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Mutants affecting amino acid cross-pathway control in *Neurospora crassa*

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SUMMARY

Arginine-requiring mutants of *Neurospora crassa* were isolated using a strain partially impaired in an enzyme of the arginine pathway (bradytroph). Among these, five strains were found which carry mutations at a new locus, *cpc-1*$^+$. The recessive *cpc-1* alleles interfere with the cross-pathway control of amino acid biosynthetic enzymes. The enzymes studied, three of arginine and one each of histidine and lysine biosynthesis, fail to derepress under conditions which normally result in elevation of enzyme concentration, namely arginine, histidine or tryptophan limitation. Enzymes not involved in amino acid biosynthesis are still able to derepress in the presence of *cpc-1*. In wild-type backgound, i.e. with the bradytroph replaced, *cpc-1* strains lose the original arginine-requirement. *cpc-1* mutations confer sensitivity of growth to 3-amino-1,2,4-triazole.

1. INTRODUCTION

Physiological studies by Carsiotis *et al.* (Carsiotis & Lacy, 1965; Carsiotis *et al.* 1970; Wesseling & Carsiotis, 1973; Carsiotis & Jones, 1974; Carsiotis, Jones & Wesseling, 1974; Wesseling & Carsiotis, 1974) have demonstrated a common regulation of tryptophan, histidine, arginine and lysine biosynthesis in *Neurospora crassa*. The phenomenon is called 'cross-pathway' or 'general' amino acid control. Starvation for any one of these amino acids leads to simultaneous derepression of the biosynthetic enzymes of all four pathways. The derepression can be reversed only by replacing the particular amino acid for which the culture was starved; increase in any of the other three is ineffective. In the presence of cycloheximide (Carsiotis, Jones & Wesseling, 1974; Flint & Kemp, 1981) derepression is prevented. This indicates that *de novo* protein synthesis is required and suggests that control may be exerted at a stage prior to translation. General control over amino acid biosynthesis has also been observed in *Saccharomyces cerevisiae* (Schürch, 1972; Schürch, Miozzari & Hütter, 1974; Wolfner *et al.* 1975) and *Aspergillus nidulans* (Piotrowska, 1980).

In *Saccharomyces* two classes of regulatory mutants that interfere with the

general control have been isolated: *tra* mutants, selected for resistance of growth to the histidine analogue triazole-alanine, lead to high constitutive levels of the enzymes of arginine, histidine, tryptophan and lysine biosynthesis (Wolfner *et al.* 1975). *aas* mutants, selected for enhanced sensitivity of growth to the tryptophan analogue 5-methyl-tryptophan, fail to derepress enzymes of the amino acid pathways mentioned under conditions of endproduct starvation (Schürch, 1972; Schürch, Miozzari & Hütter, 1974; Wolfner *et al.* 1975).

In principle, appropriate selection procedures should yield mutants involved in cross-pathway control using any of the pathways concerned. However, extensive attempts in several laboratories to find constitutively derepressed mutants of arginine biosynthetic enzymes in *Neurospora* were unsuccessful (R. Davis, personal communication, R. Weiss, personal communication, Barthelmess, unpublished). Therefore a different experimental approach was devised to detect mutants of the opposite phenotype, namely those which would fail to derepress under arginine limitation. Potential mutations could interfere, e.g. with an arginine pathway-specific control or the cross-pathway control of amino acid biosynthesis.

This report describes the discovery and physiological properties of cross-pathway control mutants at a new locus, *cpc-1*$^+$, in *Neurospora crassa*. They were shown to be allelic with mutants found in *Neurospora* by Davis (1979) just prior to this investigation. Davis mapped the new locus on the left arm of linkage group VI between the centromere and *ylo-1*.

2. MATERIALS AND METHODS

(i) *Strains*

The wild-type background of the strains used was *St. Lawrence 74 A* from D. Newmeyer. This wild type was obtained from H. Kacser. *arg-12*^{s} (5-24)A and *arg-12*^{s} (5-28)a had been backcrossed into this background. *his-2* (Y152M43A and Y175M611a) and *trp-2* (41) were obtained from the Fungal Genetics Stock Center, Humboldt State University, Arcata, California. *pyr-2* (DFC-9, RD-1)A as well as arginine biosynthetic structural gene mutants for complementation tests, among them *arg-6* (CD-25, R1), were obtained from R. Davis.

(ii) *Media and general growth conditions*

Vogel's medium N (Vogel, 1964) was used throughout. Supplements are given in the Results section. Ascospore isolation was done on 4% agar plates under a binocular microscope. For complementation tests, drops of conidial suspensions were added to 1 ml medium. Tests for growth requirements were done in 1 ml liquid cultures. Similarly amitrole (3-amino-1,2,4-triazole) sensitivity was tested in 1 ml liquid medium supplemented with 4 mM amitrole. For exponential growth, conidial suspensions were inoculated into 100 ml liquid medium and shaken overnight in 250 ml dimpled Erlenmeyer flasks on a rotary shaker at 170 rev/min and 29 °C. Cultures were harvested in general at a dry weight of 100 mg/100 ml

medium or less. For enzyme assays mycelium was collected on two layers of filter paper in a Buchner funnel, placed in liquid nitrogen, freeze dried and stored at −15 °C.

(iii) *Strains and growth condition for derepression studies*

Amounts of amino acid biosynthetic enzymes in wild type *Neurospora* grown on minimal medium represent the fully repressed level. Control properties can be studied only if starvation for endproduct with consequent enzyme derepression can be arranged. This is easily achieved by introducing an auxotrophic mutation and growing the resulting auxotrophs on limiting supplement. The disadvantage of this method is that the system is not at steady state once depletion of endproduct becomes limiting to growth. Supplement concentrations (see Figs 1 and 2) were chosen such that growth ceased when the culture reached a dry weight of 40–50 mg/100 ml medium. At 4–6 h after the cessation of growth the mycelium was harvested and enzyme activities measured. It was shown that under those conditions derepression occurred with about a 2- to 5-fold increase in enzyme concentrations. In parallel, each strain was grown on 'full supplement', a condition which allowed unrestricted growth. The supplement concentration for each amino acid was chosen such that further increases in concentration did not result in further lowering of enzyme activities.

Arginine auxotrophy was introduced by combining *cpc-1* mutations with *arg-6* by an appropriate cross. *arg-6* mutants lack acetylglutamate kinase activity (Wolf & Weiss, 1980); they represent the earliest available metabolic block in arginine biosynthesis. (j-5, *arg-6* was difficult to obtain; therefore j-5, *arg-12*^{s} was used in some investigations.) Histidine and tryptophan auxotrophies were introduced into *cpc-1* mutant strains by matings with either *his-2* (phosphoribosyl-ATP-pyrophosphorylase, Ames, 1957; Fink, 1964) or *trp-2* (anthranilic acid synthetase, Lester, 1971; Hütter & DeMoss, 1967) mutants, both affecting early steps in the respective pathways. The double mutant j-2, *trp-2* was not recovered.

(iv) *Mutant isolation and identification*

Using strain *arg-12*^{s} (5-28)a mutant isolation by filtration enrichment was carried out by Mrs G. Kruppa. Twenty ml of a conidial suspension (3×10^{7} conidia/ml) were irradiated with UV light at a survival level of 30–35 % and 10 ml inoculated into each of two 1 l flasks containing 200 ml medium supplemented with all protein amino acids (1 mM final concentrations) except arginine and histidine. At intervals over 6 days germinated conidia were removed from the shaken cultures by filtration through cheese cloth. Finally, ungerminated conidia were concentrated by centrifugation and spread on plates containing 1 % sorbose, 0·05 % glucose and 1 mM final concentrations of all 20 amino acids. Colonies were isolated for 2–6 days after plating.

The filtration and selection media were supplemented with amino acids as described, in order to recover mutants which might have lost not only the ability

to derepress but also basal enzyme levels in all or some of the pathways controlled. However, Davis (1979) isolated similar regulatory mutants using minimal medium during filtration and arginine as the only supplement on the selection plates. Therefore the additional supplements used in this investigation were unnecessary. Neither histidine nor multiple auxotrophic strains were found.

(v) *Enzyme assays*

All enzyme assays were performed with crude extract derived from freeze dried mycelium, only HPPase assays were done after passage through a short Sephadex G25 column equilibrated with 0·05 M Tris-HCl, pH 7·5. Specific enzyme activities were estimated for the following enzymes according to published methods:

AOTase. Acetylornithine aminotransferase (N^{α}-acetyl-L-ornithine:2-oxoglutarate aminotransferase EC 2.6.1.11) (Albrecht & Vogel, 1964).

AGTase. Acetylornithine-glutamate acetyltransferase (N^{α}-acetyl-L-ornithine:L-glutamate *N*-acetyltransferase, EC 2.3.1.35) (Staub & Dénes, 1966).

OTCase. Ornithine transcarbamylase (carbamoyl phosphate:L-ornithine carbamoyltransferase, EC 2.1.3.3) (Davis, 1962*b*).

HPPase. L-histidinol phosphate phosphatase (L-histidinol phosphate phosphohydrolase, EC 3.1.3.15) (Martin *et al.* 1971).

SDase. Saccharopine dehydrogenase (N^{6}-(1,3-Dicarboxypropyl)-L-lysine:NAD oxidoreductase (L-lysine forming), EC 1.5.1.7) (Saunders & Broquist, 1966).

CSase. Citrate synthase (citrate oxaloacetate-lyase (CoA-acetylating), EC 4.1.3.7) (Flavell & Fincham, 1968*b*).

IDase. NADP-linked isocitrate dehydrogenase (threo-Ds-isocitrate:NADP oxidoreductase (decarboxylating), EC 1.1.1.42) (Flavell & Fincham, 1968*b*).

ATCase. Aspartate transcarbamylase (carbamoylphosphate:L-aspartate carbamoyltransferase, EC 2.1.3.2). ATCase specific activity was estimated according to Davis (1960) with several modifications. The reaction volume was scaled down to 0·5 ml and the reaction (at 30 °C) was stopped with the 3:1:1 mixture of H_2SO_4, diacetylmonoxime and diphenylamine-*p*-sulphonate as used in the OTCase assay. All further steps were as described for the OTCase. To correct for a background reaction, the assay was performed with and without (blank) aspartate. The blank activity was subtracted from the activity found with aspartate.

Protein concentrations were measured by the Folin method (Lowry *et al.* 1951). For AOTase, AGTase, OTCase, HPPase and ATCase a mutant strain unable to carry out the reaction being analysed was included in the experiments as a control.

(vi) *Chemicals*

Enzyme reagents, amino acid supplements and 3-amino-1,2,4-triazole were obtained from the Sigma Chemical Co.

3. RESULTS

(i) *Isolation and identification of regulation deficient mutants*

Mutations were induced in strain *arg-12*^{s}(5-28)a. *arg-12*^{s} is a structural gene mutation found to reduce specific ornithine transcarbamylase (OTCase) activity to about 2–3% and the arginine pool to about 10% without imposing arginine auxotrophy (Davis, 1962*a*, *b*). All arginine biosynthetic enzymes are 2- to 3-fold derepressed in this strain including, presumably the OTCase enzyme itself (Barthelmess, Curtis & Kacser, 1974; Cybis & Davis, 1975). This allows the necessary metabolite flux through the pathway and about normal growth rate in spite of the severe restriction at the OTCase step.

Selection in this strain was based on the hypothesis that mutations causing a failure to derepress the arginine biosynthetic enzymes would reduce the OTCase activity still further than the 2–3% in the derepressed state, thus reducing the flux to arginine so drastically that an arginine requirement would result for growth. The method would also detect arginine structural gene mutations resulting in blocked arginine biosynthesis. These were likely to be present in the majority of detectable arginine-requirers.

Auxotrophs were selected by filtration enrichment (see Materials and Methods). From 368 colonies originally isolated, after retesting on minimal and arginine supplemented media, 106 tight arginine-requirers were finally obtained. Complementation of the original isolates in heterokaryon tests indicated that they fell into 12 complementation groups. Seven of these could be identified as allelic with structural loci of the arginine pathway by further complementation tests against mutants representing known loci. These were not investigated further.

Measurement of the specific activity of the arginine biosynthetic enzyme acetylornithine aminotransferase (AOTase) under arginine limitation (0·002% arginine) showed that only one out of the five unidentified complementation groups – group 'j', consisting of five independent mutants – failed to derepress this enzyme. The object of this project was to discover mutations that interfere either with arginine-specific or with cross-pathway control. To distinguish between the two possibilities, mutant strains were subjected to a preliminary, indirect test, namely inhibitor sensitivity of growth. The investigations of Schürch, Miozzari & Hütter (1974) and Wolfner *et al.* (1975) had shown that a pleiotropic effect of the *aas* cross-pathway control mutants in yeast is their sensitivity of growth to 3-amino-1,2,4-triazole (amitrole). Amitrole is a competitive inhibitor of the histidine biosynthetic enzyme imidazole glycerol-phosphate dehydratase (Carsiotis, Jones & Wesseling, 1974) and can cause a restriction of histidine biosynthesis comparable with that of the arginine biosynthesis by the mutationally altered OTCase of *arg-12*^{s}. A suitable concentration of inhibitor reduces the internal histidine concentration to such levels that it causes derepression of the biosynthetic enzymes but not sufficient to affect growth. Abolition of the ability to derepress by mutation at the control locus reduces the histidine flux below that necessary to sustain growth. Therefore, mutants defective in cross-pathway control are

expected to be unable to grow under such conditions of histidine restriction just as under arginine restriction.

It was found that a concentration of 4 mM amitrole in liquid medium distinguished unambiguously between resistant (wild type) and sensitive (mutant) strains of *Neurospora*. Sensitivity was found for all five strains belonging to complementation group j. This was taken as a first indication that j-mutants are involved in cross-pathway control. Sensitivity to amitrole became a very useful screen in genetic experiments (see below). All further investigations concentrated on three out of those five mutants, designated j-2, j-5 and j-9. The alleles carried by these three isolates differed in various respects from each other, indicating that they represented independent mutational events.

(ii) *Genetic properties of the mutants*

From the evidence given in this section it was assumed that the mutants defined by the j complementation group follow monogenic inheritance. Davis (1979) described a complementation group with phenotypically similar mutants. Using his allele CD-15, it could be shown by complementation and crosses (data not given) that the three j-mutants of this investigation were allelic with CD-15. Davis (1979) mapped the locus on the left arm of linkage group VI between the centromere and *ylo-1*. The new locus was called *cpc-1*$^{+}$ (for *c*ross-*p*athway *c*ontrol) by mutual agreement. From the complementation results it was evident, that the alleles described here were recessive and were hence designated *cpc-1*.

The original isolates j-2, j-5 and j-9 were twice backcrossed to the original *arg-12*^{s} strain. Random spore analysis of each cross resulted in two classes of segregants. As seen in Table 1 they are taken to represent prototrophic *cpc-1*$^{+}$, *arg-12*^{s} and arginine-requiring *cpc-1*, *arg-12*^{s} genotypes. Strain j-9 behaved exceptionally. Although it was originally isolated as an arginine-requirer in *arg-12*^{s} background, j-9, *arg-12*^{s} derivatives from the backcross were able to grow, albeit with a delay, on minimal medium. (In shaken liquid culture conidia of j-9, *arg-12*^{s} genotype failed to germinate.)

The second backcross of j-9, *arg-12*^{s} to *arg-12*^{s} yielded the two classes of segregants in equal frequencies as expected under the hypothesis of monogenic inheritance (Table 1). However, the second backcrosses of j-2, *arg-12*^{s} and j-5, *arg-12*^{s} deviated significantly from the expected 1 : 1 ratio (Table 1). It was assumed that the bias in favour of the *cpc-1*$^{+}$, *arg-12*^{s} genotype resulted from the low viability of mutant *cpc-1* ascospores. Crosses involving alleles j-2 or j-5 were highly infertile, yielding very few normally shaped perithecia. When these perithecia were opened, they contained mostly transparent-looking, pale ascospores; the rare dark ascospores were distributed in no regular fashion within individual asci. This prevented ascus (tetrad) analysis. A particularly high percentage of pale non-germinating ascospores were observed in all crosses involving j-5.

Apart from their arginine-requirement, segregating *cpc-1* genotypes were identified by their amitrole sensitivity (Table 1). Co-segregation of amitrole

sensitivity and inability to derepress AOTase was confirmed for all three j-alleles by investigating second backcross progenies for both characters. Furthermore, segregants carrying *cpc-1* alleles were easily recognized after ascospore isolation by their initial slow growth on slants supplemented with arginine, except in the case of j-9 which grew normally.

Table 1. *Data from second backcrosses to* cpc-1$^+$, arg-12^s *of three* cpc-1 *mutant strains in* arg-12^s *background: j*-2, arg-12^s, *j*-5, arg-12^s *and j*-9, arg-12^s

Inferred genotypes	*cpc-1*$^+$, *arg-12*^{s}	*cpc-1*, *arg-12*^{s}*		
Expected segregation frequency	0·5	0·5		
	Observed number of progeny			
cpc-1 allele			X^2(1:1)	*P*
j-2	37	21	4·41	0·04
j-5	38	16	8·96	0·003
j-9	15	15	0	1·00

* Unable to grow on minimal medium or medium containing 4 mM amitrole and 1 mM arginine. Derivatives of j-9 show delayed growth on minimal medium, no growth on amitrole + arginine.

Table 2. *Data from outcrosses to wildtype* (cpc-1$^+$, arg-12$^+$) *of three* cpc-1 *mutant strains in* arg-12^s *background: j*-2, arg-12^s, *j*-5, arg-12^s *and j*-9, arg-12^s

Class	1	2	3
Inferred genotype	*cpc-1*$^+$, *arg-12*$^+$ or *cpc-1*$^+$, *arg-12*^{s}	*cpc-1*, *arg-12*^{s}*	*cpc-1*, *arg-12*$^+$†
Expected segregation frequency	0·5	0·25	0·25
	Observed number of progeny		
cpc-1 allele			
j-2	10	8	3
j-5	17	3	2
j-9	6	0‡	(7)‡

* Arginine-requiring, amitrole-sensitive.

† Prototrophic, amitrole-sensitive.

‡ The outcross of j-9, *arg-12*^{s} to wild type yielded no arginine-requiring segregants (class 2), the amitrole-sensitive segregants of class 3 include j-9, *arg-12*^{s} segregants.

Random spore analysis from outcrosses of the arginine-requiring, amitrole sensitive strains j-2, *arg-12*^{s} and j-5, *arg-12*^{s} to wild type (*arg-12*$^+$) yielded a new class of prototrophic, amitrole sensitive segregants (Table 2, Class 3) in contrast to the amitrole resistant wild type and *arg-12*^{s} segregants (Table 2, Class 1). Representatives of the two amitrole sensitive segregant classes were tested for OTCase activity. All segregants in the arginine-requiring class (Table 2, Class 2)

had low activities (in the range of *arg-12^s*) and all prototrophic segregants had high activities (30–90 %, depending on the *cpc-1* allele, see Table 4) indicating that the two classes were *arg-12^s* and *arg-12$^+$* respectively. As observed for the second backcrosses, the outcross of j-5 deviated strongly from the expected segregation ratio. Consistent with the findings already described for the backcross, the cross of j-9, *arg-12^s* with wild type did not yield any arginine-requirers. OTCase tests on some of the amitrole sensitive segregants able to grow on minimal medium showed either low (around 2 %) or high enzyme levels, indicating the segregation of *arg-12^s* and *arg-12$^+$* genotypes in this class (Table 2, Class 3).

The finding that the arginine-auxotrophy of *cpc-1* strains was correlated with low OTCase levels typically found in *arg-12^s* genotypes was taken as a confirmation for the assumptions made at the outset of the selection procedure. The independent segregation of the new mutant class with *arg-12^s* was expected on the basis of map location and indicated that there was no genetic relationship between *cpc-1$^+$* and *arg-12*.

From the outcrosses, prototrophic single mutant strains were derived that carried the *cpc-1* alleles in wild type (*arg-12$^+$*) background. Exponentially growing cultures of these had normal growth rates but showed either a germination lag (j-2) or reduced viability of conidia (j-5). However, the single mutant strain carrying allele j-9 was in all these respects indistinguishable from wild type.

(iii) cpc-1 *mutations and cross-pathway control*

To investigate the regulation of amino acid biosynthetic enzymes, amino acid limitation had to be imposed on mutant and wild type (reference) strains. By crossing the protrotrophic *cpc-1* strains j-2, j-5 and j-9 with known arginine, histidine or tryptophan structural gene mutants, auxotrophic double mutant strains were obtained (see Materials and Methods). (Most of these double mutant strains displayed severely reduced growth rates. No explanation is offered for this observation.) Since each double mutant strain required the respective supplement for growth it was thereby possible to impose starvation for arginine, histidine or tryptophan by reducing supplement concentrations. Derepressibility of a number of arbitrarily chosen enzymes either in the pathway subjected to endproduct limitation or in any other pathway could thus be investigated. For all enzymes derepressed levels (limited supplement) and fully repressed levels (full supplement) were estimated for the *cpc-1* double mutant strains and for the appropriate auxotrophic reference strains, *arg-6*, *his-2* or *trp-2*, in *cpc-1$^+$* background. In the following sections the results of enzyme studies will be presented as 'activity ratios', i.e. specific activities relative to the relevant reference strains on full supplement. Specific activities for all reference strains on full supplements as well as for the wild type grown on minimal medium are given in Table 3 and will be discussed below.

Fig. 1 shows the activity ratios of three enzymes belonging to the arginine pathway, i.e. the aminotransferase (AOTase), acetyltransferase (AGTase) and

transcarbamylase (OTCase). From a comparison of the *cpc-1*$^+$ reference strains, *arg-6*, *his-2* and *trp-2* (first two columns in each section of Fig. 1) under limited and full supplementation (stippled versus white columns), it can be seen that under each amino acid limitation all three enzymes derepressed between 1·5 and 5 times. The activity ratio on full supplement of the respective amino acid for these reference strains equals 1 by definition. In contrast hardly any derepression was observed in the presence of the *cpc-1* alleles over all eight *cpc-1* double mutant strains and three supplement limitations (Fig. 1). In these strains the ratios of the supplement limited (stippled) to the fully supplemented (white) enzyme activities varied around a value of one. There were however a few significant exceptions from this overall lack of derepression. They occurred under arginine starvation: Strain j-9, *arg-6* showed a slightly derepressed AGTase (Fig. 1*b*) and OTCase (Fig. 1*c*) and strain j-2, *arg-6* showed a slightly derepressed AGTase (Fig. 1*b*). The small

Table 3. *Specific enzyme activities* of the reference strains*

Genotype	Supplement	AOTase	AGTase	OTCase	HPPase	SDase
Wild type	—	8·48 (5)	24·3 (10)	259 (6)	23·1 (1)	965 (2)
cpc-1$^+$, *arg-6*	Arginine 0·06 %	7·61 (3)	23·1 (4)	298 (6)	18·2 (2)	888 (4)
cpc-1$^+$, *his-2*	Histidine 0·02 %	9·66 (4)	29·2 (4)	336 (6)	26·3 (2)	808 (3)
cpc-1$^+$, *trp-2*	Tryptophan 0·01 %	11·63 (5)	39·8 (6)	603 (5)	29·7 (1)	2008 (4)
Pooled standard deviation		1·42	4·9	67	3·66	299

Abbreviations: AOTase, acetylornithineaminotransferase; AGTase, acetylornithine-glutamate acetyltransferase; OTCase, ornithine transcarbamylase; HPPase, L-histidinol phosphate phosphatase; SDase, saccharopine dehydrogenase.

* Expressed as nanomoles per minute per mg of protein with the exception of AOTase and SDase which are given as Δ O.D. per minute per mg of protein × 10^3. Brackets indicate the number of repeats.

degree of derepression of the OTCase by j-9, *arg-6* and of the AGTase by j-2, *arg-6* was reproducible if samples from starvation and full supplementation conditions were included in the same enzyme assay. It did not lead to enzyme activities above the level of the fully supplemented reference strain. However, j-9, *arg-6* was derepressed for AGTase to about half the maximum level. The AOTase was not derepressed at all in any of the *cpc-1* mutant strains investigated. j-5 was the *cpc-1* allele which most clearly showed that all three enzymes were potentially under *cpc-1*$^+$ control since none of them derepressed in the presence of this allele.

To ensure that the observed failure to derepress did not depend upon the degree of starvation at which cultures were harvested, strains j-9, *arg-6* and j-5, *arg-12*^{s} were investigated over a time course, i.e. several harvests were taken at different times under arginine limitation (0·002 % arginine). When growth had stopped for several hours and the endogenous arginine concentration had fallen below the level of detection, j-9, *arg-6* still showed only partially derepressed AGTase and fully

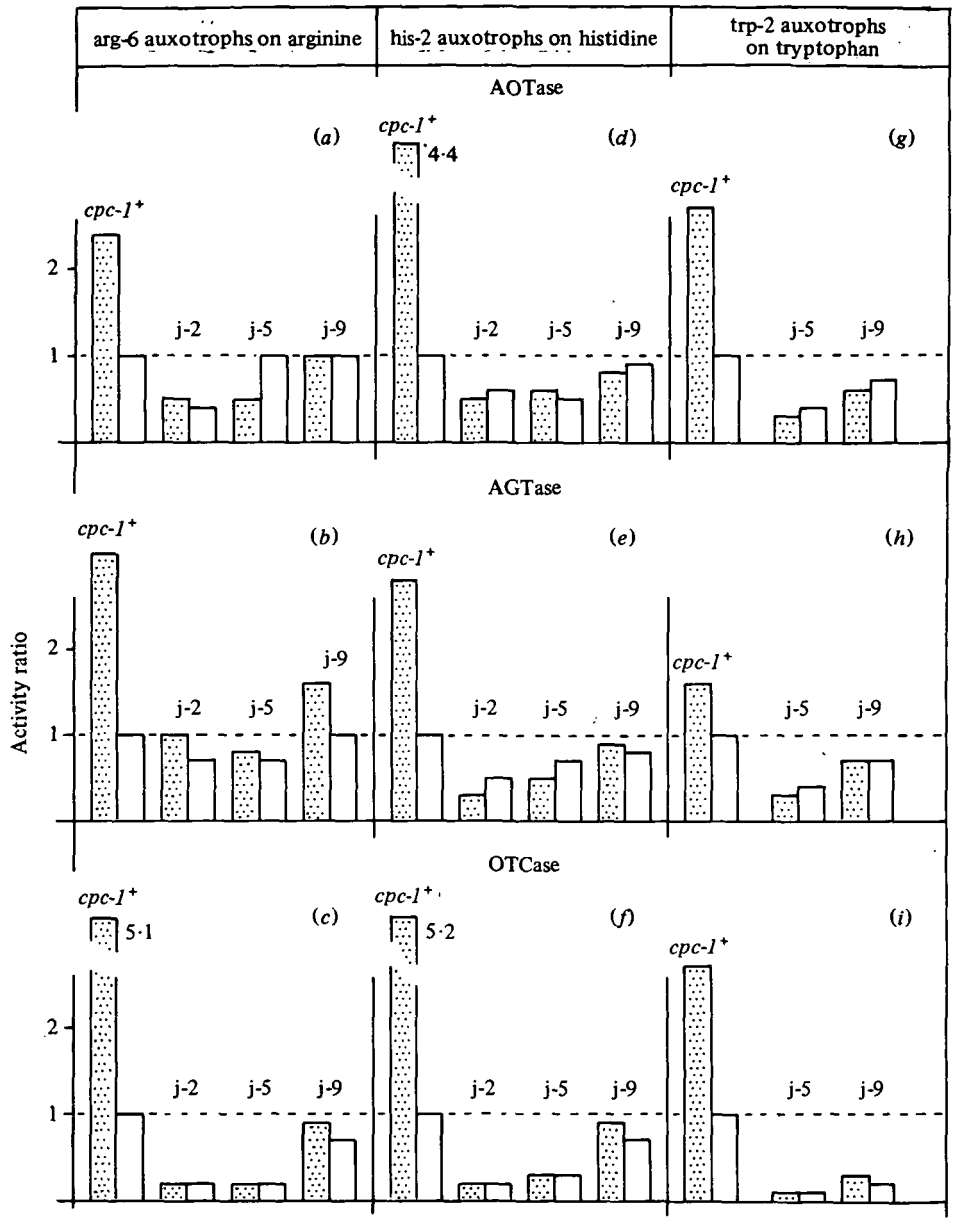

Fig. 1. (*a–i*) Activity ratios of the arginine biosynthetic enzymes AOTase, AGTase and OTCase in auxotrophic strains on different supplements. (*a–c*) (left): *cpc-1*$^+$ and *cpc-1* alleles, j-2, j-5 and j-9, combined with *arg-6* grown on arginine-supplemented (0·002 or 0·06 %) medium; (*d–f*) (middle): *cpc-1*$^+$ and *cpc-1* alleles, j-2, j-5 and j-9, combined with *his-2* grown on histidine supplemented (0·0003 or 0·02 %) medium; (*g–i*) (right): *cpc-1*$^+$ and *cpc-1* alleles, j-5 and j-9, combined with *trp-2* grown on tryptophan supplemented (0·0005 or 0·01 %) medium.

Stippled columns: limited, white columns: full supplement. Each column represents with few exceptions the average of at least 2–3 enzyme assays.

Abbreviations: AOTase, acetylornithine aminotransferase: AGTase, acetylornithine-glutamate acetyltransferase: OTCase, ornithine transcarbamylase.

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repressed AOTase levels, whilst in j-5, *arg-12*^{s} both enzymes failed to derepress at all.

In conclusion the data of Fig. 1 demonstrate that the introduction of the three *cpc-1* mutant alleles abolished the derepressibility observed in the auxotrophic reference strains. Arginine pathway enzymes failed to respond normally not only to a limitation of the endproduct of their own pathway, arginine, but also in response to either histidine or tryptophan limitation. Therefore the affected control property concerned more than one endproduct.

The next question is whether enzymes of more than one pathway are involved, i.e. whether *cpc-1* mutations have a reciprocal effect in preventing enzymes in pathways other than arginine biosynthesis from derepression upon arginine (or any other) limitation. The enzymes chosen were histidinol phosphate phosphatase (HPPase) in histidine biosynthesis and saccharopine dehydrogenase (SDase) in lysine biosynthesis.

Fig. 2 gives the activity ratios (calculated on the basis of the fully supplemented reference strains, see Table 3) for both enzymes under amino acid limitation and full supplementation in the strains as used to investigate the arginine pathway enzymes. Whereas the *cpc-1*$^{+}$ reference strains derepressed HPPase about two times (Fig. 2*a*, *c*, *e*) the double mutant strains carrying the three different *cpc-1* alleles failed completely to derepress HPPase either in direct response to histidine limitation (Fig. 2*c*) or via the cross-pathway response to arginine or tryptophan limitation (Fig. 2*a*, *e*). The same *cpc-1* double mutant strains failed also to derepress SDase in response to arginine or histidine limitation whilst the reference strains *cpc-1*$^{+}$, *arg-6* and *cpc-1*$^{+}$, *his-2* derepressed this enzyme more than twofold (Fig. 2*b* and *d*). Curiously, SDase was not derepressed in the *cpc-1*$^{+}$, *trp-2* reference strain (Fig. 2*f*). This strain already displayed a very high SDase activity on full supplement (Table 3). *cpc-1*, *trp-2* double mutants showed no sign of derepression (Fig. 2*f*).

These data show that *cpc-1* mutations affected the regulation of the enzymes of several amino acid biosynthetic pathways thereby supporting the working hypothesis derived from the amitrole sensitivity of the *cpc-1* mutant strains, namely that *cpc-1*$^{+}$ is involved in amino acid cross-pathway control. The observation that the mutational alteration of a single locus abolished the whole phenomenon suggested that there is unitary control of derepression in the pathways concerned.

(iv) cpc-1 *mutations and the control of basal enzyme levels*

Figs 1 and 2 show that *cpc-1* alleles affected not only derepressibility but the basal enzyme levels themselves. In general *cpc-1* mutant strains contained lower enzyme concentrations on full (and limiting) supplement when compared with the *cpc-1*$^{+}$ reference strains. The effect on basal enzyme levels varied between different *cpc-1* alleles; j-9 seemed to affect them least.

Figs 1 and 2 represent the relative basal enzyme levels. From Table 3 it is evident that in comparison with the wild type grown on minimal medium, the

reference auxotrophs displayed, to different degrees, elevated enzyme levels even when grown on full supplement. Highest activities were found in the *cpc-1*$^+$, *trp-2* strain grown on tryptophan. These differences between the reference strains complicate a comparison of the relative basal levels of *cpc-1* strains across different supplements. If instead the specific activities of fully supplemented *cpc-1* auxotrophs are considered, very similar values – regardless of supplement – were found for strains carrying the same *cpc-1* allele. This could indicate that the observed, elevated enzyme formation on amino acid supplements is also under *cpc-1*$^+$ influence.

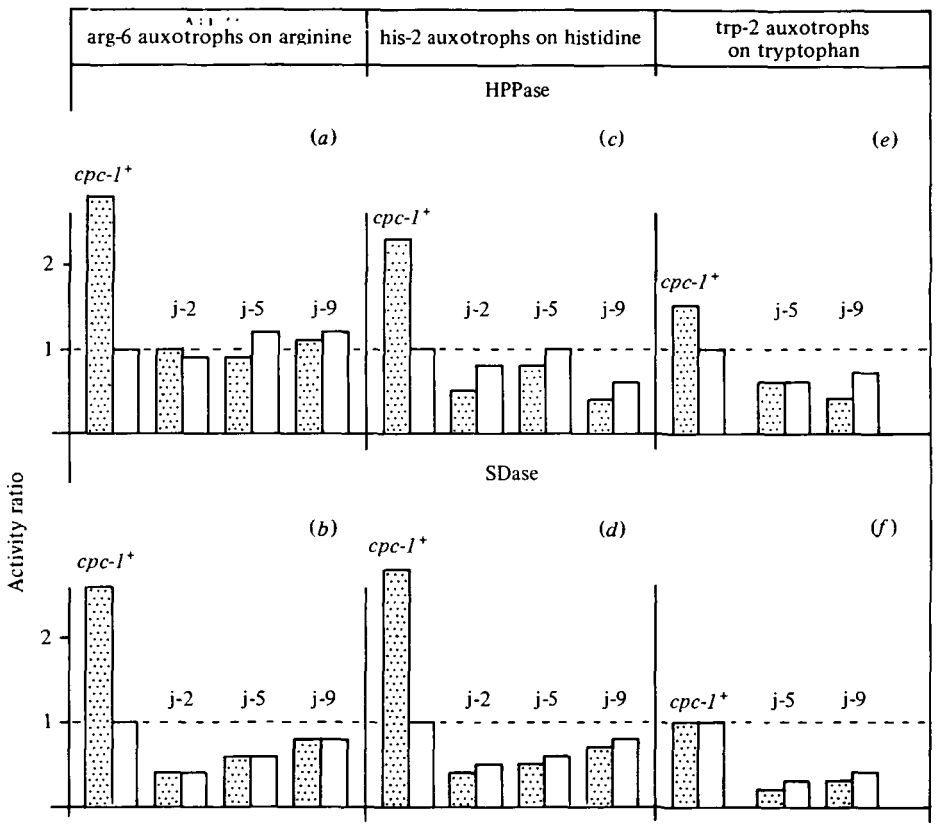

Fig. 2. (*a–f*) Activity ratios of one enzyme each of histidine (HPPase) and lysine (SDase) biosynthesis in auxotrophic strains grown on different supplements. (*a*, *b*) (left): *cpc-1*$^+$ and *cpc-1* alleles, j-2, j-5 and j-9, combined with *arg-6* grown on arginine supplemented (0·002 or 0·06 %) medium; (*c*, *d*) (middle): *cpc-1*$^+$ and *cpc-1* alleles, j-2, j-5 and j-9, combined with *his-2* grown on histidine supplemented (0·0003 or 0·02 %) medium; (*e*, *f*) (right): *cpc-1*$^+$ and *cpc-1* alleles, j-5 and j-9, combined with *trp-2* grown on tryptophan supplemented (0·0005 or 0·01 %) medium. Stippled columns: limited, white columns: full supplement. Columns in the HPPase section are based on one enzyme assay only, in the SDase section they represent with few exceptions the average of 2–3 enzyme assays. Abbreviations: HPPase, L-histidinol phosphate phosphatase; SDase, saccharopine dehydrogenase.

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To exclude an influence of the different auxotrophies and the unexplained slow growth of the auxotrophic *cpc-1* double mutants on basal enzyme levels, the same enzymes were assayed again in the prototrophic *cpc-1* strains in wild type background grown on minimal medium, where apart from viability and germination effects, the growth rates were normal. It was found that the mutants differed to various degrees from wild type, generally having lower enzyme levels (Table 4). The exception is the AGTase in j-9 which displayed a higher than wild type level.

Table 4. *Specific activity ratios* of the prototrophic* cpc-1 *strains on minimal medium*

Genotype	AOTase	AGTase	OTCase	HPPase	SDase
j-2	0·60 (8)	0·80 (4)	0·47 (6)	1·06 (1)	1·01 (4)
j-5	0·79 (8)	0·71 (4)	0·34 (6)	1·20 (1)	0·82 (4)
j-9	0·97 (3)	1·65 (7)	0·88 (6)	0·94 (1)	1·07 (4)

Abbreviations as in Table 3.

* Relative specific activities derived by dividing the specific activity of a strain by the specific activity of the wild type as given in Table 3. Parentheses indicate the number of repeats.

OTCase was chosen to investigate whether the observed decrease in basal levels was due to inhibitory effects of either small molecular effectors or inhibitory proteins. The specific activities of two strains with low OTCase levels, j-2 and j-5 (Table 4), were measured with and without overnight dialysis, and after mixing mutant and wild type homogenates in a 1:1 ratio. No influence of dialysis on enzyme activity was observed and the activity of mixtures of mutant and wild type crude extracts did not differ from the mean of the individual strains (data not given).

(v) *Control of enzymes not involved in amino acid biosynthesis*

To investigate the action spectrum of the *cpc-1*$^+$ product, the regulation of an enzyme of pyrimidine biosynthesis, aspartate transcarbamylase (ATCase), was investigated. For this purpose the mutation *pyr-2*, carrying a block in pyrimidine biosynthesis, was combined with the *cpc-1* allele j-5. Specific ATCase activity was studied in the j-5, *pyr-2* double mutant as well as the *cpc-1*$^+$, *pyr-2* reference strain under limiting and full uridine supplementation. The activity ratios are included in Table 5 and show that both strains derepressed the ATCase about two-fold. Furthermore, wild type and *cpc-1* prototrophs were grown on 0·33% acetate instead of glucose as the carbon source. Acetate leads to a rise in the levels of enzymes like NADP-linked isocitrate dehydrogenase (IDase) and citrate synthase (CSase) (Flavell & Fincham, 1968*a*). The activity ratios for wild type and *cpc-1* mutant strains when grown on glucose or acetate (relative to the wild type on glucose medium) are given in Table 5. IDase and CSase derepressed in *cpc-1* strains as well as in the *cpc-1*$^+$ wild type. The basal enzyme levels of the mutant strains varied around wild type levels.

Table 5. *Activity ratios* of the enzymes aspartate transcarbamylase (ATCase) NADP-linked isocitrate dehydrogenase (IDase) and citrate synthase (CSase)*

	ATCase			
Supplement	Uridine, 0·03 %		Uridine, 0·001 %	
cpc-1$^+$, *pyr-2* (reference)	1	(4)	2·1	(1)
j-5, *pyr-2*	0·9	(4)	2·4	(3)

	IDase		CSase	
Carbon source	Glucose, 2 %	Acetate, 0·33 %	Glucose, 2 %	Acetate, 0·33 %
Genotype				
Wild type (reference)	1 (4)	5·15 (4)	1 (4)	2·06 (4)
cpc-1 strain:				
j-2	1·18 (1)	5·92 (1)	0·83 (2)	2·15 (2)
j-5	1·22 (4)	5·24 (4)	1·02 (4)	1·88 (4)
j-9	0·98 (4)	6·00 (4)	0·80 (4)	2·39 (4)

* Relative specific activities derived by dividing the specific activity of a strain by that of the reference strain. Specific activities of the reference strains in Δ O.D. per minute per mg of protein × 10^3: *cpc-1*$^+$, *pyr-2* on 0·03 % uridine, ATCase 210; wild type on glucose, IDase 106 and CSase 5025. Brackets indicate the number of repeats.

The unimpaired regulation of the enzymes ATCase, IDase and CSase in the presence of *cpc-1* alleles was taken as an indication that the *cpc-1*$^+$ gene product has a specific function in the control of amino acid biosynthesis.

4. DISCUSSION

Using the same approach – though with different objectives – this investigation and the work of Davis (1979) uncovered a number of alleles at a new locus, *cpc-1*$^+$, of *Neurospora crassa*. Recessive *cpc-1* alleles affect amino acid cross-pathway control: Enzymes of arginine, histidine and lysine biosynthesis, unlike normal strains, fail to derepress in response to limitations of either arginine, histidine or tryptophan. (Derepression or repression are used as operational terms without implying a particular mechanism.) The unimpaired regulation of enzymes not involved in amino acid biosynthesis, such as two citric acid cycle enzymes and one of pyrimidine biosynthesis supports the hypothesis that *cpc-1*$^+$ is a genetic element which is specifically involved in amino acid cross-pathway control. Alleles like j-5 indicate that *cpc-1*$^+$ specifies a crucial element.

Five regulation defective mutants found in this work as well as Davis' fifteen mutants are due to lesions at the same gene, *cpc-1*$^+$. This is at variance with the findings in yeast, where two groups of investigators (Schürch, Miozzari & Hütter, 1974; Wolfner *et al.* 1975) each had to attribute mutants of similar phenotype to

three different loci, namely *AAS-1*, *AAS-2* and *AAS-3*. A possible reason for finding only one complementation group in *Neurospora* could be seen in the different selection schemes employed. All yeast mutants were selected by virtue of their sensitivity of growth to the tryptophan analogue 5-methyl-tryptophan, whilst this and Davis' investigation relied on their interference with the control of the arginine pathway enzyme OTCase. Studying alleles of two of the different yeast complementation groups Messenguy (1979) observed normal derepression of this particular enzyme in double mutants containing an *aas* allele and different arginine bradytrophs. Therefore, whatever the mechanism of selection via 5-methyl-tryptophan in yeast, it uncovered two complementation groups which would most probably not have been detected by the selection method used in this investigation.

According to currently available models, *cpc-1*$^+$ could specify an enzyme responsible for the synthesis, modification or spatial distribution of a general co-repressor(s). Alternatively the observed control of a number of unlinked structural genes by the *cpc-1*$^+$ locus could be mediated via the specification of a positive or negative acting control element, e.g. an activator or repressor protein. Since the recessive *cpc-1* mutations lead to the loss of derepressibility under amino acid limitation the simplest assumption would be that the mode of action of the *cpc-1*$^+$ product would be a positive one. Yet similar phenotypes could also result from mutations in a penultimate element of a double negative control cascade (Littlewood, Chia & Metzenberg, 1975). Wolfner *et al.* (1975) suggest that the three *AAS* genes found in yeast exert an indirect influence via the (negative) control of the *TRA* genes or their product to account for the finding that *aas*, *tra* double mutants are constitutive like the *tra* mutant itself.

So far the data presented in this paper do not justify detailed speculation as to the molecular mechanism by which control is exerted nor to the effector molecule(s) that brings about the cross-pathway phenomenon. Any model will have to take into account that the *cpc-1* mutants imply that at least as many as four amino acid biosyntheses are involved. Lester (1971) proposed a 'polyrepressor' model. A model would have to account for the pleiotropic effect of *cpc-1* alleles on basal enzyme levels. Using dialysis and extract mixing, possible enzyme inhibition as a secondary consequence of the *cpc-1* lesion was excluded. It appears therefore that the effect of *cpc-1*$^+$ is on amounts of enzyme synthesized.

The question arises whether other alleles, not discovered, could reduce basal levels to zero. Such alleles might have severe adverse effects on viability, particularly if they lowered all the biosynthetic enzymes controlled to zero and therefore might not be recovered with the selection system used. Thus the isolated *cpc-1* alleles could represent a selected sample, being a compromise between the selection for low (not derepressed) OTCase levels and the need for high enough levels of all other potentially affected enzymes. Possible support for this suggestion may be seen in the differences found between the three *cpc-1* alleles investigated, which suggest that they are not simply 'loss of function' mutations.

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