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Pyrimidine-Specific Carbamyl Phosphate Synthetase in *Neurospora crassa*

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Two carbamyl phosphate synthetases, the first an arginine-synthetic enzyme (CPS_{arg}) and the second a pyrimidine-synthetic enzyme (CPS_{pyr}), are shown to be present in *Neurospora*. The two enzymes can be separated on the basis of size and are distinguished by several different properties. Both CPS_{pyr} and CPS_{arg} have substrate requirements of adenosine triphosphate, HCO_3^-, and L-glutamine, although NH_4^+ in high concentration will partially replace glutamine. CPS_{pyr} activity can be completely inhibited by 5×10^{-4} to 10×10^{-4} M uridine triphosphate (UTP). CPS_{pyr} is cold-labile and can be protected against cold inactivation by UTP. The synthesis of CPS_{pyr} and aspartate transcarbamylase (ATC), the initial enzymatic steps of the pyrimidine pathway, are co-derepressed by pyrimidine starvation. Mutations affecting CPS_{pyr} and ATC all map at the same locus, *pyr-3*. Three classes of mutants with respect to the two activities were found: CPS^+ATC^-, CPS^-ATC^+, and CPS^-ATC^-. The distribution of these mutants on the genetic map, together with other data, indicate that the two activities are carried by a bifunctional protein.

Carbamyl phosphate (CAP) is an intermediate in both arginine and pyrimidine synthesis (Fig. 1). *Neurospora* appears to have two carbamyl phosphate synthetases, one specific for arginine synthesis (CPS_{arg}) and one specific for pyrimidine synthesis (CPS_{pyr}; references 6 and 7). Mutations affecting CPS_{arg} (at the *arg-2* and *arg-3* loci) do not affect pyrimidine synthesis, and mutations presumed to affect CPS_{pyr} (at the *pyr-3* locus) do not affect arginine synthesis (4). Because single CPS mutants have absolute requirements for the end product of their pathway, the CAP produced by the remaining CPS is evidently channelled to a particular fate and cannot be used in the deficient pathway (4).

CPS_{arg} has been briefly characterized as a glutamine-dependent CPS under control of the unlinked *arg-2* and *arg-3* loci (4, 5). The evidence indicates that CPS_{arg} is a two-polypeptide enzyme involving the *arg-2*$^+$ and *arg-3*$^+$ products. Although the *arg-3*$^+$ protein does possess NH_4^+-dependent activity, it completely lacks the biologically significant glutamine-dependent activity of the complete enzyme. In spite of considerable evidence indicating its presence, the demonstration of in vitro CPS_{pyr} activity had proved negative until recently. We report here the demonstration of a glutamine-dependent CPS_{pyr}, factors necessary for its stabilization, and some of its properties.

A major point of interest with respect to pyrimidine synthesis in *Neurospora* has been that mutations affecting the postulated CPS_{pyr} and those affecting aspartate transcarbamylase (ATC; carbamoylphosphate: L-aspartate carbamoyltransferase, EC 2.1.3.2.) all map at the *pyr-3* locus (9). Mutations at this locus appear to affect either one or both enzymatic activities. We report here the enzymatic tests which substantiate the hypothesis that CPS_{pyr}, like ATC, is under control of the *pyr-3* locus.

MATERIALS AND METHODS

Strains. Mutant strains used in this work are as follows: *arg-3* (30300); *arg-12*^{s} (s); *pyr-1* (H263); *pyr-3a* (37301, KS-20, and KS-16); *pyr-3d* [45502 and KS-43; 43-105 (Courtesy of Kenneth McDougall) and AR-618 (Courtesy of Alan Radford)]; and *pyr-3ad* (KS-11 and KS-23). Unless otherwise indicated, the strains came from our stock collection. For purposes of derepression of the enzymes of the pyrimidine and arginine synthetic pathways and for the selective elimination of one of the two CPS activities, various *arg, pyr* double mutants were required. These double mutants were isolated from the appropriate crosses. The sites of action of the mutant loci are shown in Fig. 1. The *arg-12*^{s} mutation causes greatly reduced (3 to 5% of normal) ornithine transcarbamylase activity (carbamoyl-phosphate: L-ornithine carbamoyltransferase, EC 2.1.3.3), but still permits vigorous growth on minimal medium (8).

Chemicals. Dilithium carbamyl phosphate was synthesized by the method of Spector et al. (26). Adenosine triphosphate (ATP), pyrimidine nucleosides and nucleotides, L-arginine, L-glutamine, and L-aspartic acid were purchased from the Sigma Chemi-

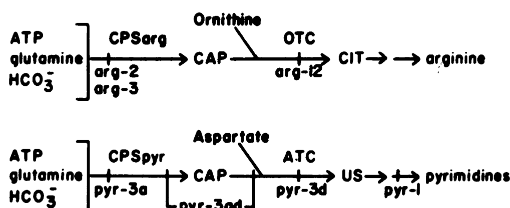

FIG. 1. *Schematic diagram of carbamyl phosphate (CAP) formation by path-specific synthetases (CPS_{arg} and CPS_{pyr}) and conversion by ornithine transcarbamylase (OTC) and aspartate transcarbamylase (ATC) to the arginine and pyrimidine precursors, citrulline (CIT), and ureidosuccinate (US).*

cal Co. Agarose 1.5 gel (Biogel) was obtained from Calbiochem.

Growth and enzyme derepression. The growth medium used was medium N of Vogel (28). It was supplemented with 1.5% sucrose as a carbon source and, where required for mutant strains, with 200 μg of arginine and 100 μg of uridine per ml. Mycelia for enzyme extraction were grown in 2,500-ml low-form culture flasks containing 700 ml of medium. The flasks were inoculated with approximately 2×10^5 conidia/ml of medium and placed on a reciprocal shaker at 25 C. The flasks were harvested after 18 to 24 hr and yielded 0.5 to 1.5 g (dry weight) per flask.

After 18 to 24 hr of growth in supplemented medium, derepression of CPS_{pyr} and ATC synthesis could be brought about in strains carrying a *pyr* mutation by transfer of the washed mycelia to an equal volume of medium lacking uridine. Incubation for 4 to 5 hr in the absence of uridine leads to a three- to fivefold increase in CPS_{pyr} and ATC activities and no increase in CPS_{arg} activity. Synthesis of CPS_{arg} was derepressed (without increase in the activities of CPS_{pyr} and ATC) by growth of strains carrying the *arg-12*^{s} mutation on minimal medium. Extracts were made from damp mycelial pads or from powders prepared from acetone-dried mycelia (R. H. Davis and F. J. de Serres, Methods in Enzymology, vol. 17A, *in press*). Enzyme activities of acetone-dried pads were stable for several weeks at −15 C.

Enzyme preparation. Unless otherwise indicated, the buffer used for enzyme extraction and equilibration of gel filtration columns was 0.05 M potassium phosphate (*p*H 7.3), containing 10^{-3} M L-glutamine. For extraction of fresh mycelia, buffer, damp mycelial pad, and fine sand (5:5:1) were ground rapidly in a mortar at 0 C. For extraction of acetone-dried mycelial powder, 1 ml of buffer was added for each 25 to 100 mg of powder, and the suspension was stirred slowly for 5 min at 15 C. The suspensions resulting from either extraction method were centrifuged for 20 min at $12{,}000 \times g$ at 10 to 15 C and the supernatant solution was recovered. For CPS_{pyr} of higher specific activity, a $(NH_4)_2SO_4$ precipitation was carried out. Solid $(NH_4)_2SO_4$ was dissolved in the crude supernatant (2.5 g/10 ml at 15 C). The precipitate was collected by 25 min of centrifugation at $12{,}000 \times g$ at 10 to 15 C. The precipitate, which contained all the recoverable CPS_{pyr} and ATC activity (threefold purified) was redissolved in buffer. Crude supernatant and $(NH_4)_2SO_4$ fractions were desalted by passage through short Sephadex G-25 columns at room temperature before assay of activity. Enzyme preparations from acetone-dried and fresh mycelia gave similar specific activities for CPS_{pyr} and ATC. Preparations from fresh mycelia were feedback inhibitable, those from acetone-dried mycelia were not.

Enzyme assays. The standard reaction mixture for CPS activity contained: 50 μmoles of tris(hydroxymethyl)aminomethane (Tris)-acetate buffer (*p*H 7.5), 3 μmoles of L-glutamine, 15 μmoles of $KHCO_3$, 6 μmoles of ATP, 6 μmoles of $MgCl_2$, and 0.10 or 0.20 ml of enzyme in a final volume of 0.50 ml. The reaction mixture was incubated for 30 min at 25 C. The reaction was stopped by the addition of 0.30 ml of 1 M NH_4Cl, followed by 10 min in a boiling water bath. By stopping the reaction in this manner, about 90% of the labile CAP product is converted, via cyanate, to the more stable urea (29). Control experiments in which CAP was trapped as ureidosuccinate (US) by the addition of 5 μmoles of aspartate and excess ATC to the assay system gave CPS specific activities close to those obtained when CAP was trapped as urea.

The standard assay system for ATC activity contained 150 μmoles of glycine buffer (*p*H 9.0), 10 μmoles of aspartic acid, 5 μmoles of freshly dissolved dilithium carbamyl phosphate, and 0.20 ml of enzyme in a final volume of 1.00 ml. After 15 min of incubation at 25 C, the reaction was stopped by the addition of 0.20 ml of 2 M $HClO_4$ and the product, US, was measured. Control incubation mixtures, lacking aspartate, were run with each ATC determination.

Urea and US were determined by a modified Gerhart-Pardee colorimetric assay (10). The final incubation for color development was at 25 C. The development time was 20 min for urea and 30 min for US. The color reaction tubes were then placed in ice water to prevent the loss of developed color and the later formation of spurious color. Color was read in a Klett-Summerson colorimeter with a no. 54 filter. The procedure will detect amounts of urea as low as 0.005 μmoles.

Agarose gel filtration. An agarose 1.5 gel column (37.5 by 1.2 cm) was used. After pouring, the column was equilibrated by the overnight passage of 100 to 200 ml of phosphate buffer. The void volume, as determined by the elution volume of high-molecular-weight protein, was 19 ml. Volumes of extract of 1.0 to 1.5 ml were applied to the top of the column, and a constant-flow rate of 0.25 ml per min was maintained by use of a Mariotte flask. Fractions of 1.25-ml volume were collected.

RESULTS

Demonstration of two CPS enzymes. The presence in *Neurospora* of two species of CPS, one controlled by the *arg-3* locus and the other by the *pyr-3* locus, can be shown clearly by gel filtration of mutant extracts. Crude extracts of strains carrying the four possible combinations of mutant and wild-type alleles at the *arg-3* and *pyr-3a* loci were applied to the agarose column, and the

eluant fractions were assayed for CPS activity. The *arg-3*+-*pyr-3a*+ strain has two peaks of CPS activity, one peak which is eliminated by mutation at the *arg-3* locus and the other by mutation at the *pyr-3a* locus (Fig. 2). In experiments devised to characterize CPS_{pyr}, strains carrying the *arg-3* mutation were used routinely to eliminate CPS_{arg} (Fig. 2, part B).

Substrate and pH dependency. CPS_{pyr} as extracted is a soluble enzyme found in the supernatant of extracts centrifuged at 100,000 × *g* for 1 hr. The enzyme has an absolute requirement for ATP, HCO_3^-, and L-glutamine (Table 1).

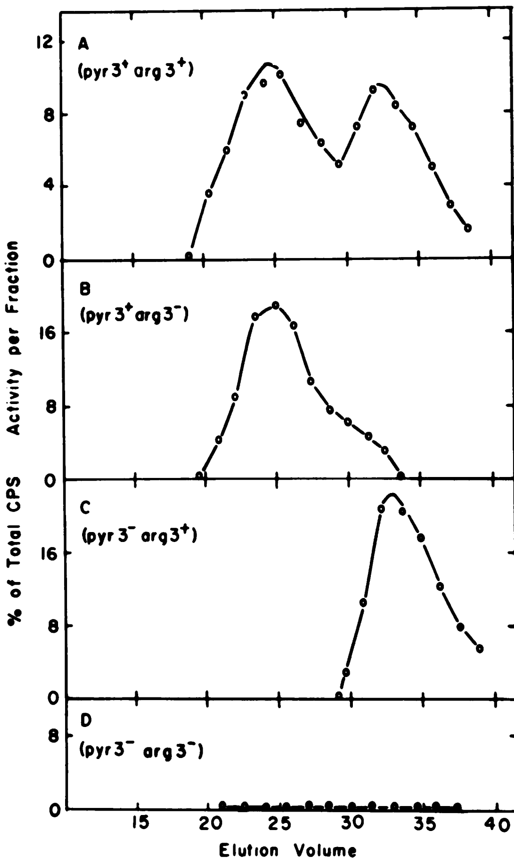

FIG. 2. *Distribution of L-glutamine-dependent CPS activity on agarose gel filtration columns. The chromatography procedure is described in the text. The columns used here were equilibrated with phosphate buffer (lacking L-glutamine) and run at 15 C, a compromise between conditions conferring optimal stabilization of the two CPS enzymes. Extracts of strains partially derepressed for the enzymes of both the arginine and pyrimidine pathways were applied to the column. Strains used were (A) arg-12ˢ, pyr-1; (B) arg-3, pyr-1; (C) arg-12ˢ, pyr-3a; and (D) arg-3, pyr-3a. The status of the pyr-3 and arg-3 loci, either mutant (−) or wild (+), is indicated.*

TABLE 1. *Requirements of CPS_{pyr} reaction*[a]

Substrate omissions	CAP formed (nmoles)
Complete	41
Complete minus glutamine + NH_4Cl	10
Complete minus glutamine	<2
Complete minus adenosine triphosphate	<2
Complete minus $KHCO_3$	<2
Complete minus enzyme	<2

[a] The enzyme preparation, an $(NH_4)_2SO_4$ fraction from an acetone powder extract of a derepressed *arg-3*, *pyr-1* strain, was desalted on a Sephadex column equilibrated with phosphate buffer. The assay system and incubation conditions are described in the text with the following changes: (i) the Tris buffer in the standard reaction mixture was replaced with potassium phosphate buffer (*p*H 7.8) and (ii) NH_4Cl, where present, was added to a final concentration of 60 mM.

NH_4^+ at high concentrations will partially replace L-glutamine. At *p*H 7.0, 60 mM NH_4^+ gives only about 10% the CPS activity given by 1 mM L-glutamine; at *p*H 8.9, 60 mM NH_4^+ and 1 mM L-glutamine are about equally effective as N donors. The optimal *p*H level for enzyme activity is 7.5, activity being about two-thirds the maximum at *p*H 6.5 and 8.5. CPS_{pyr} is rapidly inactivated at *p*H levels below 6.0 (50% loss of activity in 5 min at *p*H 6.0).

CPS_{pyr} stabilization. Upon demonstration of a highly labile CPS activity in *arg-3* extracts, provisionally designated CPS_{pyr}, attempts were made to stabilize it. The most dramatic finding was the cold lability of this enzyme. CPS_{pyr} activity is rapidly lost at 0 C, whereas it retains considerable activity at 25 C (Fig. 3). The cold inactivation is irreversible; upon rewarming to 25 C, no activity is restored. L-Glutamine, a substrate, and UTP, the feedback inhibitor of CPS_{pyr}, were found to stabilize the enzyme activity under certain conditions. L-Glutamine does not alter the rapid loss of activity at 0 C but it does retard loss of activity that occurs above 15 C (cf. Fig. 3A and 3B). After 3 hr at 25 C in the presence of 10^{-3} M L-glutamine, 95% of the enzyme activity remains, whereas in the absence of glutamine only 50% of the activity remains. Cold inactivation can be greatly slowed by the presence of 1×10^{-4} to 5×10^{-4} M uridine triphosphate (UTP). The effect of UTP is greatest at 0 C and becomes slight at 15 C. No interactions between UTP and L-glutamine were noted. L-Glutamine does not affect the protective action of UTP at 0 C, and UTP does not alter the action of L-glutamine at higher temperatures.

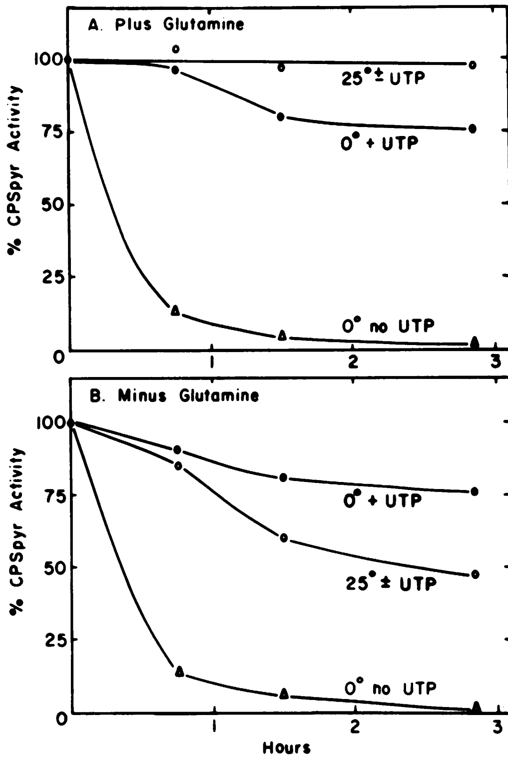

FIG. 3. *Effect of temperature and stabilizing agents on* CPS_{pyr} *activity. The enzyme preparation, an* $(NH_4)_2SO_4$ *fraction from a fresh mycelial extract of a derepressed arg-3, pyr-1 strain, was desalted on a Sephadex column equilibrated with phosphate buffer. Preparations were incubated at 0 C or 25 C with or without* L*-glutamine* (10^{-3} M) *and with or without UTP* (5×10^{-4} M).

CPS_{pyr} is also heat-sensitive. In the absence of L-glutamine, it loses 50% of its activity after 15 min at 37 C. CPS_{pyr} can be stored at high protein concentrations for several weeks after rapid freezing at −80 C in the presence of 5×10^{-4} M UTP.

Derepression and feedback inhibition of CPS_{pyr}. Because ATC is derepressed by pyrimidine starvation, experiments were carried out to determine whether CPS_{pyr} was derepressed under similar conditions. The co-derepression of the two enzymes (Fig. 4) demonstrates that CPS_{pyr} has a property expected of pyrimidine synthetic enzymes. The very close correlation of the curves indicates a close relationship between the synthesis of CPS_{pyr} and ATC. Dihydroorotase, an enzyme in the pyrimidine synthetic pathway, changes only slightly in activity over the same time course.

Because the synthesis of CAP is the initial step in pyrimidine synthesis, this enzyme is a likely candidate for feedback inhibition. CPS_{pyr} extracts prepared from acetone powders were not feedback-sensitive. The CPS_{pyr} activity in extracts of fresh mycelia, however, was inhibited by UTP and to a lesser extent by uridine monophosphate (UMP). At the standard concentration of substrates in the incubation mixture, 5×10^{-4} M UTP causes 90 to 100% inhibition of the CPS_{pyr} activity. The same concentration of UMP causes 65% inhibition. Changes in the concentration of L-glutamine, $KHCO_3$, and aspartate from one-third to four times those normally used in assays did not affect the inhibition of CPS_{pyr} by UTP. However, increases in ATP concentration markedly lessened the inhibition by UTP (Fig. 5).

CPS_{pyr} activity was not inhibited by uridine, cytidine, CMP, CTP, or arginine at concentrations as high as 2.0×10^{-3} M. In yeast, both CPS_{pyr} and ATC are subject to feedback inhibition by UTP (16). Although a variety of assay conditions were tried, *Neurospora* ATC activity was not inhibited by UTP, UMP, nor any of the other pyrimidines mentioned above.

CPS_{pyr} and ATC activities of pyr-3 mutants. In previous work (9) mutants arising from single mutational events at the *pyr-3* locus were classified into three groups. (i) Mutants of *pyr-3a* possess ATC activity and are suppressed by the *arg-12^s* mutation by diversion of arginine specific CAP into the pyrimidine pathway. They presumably lack CPS_{pyr} (4). (ii) Mutants of *pyr-3d* lack ATC and act as suppressors of *arg-2* and

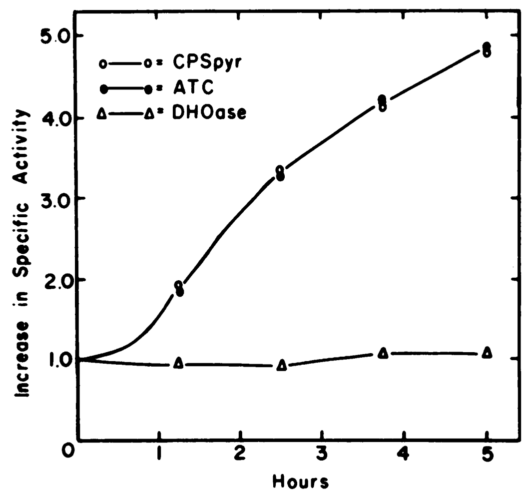

FIG. 4. *Response of* CPS_{pyr}*, ATC, and dihydroorotase (DHOase) activities to uridine starvation. Arg-3, pyr-1 mycelia were transferred to medium lacking uridine. At 1.25-hr intervals, samples were withdrawn and ATC,* CPS_{pyr}*, and DHOase levels were determined.*

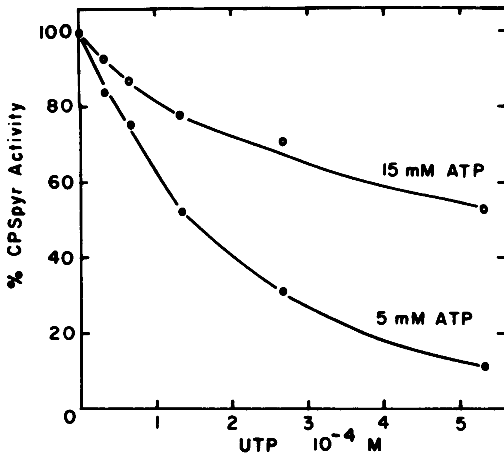

FIG. 5. *Effect of ATP on UTP inhibition of* CPS_{pyr}. *Enzyme preparation was the same as described in Fig. 3 except that the phosphate buffer system contained* L-*glutamine. Substrate levels in assay system were those used previously with the exception of ATP and* $MgCl_2$. ●, *5 mM ATP and 10 mM* $MgCl_2$. ○, *15 mM ATP and 30 mM* $MgCl_2$.

arg-3 by diverting unused, pyrimidine-specific CAP into the arginine pathway (6, 25). The *pyr-3a* and *pyr-3d* mutants complement in heterocaryons. (iii) Mutants of *pyr-3ad* do not complement with *pyr-3a* or *pyr-3d* mutants, are not suppressed by *arg-12*ˢ, nor are they suppressors of *arg-2* and *arg-3*. *Pyr-3ad* mutants lack ATC and, presumably, CPS_{pyr}. These conclusions are supported by the data of Table 2, which show the CPS_{pyr} and ATC activities of eight *pyr-3* isolates and their ability to suppress *arg-3*. Three classes of *pyr-3* mutants exist with respect to the two enzyme activities: (i) those possessing ATC and lacking CPS_{pyr} activities (*pyr-3a*), (ii) those possessing CPS_{pyr} and lacking ATC (*pyr-3d*), and (iii) those lacking both activities (*pyr-3ad*). Only those mutants having CPS_{pyr} activity act as suppressors of *arg-3*. It should also be noted that a *pyr-3* strain carrying allele 45502, which is a rather poor suppressor of *arg-3*, also has considerably lower CPS_{pyr} activity than normal.

DISCUSSION

The work reported here clearly demonstrates that *Neurospora* has two CPS enzymes. The two enzymes can be physically separated on the basis of molecular size and can be differentiated by a number of properties (Table 3). There are three lines of evidence which indicate CPS_{pyr} is a pyrimidine-synthetic enzyme. (i) Strains carrying mutations which eliminate this enzyme require a pyrimidine supplement for growth. (ii) Synthesis of the enzyme is derepressed by pyrimidine starvation. (iii) The enzyme activity can be completely inhibited by the pyrimidine end product, UTP, at concentrations of 5×10^{-4} to 10×10^{-4} M.

Three types of enzymes have been found which synthesize CAP in vitro from ATP, HCO_3^-, and glutamine or NH_3. One of these, carbamate kinase, first found in *Streptococcus faecalis* (14), uses only NH_3 as the nitrogen source and produces 1 mole of CAP per mole of ATP utilized (13). In vivo carbamate kinase probably functions as a catabolic enzyme (13). The remaining two types are classified as carbamyl phosphate synthetases and require 2 moles ATP per mole of CAP synthesized (2, 21). The first class of CPS, found in animal tissue, uses NH_3 as the nitrogen source and requires *N*-acetyl-glutamate as a cofactor (21). The second class of CPS utilizes glutamine as the preferred nitrogen donor and does not require the cofactor. The second class of CPS has been found in *Agaricus* (18), yeast (17), *Escherichia coli* (22), *Neurospora* CPS_{arg}

TABLE 2. CPS_{pyr} *and ATC activities of arg-3, pyr-3, double mutants*[a]

Mutant	*Pyr-3* allele	Specific activity[b]		Growth on medium supplemented with		
		CPS_{pyr}	ATC[c]	arg	uri	arg + uri
arg-3, pyr-3a	37301	0.00	76.5	−	−	+
	KS-20	0.00	62.0	−	−	+
arg-3, pyr-3d	KS-43	2.50	0.0	−	+[d]	+
	43-105	3.08	0.0	−	+	+
	AR-618	5.00	0.0	−	+	+
	45502	0.33	0.0	−	±	+
arg-3, pyr-3ad	KS-11	0.00	0.0	−	−	+
	KS-23	0.00	0.0	−	−	+
arg-3, pyr-1	+	2.44	72.5	−	−	+
arg-3, pyr-1 (repressed)	+	1.00	30.8	−	−	+

[a] Strains carried the *arg-3* mutation to eliminate CPS_{arg}. Enzyme preparations were from fresh mycelial extracts of strains partially derepressed for pyrimidine synthetic enzymes.

[b] Specific activity is expressed as 10^{-3} micromoles of product (carbamyl phosphate or ureidosuccinate) per milligram of protein per minute.

[c] Aspartate transcarbamylase.

[d] Growth of this double mutant on uridine-supplemented medium indicates suppression of *arg-3*.

TABLE 3. *Comparison of CPS_{arg} and CPS_{pyr} of Neurospora*

	CPS_{arg}	CPS_{pyr}
Substrates	ATP, HCO_3^-, glutamine	ATP, HCO_3^-, glutamine
Controlling genetic loci	*arg-2* and *arg-3*	*pyr-3*
Derepression by	Arginine starvation	Pyrimidine starvation
Feedback inhibitor		UTP
Molecular weight[a]	250,000	650,000
Incubation at 0 C	Stable	Labile
NH_4^+ as substitute substrate for L-glutamine	Fair	Poor

[a] Rough estimate on basis of elution pattern from agarose gel columns.

(4), and in animal tissue (11, 27). *Neurospora* CPS_{pyr} is of the latter type in that it functions as an anabolic enzyme in vivo, preferentially uses glutamine over NH_3 as a nitrogen donor, and does not require *N*-acetylglutamate as a cofactor. The property of feedback inhibition is found in the CPS of a number of species (17, 22, 27) including *Neurospora* CPS_{pyr}. The aggregation state of *E. coli* CPS is affected both by activator and inhibitor (1). The *Neurospora* CPS_{pyr} is unique in its cold lability.

CPS_{pyr} activity is determined by the *pyr-3* locus and is not affected by mutation at the *arg-2* and *arg-3* loci, which determine CPS_{arg}. It is probable that *pyr-3* is the structural gene for CPS_{pyr} although structural variants have not been identified. Temperature-sensitive *pyr-3a* mutants (37815 and 67602) were tested for altered CPS_{pyr}, but unfortunately extracts had little or no detectable activity.

Mutants lacking only ATC activity or both ATC and CPS_{pyr} activity also lie at the *pyr-3* locus (Table 2). This suggests that *pyr-3* may code for an enzyme with two catalytic activities. In fact, Hill and Woodward have found that the ATC enzymes of *pyr-3a* mutants (CPS^- ATC^+) have altered kinetic properties (12). This supports the idea that the *pyr-3* locus has a structural role in the determination of such a bifunctional protein. A similar situation prevails in yeast (15, 16). There is no in vitro evidence, in either yeast or *Neurospora*, of the association of other pyrimidine enzymes with ATC or CPS_{pyr}. Pyrimidine enzymes for the rest of the pathway, in both yeast and *Neurospora*, are specified by loci nonallelic to the CPS-ATC determinant (3, 16).

Physical evidence which demonstrates *Neurospora* ATC and CPS_{pyr} activities associated as a bifunctional protein will be presented elsewhere. However, the question of whether the *pyr-3* locus specifies one, two, or more different polypeptides remains open. The genetic map of the *pyr-3* locus (Fig. 6; reference 9) does not resolve this question, but does support the idea of a bifunctional

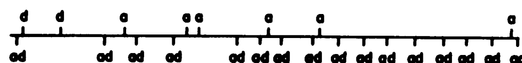

FIG. 6. *Schematic map of the pyrimidine-3 locus, showing locations of the three enzymatic classes of mutants. d = CPS^+ ATC^-, a = CPS^- ATC^+, ad = CPS^- ATC^-.*

protein. Two features are evident in the map. First, the scatter of *pyr-3ad* mutations (CPS^--ATC^-) throughout the length of the map suggests a bifunctional protein. Second, the fact that singly deficient mutations do not overlap suggests an internal differentiation of the locus. The latter possibility is given further support by genetic studies with additional *pyr-3d* mutant isolates (K. J. McDougall, R. Ostman, and V. W. Woodward, Genetics **60**: 203, 1968). Clearly a detailed study of the subunits of the enzyme is needed.

More recently, Radford in complementation and mutational studies of the *pyr-3* locus has given evidence suggesting the entire bifunctional protein is translated from a common messenger (23, 24). Frameshift mutations giving rise to singly deficient types were rare, but such mutations were always of the ATC^- type, indicating a polarized translation from CPS to ATC (right to left in map of Fig. 6). Radford has also detected a pattern of polarized complementation which suggests a reading of a single messenger from CPS_{pyr} to ATC. A similar situation with respect to a bifunctional protein is seen in tryptophan synthetase of yeast (19, 20).

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