
ser-1	*C127*	b	Serine	0·0005	*CD-86*
ser-2	*65004*	a	Serine	0·0005	*CD-86*
thr-2	*35423*	b	Threonine	0·002	*CD-86*
thr-3	*44104(t)*	b	Threonine	0·0003	—
trp-2	*41*	a	Tryptophan	0·0005	*j-5*
tyr-1	*T145*	b	Tyrosine	0·001	*CD-86*

[a] *cpc-1* allele with which the respective auxotrophic mutation was combined in double-mutant strains.

(ii) *Media and limitation conditions*

Where not otherwise stated (Figs. 1, 2) Vogel's medium N (Vogel, 1964) was used. For each strain 5×10^6 conidia were directly inoculated into medium containing a limiting amount of the required L-amino acid (Table 1). The concentration of supplement was chosen such that auxotrophic strains could not achieve more than about 80 mg dry weight/100 ml medium at 29 °C in an overnight grown shaken culture (about 20 h). Starvation could be estimated from the increment in dry weight.

(iii) *Enzyme assays*

These were in general performed with crude extracts derived from freeze-dried mycelium. Specific enzyme activities were estimated as follows: OCT, ornithine carbamoyl-transferase (carbamoylphosphate:L-ornithine carbamoyl transferase, EC 2.1.3.3) according to Davis (1962). LAT, leucine amino transferase (branched-chain amino acid-amino transferase, EC 2.6.1.6), according to Jenkins & Taylor (1970) except that the mycelium was extracted with 0·067 M phosphate buffer at pH 7·4 and pyridoxal-5-phosphate added (Fincham & Boulter, 1956). SD, saccharopine dehydrogenase (N^6-(1,3-dicarboxypropyl)-L-lysine:NAD oxidoreductase (L-lysine forming), EC 1.5.1.7) according to Saunders & Broquist (1966). PAT, phenylalanine transaminase was measured by adapting an assay described by Bode & Birnbaum (1984). Mycelium was extracted in 0·1 M Tris buffer, pH 8·6. The reaction mixture contained 25 mM phenylalanine, 10 mM 2-oxoglutarate and 0·1 mM pyridoxal-5-phosphate (prepared fresh daily). The reaction proceeded for 15 min at 37 °C and was terminated by the addition of 1·5 ml 1·6 N-NaOH. Optical density was read at 320 nm. GS, glutamine synthetase (L-glutamate:ammonia ligase (ADP), EC 6.3.1.22) was studied by the transferase activity according to Ferguson & Sims (1971). Dialysis of selected samples ensured that the high activities found for this enzyme on glutamate as the nitrogen source were not due to enzyme activation.

3. Results

(i) *Derepression in regulation-competent strains (cpc-1$^+$)*

The specific activities of the enzymes ornithine carbamoyl-transferase (OCT, arginine biosynthesis), leucine amino transferase (LAT, leucine biosynthesis), saccharopine dehydrogenase (SD, lysine biosynthesis), phenylalanine amino transferase (PAT, phenylalanine biosynthesis), and glutamine synthetase (GS, glutamine biosynthesis) were investigated in a wide range of mutant strains altered in amino-acid biosynthesis.

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Table 2. *Relative*[a] *specific activities of ornithine carbamoyltransferase (OCT), leucine amino transferase (LAT), saccharopine dehydrogenase (SD), phenylalanine amino transferase (PAT) and glutamine synthetase (GS) in auxotrophic strains grown on limiting supplement concentrations (see Table 1) (average of two or more estimates)*

Mutant strain	*cpc-1*$^+$					*cpc-1*				
	OCT	LAT	SD	PAT	GS	OCT	LAT	SD	PAT	GS
arg-6	4·9	2·4	2·7	3·0	1·5	0·5	0·6	0·7	0·6	n.d.[b]
arg-8	2·5	1·4	1·2	n.d.	n.d.	—	—	—	—	—
asn	8·1	3·4	4·0	3·9	2·2	0·5	0·4	n.d	0·3	0·1
cys-4	8·8	2·3	1·6	3·8	n.d	0·5	0·8	0·9	n.d.	n.d.
gln-1	3·4	1·6	n.d.	n.d.	0	0·4	0·5	n.d.	n.d.	0
his-2	4·6	3·6	2·3	2·9	0·6	0·2	0·3	0·5	0·6	0·3
ile-1	10·0	n.d	n.d.	3·7	1·4	—	—	—	—	—
ilv-2	7·5	2·3	2·6	4·8	1·0	0·5	0·5	0·4	1·4	0·5
leu-1	6·7	6·6	n.d.	4·8	n.d.	0·5	0·8	0·4	n.d.	n.d.
leu-2	8·4	4·0	2·6	8·5	0·4	0·3	1·0	0·4	1·2	0·2
leu-3	10·3	1·3	2·3	1·7	1·3	0·3	0·7	0·2	1·7	n.d.
leu-4	4·0	1·8	1·9	2·9	n.d.	0·4	0·9	0·3	n.d.	n.d.
lys-4	3·1	1·5	0	1·0	n.d.	0·7	0·7	0	0·4	n.d.
lys-5	9·2	5·4	4·6	6·0	0·6	—	—	—	—	—
met-5	5·4	1·8	2·0	2·2	1·4	—	—	—	—	—
phe-2	5·9	1·9	2·0	1·6	0·7	0·4	0·7	0·4	1·9	n.d.
pro-1	2·6	0·7	1·1	0·9	n.d.	0·5	0·2	1·0	0·3	n.d.
ser-1	7·7	4·8	2·1	3·6	1·3	0·6	0·9	0·3	1·2	0·2
ser-2	7·9	1·6	3·1	5·0	2·0	0·9	0·9	0·6	0·6	0·6
thr-2	3·8	1·1	1·4	0·8	2·4	0·4	1·1	n.d.	0·8	0·4
thr-3	10·7	1·7	1·8	1·3	1·4	—	—	—	—	—
trp-2	10·5	2·8	2·0	9·0	0·9	0·3	0·2	0·4	0·5	0·2
tyr-1	13·5	3·4	2·6	6·4	2·1	0·4	1·1	0·7	0·9	0·4

[a] Related to the following activities found in wild type grown on minimal medium (means, based on 20 estimates, parentheses indicate relative standard error): OCT 0·166 μmol citrulline min^{-1} mg^{-1} protein ($\pm$0·04); LAT 0·145 ΔOD min^{-1} mg^{-1} protein ($\pm$0·10); S.D. 1·641 ΔOD min^{-1} mg^{-1} protein ($\pm$0·08); PAT 33·180 μmol phenylpyruvate min^{-1} mg^{-1} protein ($\pm$0·07); GS 0·178 μmol glutamyl hydroxamate min^{-1} mg^{-1} protein ($\pm$0·02).
[b] n.d., not determined.

Most of the mutations cause lesions in the structural genes for amino-acid synthetic enzymes, only *leu-3* affects a regulatory element of leucine, isoleucine and valine biosynthesis (Polacco & Gross, 1973; Olshan & Gross 1974). Conidia of the mutant strains were inoculated and grown overnight on limiting concentrations of the appropriate amino-acid supplements (see Table 1). Since, under these conditions, growth rates were substantially lower than the 2 h doubling time of the wild type (*arg-8* and *pro-1*, both affecting proline biosynthesis, were least affected), it was assumed that this procedure resulted in reduced endogenous concentrations for any one of the 16 amino acids concerned, i.e. arginine, asparagine, cysteine, glutamine, histidine, isoleucine, valine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, and tyrosine, respectively.

The left panel of Table 2 displays the relative specific enzyme activities found under these conditions, i.e. the derepression ratio with reference to the wild-type strain grown on unsupplemented Vogel's minimal medium. Highly derepressed activities were found for the enzyme OCT, on average 3- to 10-fold over all mutant strains. Derepression was less, on average 2- to 4-fold over all strains, for the enzymes LAT, SD and PAT, occasionally not differing from the level found in the wild-type strain on minimal medium. Where no increase in specific activity for an enzyme was found, several fold derepression of the OCT in the particular batch of mycelium in all cases confirmed that, by this criterion, limitation conditions were met. With the exception of the low derepression ratio in the leaky *arg-8* and *pro-1* strains no obvious difference was found between complete and leaky auxotrophs. The behavior of GS will be discussed below.

(ii) *Enzyme activities in cpc-1 genetic background*

The right panel of Table 2 shows enzyme activities in double mutants containing a *cpc-1* mutation in addition to the allele confering an amino-acid re-

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quirement under amino-acid limitation. A comparison with the enzyme activities found in *cpc-1*$^+$ background (left panel of Table 2) demonstrates that in the presence of a *cpc-1* mutation no derepression occurred, regardless of the enzyme tested or the amino acid limitation. The repeatedly observed about 2-fold increase in PAT activity in the *phe-2, cpc-1* and *leu-3, cpc-1* strains might be explained by induction of a catabolic phenylalanine transaminase activity on phenylalanine supplement (Bode & Birnbaum, 1984) in *phe-2, cpc-1*. We have no explanation in the other case. *cpc-1* mutations had the general effect (already previously reported; Barthelmess, 1982) of actually lowering the remaining basal enzyme activities. Consistent with earlier findings (Davis, 1979; Barthelmess, 1982), the loss of derepression and lowered basal activities turned double mutants carrying leaky auxotrophic and *cpc-1* mutations into complete auxotrophs (exception *arg-8* and *pro-1*).

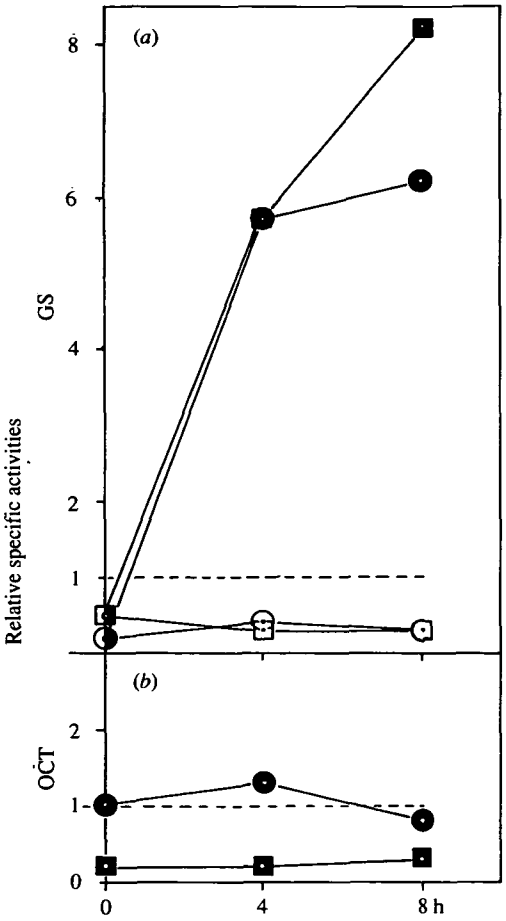

Fig. 1. Relative specific activities of (*a*) glutamine synthetase, GS, and (*b*) ornithine carbamoyltransferase, OCT, under glutamine limitation. Wild type (circles) and *cpc-1(CD86)* (squares) strains were grown on 10 mM glutamine and shifted at 0 h to medium containing a final concentration of either 10 mM glutamate + 10 mM glutamine (open symbols) or 10 mM glutamate (filled symbols) as the only nitrogen sources. In both strains OTC activities on glutamate + glutamine were almost identical with those on glutamate, and are therefore not shown. (Activity of the wild type on minimal medium set equal to 1---.)

(iii) *Regulation of glutamine synthetase (GS)*

GS activities are found to be about 5 times higher on minimal medium than on glutamine as the only nitrogen source (Figs. 1*a*, 2*a*). Further studies of GS showed that conditions exist under which this enzyme is able to derepress even in *cpc-1* mutant background (Fig. 1*a*). In this case derepression was achieved by shifting mycelia after growth on glutamine-supplemented medium to medium containing glutamate as the only nitrogen sources (Mitchell & Magasanik, 1984). Following the shift, wildtype and *cpc-1* strains reached an 8- to 10-fold elevated GS level (compared with that of the wild type grown on minimal medium) over an 8 h time course. The OCT level remained unchanged under these conditions (Fig. 1*b*).

The finding raised the question whether derepression of GS, as reported in Table 2, was signaled by glutamine rather than other specific amino acid limitations, since the activation of general amino-acid control and the consequently increased biosynthetic capacity was likely to cause a drain on the glutamine pool as amino nitrogen donor for the biosynthesis of several other amino acids. Therefore GS was measured in wild-type and *cpc-1* strains under histidine limitation in the presence of non-limiting concentrations of glutamine as the only nitrogen source. Histidine

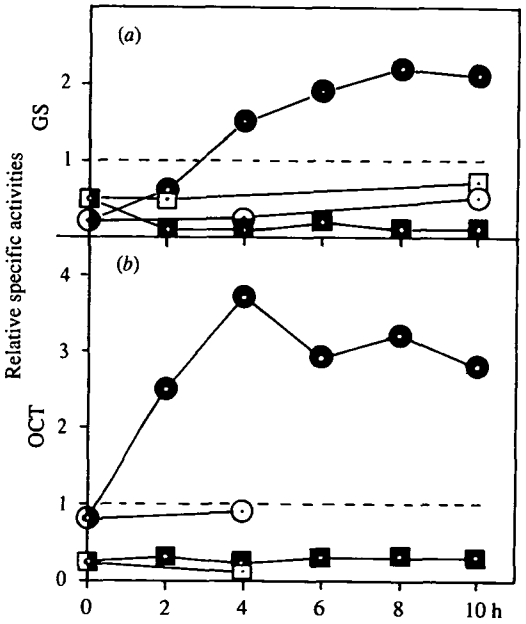

Fig. 2. Relative specific activities of (*a*) glutamine synthetase, GS, and (*b*) ornithine carbamoyltransferase, OCT, under histidine limitation. Wild type (circles) and *cpc-1(CD86)* (squares) strains were grown on 10 mM glutamine as the only nitrogen source to which at 0 h either 10 mM glutamine (open symbols) or 6 mM 3AT and 10 mM glutamine (filled symbols) were added (final concentrations). (Activity of the wild type on minimal medium set equal to 1---.)

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limitation was mediated via the addition of 3-amino-1,2,4-triazole (3AT), an inhibitor of the enzyme imidazol glycerol-phosphate dehydratase in histidine biosynthesis (Hilton *et al.* 1965). The result indicated that GS is under general amino acid control (Fig. 2*a*), since in the wild type grown on 3AT and glutamine a 10-fold rise of activity over that found after growth on glutamine alone was observed, whereas in the *cpc-1* strain no increase in enzyme activity occurred under the same conditions. Simultaneously the OCT activity increased about 4-fold in the wild-type mycelium grown on 3AT and glutamine and displayed a constant low level in the *cpc-1* strain (Fig. 2*b*).

4. Discussion

The data enlarge the range of known amino acids for which limitation elicits enzyme derepression. Tests could not be made on the role of alanine, aspartate, glutamate or glycine because of lack of appropriate mutant strains; but it seems likely that general amino-acid control is activated by all 20 amino acids represented in proteins.

Interpretation of the results is based on the assumption that deprivation of an auxotroph for an amino acid leads to a reduced endogenous amino-acid pool, i.e. amino-acid limitation. This has been shown to apply (e.g. Flint & Kemp, 1981; Flint *et al.* 1985; Barthelmess *et al.* 1974; Carsiotis, pers. comm., Barthelmess, unpubl.), but has not been verified in every case. Such reduction could affect the reaction with tRNAs and thereby raise the level of uncharged tRNA which is considered to be the relevant signal to elicit derepression (Wek *et al.* 1989).

It is evident from Table 2 that the degree of derepression of different enzymes as measured *within* a particular auxotroph is different; OCT showed always the highest amplitude. Other workers (Carsiotis *et al.* 1970; Flint & Kemp, 1981) have observed correlations between the derepression levels of different enzymes within the same pathway which this study did not address. In yeast it is a common phenomenon that derepression ratios of enzymes even within pathways differ (see review by Hinnesbusch, 1988).

The investigation confirms the central role of the *cpc-1* locus for general amino-acid control in *Neurospora crassa*. Glutamine synthetase, which is subject to repression by glutamine, is the only enzyme involved in amino-acid biosynthesis so far encountered able to exhibit a high degree of derepression in a *cpc-1* mutant strain. The signal for derepression was in this case given by a shift from glutamine to glutamate as the only nitrogen source in the medium. This suggests that GS is in *Neurospora*, as in yeast (Mitchell & Magasanik, 1984), under general amino-acid as well as being affected by other independent controls.

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