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DISSOCIATION OF AN ENZYME COMPLEX FROM *NEUROSPORA CRASSA*

EVIDENCE FOR A PROTEASE *

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Summary

Purification of the *pyrimidine—3* gene product (an enzyme catalysing the first two steps of the pyrimidine biosynthetic pathway) of *Neurospora crassa* has proved to be complicated by the extreme lability of one of the two activities and by changes in the molecular weight of the other. The native enzyme, which possesses both pyrimidine-specific carbamoyl-phosphate synthase (ATP: carbamate phosphotransferase (dephosphorylating), EC 2.7.2.5) and aspartate carbamoyltransferase (carbamoylphosphate:L-aspartate carbamoyltransferase, EC 2.1.3.2) activities has a molecular weight of approx. $6.5 \cdot 10^5$, as does a form which has lost its carbamoyl-phosphate synthase activity. This form is capable of undergoing aggregation to a species which may well be heterogeneous and has a molecular weight greater than $2 \cdot 10^6$. A form of lower molecular weight than the native form, and also lacking carbamoyl-phosphate synthase activity, has also been found. The formation of this low molecular weight species has been shown to be accelerated by the removal of a substance of approx. $1.2 \cdot 10^4$ daltons and retarded by its replacement. Lowering the pH below 7.3 also significantly accelerated the molecular weight change, while raising it had the opposite effect. Retardation was also brought about by heat treatment or the serine protease inhibitor, phenylmethylsulphonylfluoride. Addition of the endopeptidases, chymotrypsin, elastase, thermolysin, or trypsin, also brought about this dissociation of aspartate carbamoyltransferase, whereas exopeptidases had no effect. These results are interpreted in terms of the dissociation of aspartate carbamoyltransferase being mediated by an endopeptidase which, on extraction, is initially inactive due to its being complexed with an inhibitor.

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Introduction

Although the existence of proteases in all organisms has long been recognised, it is only comparatively recently that the effects they might have on enzyme extraction and purification have been fully appreciated. In *Neurospora*, it has been shown that the four subunits previously reported in the *arom* enzyme complex are in fact fragments resulting from proteolysis of a single polypeptide species, and their appearance could be inhibited by phenylmethylsulphonylfluoride [1]. The various enzyme activities, anthranilate synthase, chorismate synthase, and kynureninase from *Neurospora* are also very unstable, but can be stabilised by phenylmethylsulphonylfluoride [2,3]. Similar observations have been reported for tryptophan synthase, and in this instance direct evidence of protease involvement has been obtained, as the lability of the enzyme correlated well with the level of protease activity [4]. Many similar examples have been reported in yeast [5].

In *Neurospora*, five intracellular proteases have been isolated and purified: one carboxypeptidase, two aminopeptideases, and two endopeptidases. Each exists as an inactive complex with a specific inhibitor, and activation is achieved by dissociation of the complex. Properties of each of the proteases, heat stability, pH dependence, and sensitivity to serine protease and sulphydryl inhibitors, allowed each to be uniquely characterised [6]. One of the two endopeptidases appears to be the same as that reported by Gaertner's group, in view of its low heat-stability and its sensitivity to phenylmethylsulphonylfluoride [1]. This endopeptidase has been shown to have a molecular weight of $3.5 \cdot 10^4$ when complexed with its specific inhibitor, and this form is reversibly dissociated into the protease ($2.4 \cdot 10^4$) and the inhibitor ($1.0 \cdot 10^4$) [7].

The first two steps in the pyrimidine biosynthetic pathway in *Neurospora*, pyrimidine-specific carbamoyl-phosphate synthase (ATP:carbamate phosphotransferase (dephosphorylating), EC 2.7.2.5) and aspartate carbamoyltransferase (carbamoylphosphate:L-aspartate carbamyltransferase, EC 2.1.3.2) are both specified by the *pyrimidine-3* gene on the right arm of linkage group IV [8]. A similar situation, with both activities specified by a single locus, is found in *Saccharomyces cerevisiae* and *Aspergillus nidulans* [9,10]. In all these organisms, it is possible to obtain mutants lacking pyrimidine-specific carbamoyl-phosphate synthase, aspartate carbamoyltransferase, or both activities. The gene may produce a single cistron message which is translated into a bifunctional polypeptide, which may or may not be subsequently cleaved to give two secondary polypeptide chains, or, alternatively, the message may be polycistronic. Polar mutants have been used to order the two activities with respect to translation, but this does not resolve the problem of the number of cistrons involved [11—14].

Inter-allelic complementation studies have shown that high levels of complementation only occur between *CPSpyr*$^+$*ACT*$^-$ * and *CPSpyr*$^-$*ACT*$^+$ ** mutants. However, this again cannot differentiate between a situation in which there are

* *CPSpyr*$^+$*ACT*$^-$, mutant containing pyrimidine-specific carbamoyl-phosphate synthase activity but lacking aspartate carbamoyltransferase activity.

** *CPSpyr*$^-$*ACT*$^+$, mutant containing aspartate carbamoyltransferase activity but lacking pyrimidine-specific carbamoyl-phosphate synthase activity.

two polypeptides, one with pyrimidine-specific carbamoyl-phosphate synthase activity and the other with aspartate carbamoyltransferase activity, and a single polypeptide with two domains, one with each activity.

The native pyrimidine-specific carbamoyl-phosphate synthase/aspartate carbamoyltransferase enzyme of *Neurospora* has a molecular weight of $6.5 \cdot 10^5$, with an alternative form approximately half that size [15]. The homologous enzyme in *Saccharomyces* is $8.0 \cdot 10^5$, dissociating in the absence of UTP to approximately $3.8 \cdot 10^5$, a form still possessing both pyrimidine-specific carbamoyl-phosphate synthase and aspartate carbamoyltransferase activities [16]. From this form, two others could be derived: passage through DEAE-Sephadex, or heat treatment, gave a form at $1.4 \cdot 10^5$ with only aspartate carbamoyltransferase activity; removal of UTP, L-glutamine, and Mg^{2+} gave a form at $2.5 \cdot 10^5$ with only pyrimidine-specific carbamoyl-phosphate synthase activity [16]. The origin of these two forms, as different polypeptide products of two separate cistrons, or as secondary products of the same cistron, was unclear.

As a first step in differentiating between the alternative models of the pyrimidine-specific carbamoyl-phosphate synthase · aspartate carbamoyltransferase complex, attempts were made to purify the enzyme. However, as Williams and Davis have already reported, the pyrimidine-specific carbamoyl-phosphate synthase activity is unstable [19]. This was, therefore, also investigated.

Materials and Methods

The strains of *Neurospora crassa* used in this work were *arginine—2* (33442), *arginine—3* (30300) and the double mutant strain *pyrimidine—1* (KS12p1)/ *arginine—3* (30300). Both *arg*$^-$ mutants lack arginine-specific carbamoyl-phosphate synthase activity.

Uridine triphosphate (UTP), L-glutamine, dithiothreitol (Cleland's reagent), phenylmethylsulphonylfluoride, *p*—chloromercuribenzoate, elastase, carboxypeptidase B (hog pancreas), carboxypeptidase A (bovine pancreas) and trypsin were obtained from Sigma, imidopeptidase (prolidase, pig kidney) was purchased from Miles-Serovac, and thermolysin (*Bacillus thermoproteolyticus*) from Calbiochem.

Growth and assay procedures for both pyrimidine-specific carbamoyl-phosphate synthase and aspartate carbamoyltransferase have been described elsewhere [20,21]. Except where otherwise stated, the assay procedure for protein of Lowry et al. was used [22]. Details of the alternative method, a modification of the biuret method, have been published elsewhere [20]. Ornithine carbamoyltransferase (EC 2.1.3.3) was assayed by production of citrulline [19—21].

Mycelial extracts were prepared from either fresh or lyophilised mycelia, the former method as previously described [20,21]. Freeze-drying was carried out on mycelia which had been frozen for less than 24 h in order to minimise any changes which might have been caused by storage in the presence of water. The mycelia were then stored, frozen, in an air-tight container, until required. The mycelia were ground as required, and extracted with 8 ml buffer/g. Preparation of crude extracts was as previously described [21].

As preliminary experiments had shown that most aspartate carbamoyltransferase activity precipitated between 25% and 40% $(NH_4)_2SO_4$, and this step re-

sulted in approx. 3-fold purification of the enzyme, such extracts were regularly used in the experiments described in this paper.

Gel filtration was carried out using various grades of Sephadex and Sepharose 6B (Pharmacia). The dimensions of the columns differed from experiment to experiment, and details are given for each. Rates of flow, and pressures, were always kept within the recommended limits.

Bacterial growth was a problem in any extracts stored at 0—5° C for prolonged periods. This was overcome by the inclusion of chloramphenicol (Sigma) at 25 μg/ml in all buffers used in such experiments. It had previously been shown that chloramphenicol does not interfere with aspartate carbamoyltransferase or pyrimidine-specific carbamoyl-phosphate synthase activity.

Results

Stability of pyrimidine-specific carbamoyl-phosphate synthase and aspartate carbamoyltransferase

Preliminary experiments were performed to test the stability of the two enzyme activities. Fresh mycelia from two strains, *arg—2* and *arg—3*, were extracted in the presence of UTP, L-glutamine, and dithiothreitol, three compounds which had previously been reported to stabilise pyrimidine-specific carbamoyl-phosphate synthase [15]. Samples of the extract were stored at 0—5° C, and at various times were de-salted on a Sephadex G25 column and assayed for pyrimidine-specific carbamoyl-phosphate synthase and aspartate carbamoyltransferase activities. Protein was estimated by the modified biuret method. The changes in specific activity are shown in Fig. 1. As can be seen, under these conditions pyrimidine-specific carbamoyl-phosphate synthase was very unstable, being completely inactivated after 30 h. The pyrimidine-specific carbamoyl-phosphate synthase activity was assayed via citrulline, and depended upon ornithine carbamoyltransferase activity in the extract, but control experiments had shown this enzyme to be stable under the experimental conditions. There was also a marked tendency for the specific activity of aspartate carbamoyltransferase to increase during storage. Loss of pyrimidine-specific carbamoyl-phosphate synthase activity and increase in aspartate carbamoyltransferase activity was repeatedly observed, both in the presence and absence of UTP. Yet Williams and

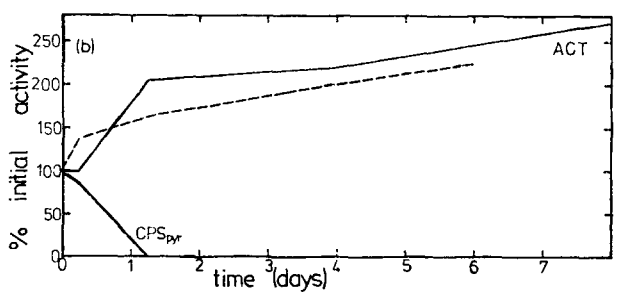


Fig. 1. The stability of pyrimidine-specific carbamoyl-phosphate synthase (CPSpyr) and aspartate carbamoyltransferase (ACT) at 0—5°C in the presence of 0.75 mM UTP. (———) from *arg-2* strain, (- - - - -) from *arg-3* strain.

Davis [19] found that UTP stabilised pyrimidine-specific carbamoyl-phosphate synthase against cold-inactivation.

The experiments of Williams and Davis were therefore repeated in order to resolve this contradiction. In these experiments, freeze-dried mycelia from a derepressed *arg—3 pyr—1* strain were extracted in a buffer containing 1 mM L-glutamine and 1 mM UTP. The 25—40% $(NH_4)_2SO_4$ fraction was divided into batches. Each was eluted through a Sephadex G25 column which had been equilibrated with either buffer alone, buffer plus 1 mM UTP, buffer plus 1 mM L-glutamine, or buffer plus 1 mM UTP and 1 mM L-glutamine. Each batch was further subdivided, and incubated at either 0 or 25°C. Pyrimidine-specific carbamoyl-phosphate synthase assays were performed at various times and the results are shown in Fig. 2. In contrast to the findings of Williams and Davis, these results show that UTP did not stabilise pyrimidine-specific carbamoyl-phosphate synthase at 0°C, and neither did L-glutamine at 25°C. The experiment was repeated, and further possible stabilisers, 10 mM ATP plus Mg^{2+}, 10 mM uridine, 10 mM L-aspartate, 1 mM carbamoyl aspartate, and 1 mM dithiothreitol were also tested, all without significant effect on the loss of pyrimidine-specific carbamoyl-phosphate synthase activity at either 0 or 25°C.

In all our experiments, whenever pyrimidine-specific carbamoyl-phosphate synthase activity decreased, aspartate carbamoyltransferase activity was observed to increase. These two processes clearly did not parallel one another, as aspartate carbamoyltransferase activity continued to rise long after pyrimidine-specific carbamoyl-phosphate synthase had reached zero. Other conditions leading to loss of pyrimidine-specific carbamoyl-phosphate synthase activity were examined for their effects on aspartate carbamoyltransferase (Table I). Raising the pH and the addition of ethanol to a final concentration of 10% both caused this same effect. *p*-Chloromercuribenzoate, after 1 h of incubation, inactivated both pyrimidine-specific carbamoyl-phosphate synthase and aspartate carbamoyltransferase, but not co-ordinately. As the *p*-chloromercuribenzoate concentration was raised, pyrimidine-specific carbamoyl-phosphate synthase activity was progressively lost whereas aspartate carbamoyltransferase activity initially rose before falling off, at much higher concentrations. As pyrimidine-specific

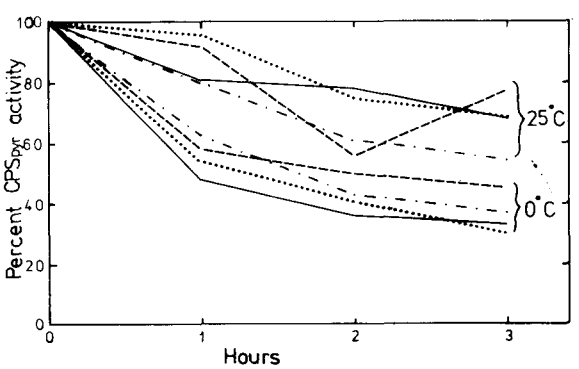


Fig. 2. The effect of UTP and L-glutamine on the stability of pyrimidine-specific carbamoyl-phosphate synthase (CPSpyr) at 25 and 0°C. (———) control (- - - - - -) + UTP, (· · · · · ·) + L-glutamine, (- · - · -) + UTP and L-glutamine.

TABLE I

THE EFFECT OF VARIOUS TREATMENTS ON PYRIMIDINE-SPECIFIC CARBAMOYL-PHOSPHATE SYNTHASE, ASPARTATE CARBAMOYLTRANSFERASE AND ORNITHINE CARBAMOYLTRANSFERASE ACTIVITIES

Treatment	Pyrimidine-specific carbamoyl-phosphate synthase (units/ml)	Aspartate carbamoyl-transferase (units/ml)	Ornithine carbamoyl-transferase (units/ml)
Control (pH 7.3)	0.028	0.60	4.12
pH 8.1	0.018	0.65	4.02
10% ethanol	0.000	0.98	3.91
0.2 mM *p*-chloromercuribenzoate	0.013	0.68	2.19
0.4 mM *p*-chloromercuribenzoate	0.001	0.74	0.51
1.0 mM *p*-chloromercuribenzoate	0.000	0.00	0.01

carbamoyl-phosphate synthase was assayed via citrulline in these experiments, there was a possibility that the observed inactivation of *p*-chloromercuribenzoate was due in reality to inactivation of ornithine carbamoyltransferase. This was shown not to be the case by repeating the experiment, this time assaying the pyrimidine-specific carbamoyl-phosphate synthase product as urea [19]. Assayed in this way, the pyrimidine-specific carbamoyl-phosphate synthase activity followed the same course as when assayed via citrulline.

It, therefore, appeared that inactivation of pyrimidine-specific carbamoyl-phosphate synthase by a variety of different means was associated with a conformational change in the molecule giving rise to activation of aspartate carbamoyltransferase. The possibility that these conformational changes were accompanied by changes in molecular weight was then investigated. A crude extract, prepared from de-repressed fresh mycelia of an *arg—3/pyr—1* strain, was de-salted and divided into two batches. One was immediately eluted through a Sepharose 6B column (70 × 1.5 cm) after an aliquot had been assayed for pyrimidine-specific carbamoyl-phosphate synthase and aspartate carbamoyltransferase activities, and protein (modified biuret method). Fig. 3a shows the aspartate carbamoyltransferase activity and protein profile of the elution. After two days at 0—5°C, the other batch was similarly treated, and the results are given in Fig. 3b. During the two days, aspartate carbamoyltransferase activity had risen from 0.041 to 0.061 units/mg and pyrimidine-specific carbamoyl-phosphate synthase had fallen from 0.0016 to 0.0003 units/mg, yet the main aspartate carbamoyltransferase peak was unaltered on the Sepharose 6B profile. There was, however, a very small accumulation of aspartate carbamoyltransferase activity with a very high molecular weight ($2 \cdot 10^6$) eluting in the void volume. This result was reproducible, and independent of the presence or absence of UTP. The high molecular weight form was termed aspartate carbamoyltransferase-H in order to distinguish it from the native form, aspartate carbamoyltransferase-N.

Dissociation of aspartate carbamoyltransferase

After elution through Sepharose 6B, the aspartate carbamoyltransferase-N peak (molecular weight approx. $6.5 \cdot 10^5$) was pooled, and after concentration

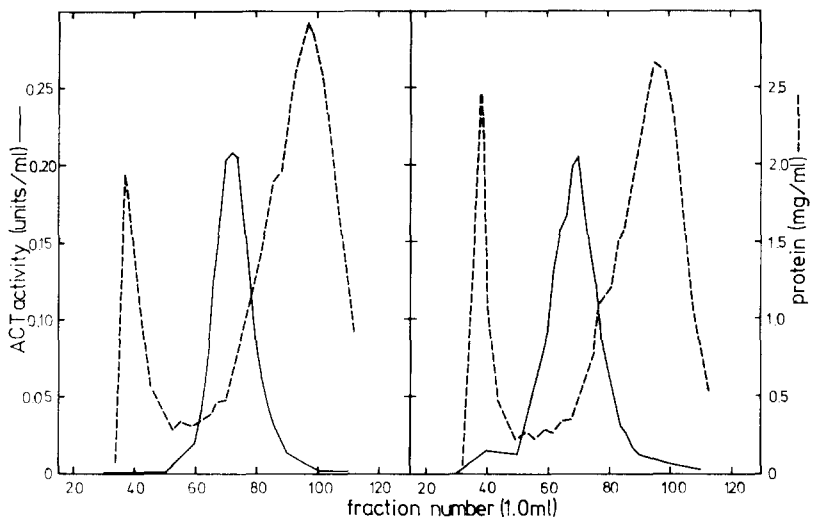


Fig. 3. Elution profiles before and after loss of pyrimidine-specific carbamoyl-phosphate synthase (CPSpyr) activity. (a) Elution profile of a crude extract (0.041 units aspartate carbamoyltransferase and 0.0016 units pyrimidine-specific carbamoyl-phosphate synthase/mg protein) on a Sepharose 6B column (70 × 1.5 cm) on the same day as extraction. (b) Elution profile of the crude extract from (a) (now 0.061 units aspartate carbamoyltransferase and 0.0003 units pyrimidine-specific carbamoyl-phosphate synthase/mg protein) on the same column after 2 days at 0—5°C. (———) aspartate carbamoyltransferase activity, (- - - - -) protein concentration.

by $(NH_4)_2SO_4$ precipitation and resuspension, was re-applied to Sepharose 6B (75 × 1.5 cm). In this elution, there were also two aspartate carbamoyltranferase peaks, one corresponding to aspartate carbamoyltransferase-H, and a new one, retarded to a much greater extent than aspartate carbamoyltransferase-N. This form of the enzyme, significantly smaller than aspartate carbamoyltransferase-N, will be referred to as aspartate carbamoyltransferase-L. Both aspartate carbamoyltransferase-H and aspartate carbamoyltransferase-L were pooled separately, as shown in Fig. 4a, and, after being concentrated, were re-applied to the column. The re-run of aspartate carbamoyltransferase-H (pool A) showed formation of more aspartate carbamoyltransferase-L (Fig. 4b), while the re-run of aspartate carbamoyltransferase-L showed no further change (pool B, Fig. 4c). These observations were reproducible.

The generation of aspartate carbamoyltransferase-L or aspartate carbamoyltransferase-H from aspartate carbamoyltransferase-N occurred whether or not the 'stabilisers' UTP, L-glutamine, and dithiothreitol were present. It also occurred with extracts from both fresh and lyophilised mycelia. Although the relative quantities of each kind of aspartate carbamoyltransferase varied with each experiment, aspartate carbamoyltransferase-N could be converted to aspartate carbamoyltransferase-H, but not vice-versa. Both aspartate carbamoyltransferase-H and aspartate carbamoyltransferase-N gave rise to aspartate carbamoyltransferase-L, but these conversions also appeared to be irreversible. In fact, a number of treatments were employed in order to effect this latter conversion. Several samples of aspartate carbamoyltransferase-L were incubated in the presence of L-aspartate, ATP, EDTA, or high ionic strength (250 mM phosphate

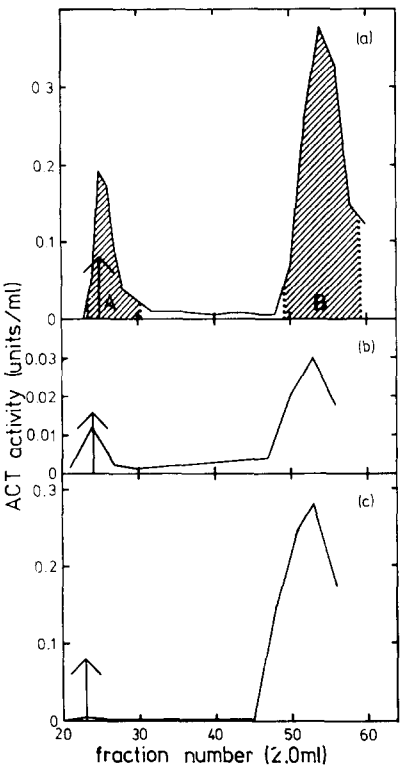


Fig. 4. Generation of two new forms of aspartate carbamoyltransferase (ACT). (a) aspartate carbamoyltransferase elution profile of an extract from a prior elution through Sepharose 6B on a Sepharose 6B column (75 × 1.5 cm). Pools A and B, as indicated, were retained. (b) aspartate carbamoyltransferase elution profile of pool A (aspartate carbamoyltransferase-H) on the same column. (c) aspartate carbamoyltransferase elution profile of pool B (aspartate carbamoyltransferase-L) on the same column. (The broad arrow in each case indicates the position of the first protein peak, the void volumer marker.)

buffer), but subsequent elution through Sepharose 6B revealed no change in the aspartate carbamoyltransferase-L.

So far the only condition which led to dissociation of aspartate carbamoyltransferase-N was elution through Sepharose 6B, suggesting that a molecular weight change might result from the removal of some factor from the crude aspartate carbamoyltransferase preparation. Further investigations were carried out, replacing Sepharose 6B by Sephadex G150 in some steps, as the latter voids both aspartate carbamoyltransferase-H and aspartate carbamoyltransferase-N, while well-retarding aspartate carbamoyltransferase-L. The first protein peak acts as a void volume marker.

An $(NH_4)_2SO_4$-fractionated extract was applied to a Sepharose 6B column (80 × 1.5 cm), and the resulting aspartate carbamoyltransferase-N peak was divided into three pools, A, B, and C as shown in Fig. 5a. Each pool was concentrated after storage for 7 days at 0—5° C, and applied in turn to a Sephadex G150 column (20 × 1.5 cm). A mixture of the three pools was run also. These four runs are shown in Fig. 5 b—e, and, of them, only pool A shows generation of aspartate carbamoyltransferase-L. This strongly suggests that elution through Sepharose 6B separates from aspartate carbamoyltransferase-N a stabiliser,

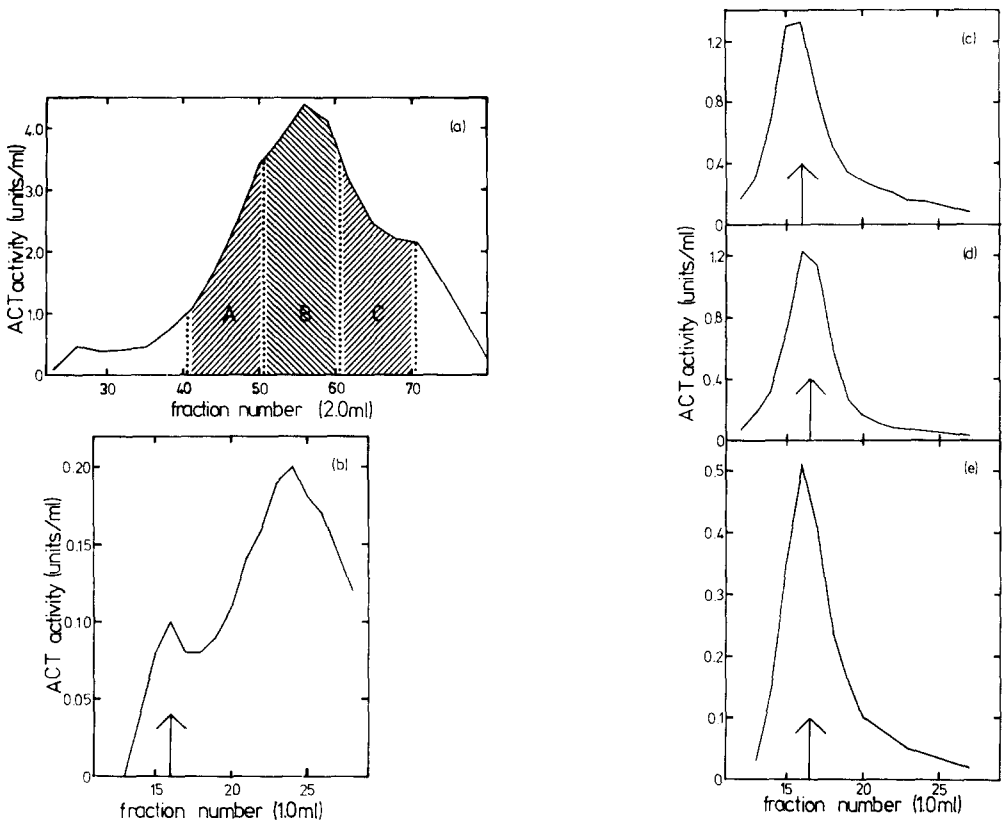


Fig. 5. The effect of gel filtration through Sepharose 6B on the rate of dissociation of aspartate carbamoyltransferase (ACT). (a) aspartate carbamoyltransferase elution profile of an ammonium sulphate-fractionated extract on a Sepharose 6B column (80 X 1.5 cm). Pools A, B, and C, as indicated, were retained. (b) aspartate carbamoyltransferase elution profile of pool A, after storage at 0—5°C for 7 days, on a Sephadex G150 column (20 X 1.5 cm). (c) aspartate carbamoyltransferase elution profile of pool B obtained as in (b). (d) aspartate carbamoyltransferase elution profile of pool C obtained as in (b). (e) aspartate carbamoyltransferase elution profile of a mixture of all three pools obtained as in (b).

which slows down the rate of dissociation, and that its molecular weight is less than that of aspartate carbamoyltransferase-N. This is supported by the finding that, after a further 5 days of storage, aspartate carbamoyltransferase-L appeared in pool B, but still not in pool C.

Attempts were then made to remove the putative stabiliser from aspartate carbamoyltransferase-N by other means. $(NH_4)_2SO_4$-fractionated extracts were applied to columns of various grades of Sephadex, the void volumes were pooled and analysed for the presence of aspartate carbamoyltransferase-L. The partially-purified extract was divided into four batches, one of which was diluted with buffer as a control, while the other three were eluted through the various Sephadex grades. On all occusions, Sephadex G150, G100, and G75 removed the stabiliser from aspartate carbamoyltransferase, enabling the dissociation to occur much faster than in the control. Sephadex G50 gave inconsistent results, sometimes, but not always, causing faster dissociations; with Sephadex G25, dissociation would not occur even after it had taken place in the control. These results suggest that the stabiliser is well-retarded by Sephadex G150, G100, and

G75, but not retarded by G25. Sephadex G50 probably retards the stabiliser so that it is partially separated from the void volume. As Sephadex G25 retards molecules smaller than $5 \cdot 10^3$ daltons while for G50 the limit is $3 \cdot 10^4$ daltons, the stabiliser must be in the range $5 \cdot 10^3$ to $3 \cdot 10^4$ daltons. There may also be a small molecular weight ($<5 \cdot 10^3$) activator which accelerates the dissociation of aspartate carbamoyltransferase.

The separation of the stabiliser from aspartate carbamoyltransferase-N by Sephadex G150 was studied in more detail. Using an $(NH_4)_2SO_4$-fractionated extract, this was run on a large column of Sephadex G150 (77 × 1.5 cm). The void volume and six subsequent fractions (A—F) were collected (Fig. 6a). Samples from each fraction were mixed with an equal volume from the void volume pool and stored at 0—5°C. Aliquots of each mixture were then analysed by elution through a small Sephadex G150 column (20 × 1.5 cm). After 3—4 days storage, the fraction A mixture had some aspartate carbamoyltransferase-L, but not nearly so much as the fraction F mixture (Figs. 6b and g). Fractions

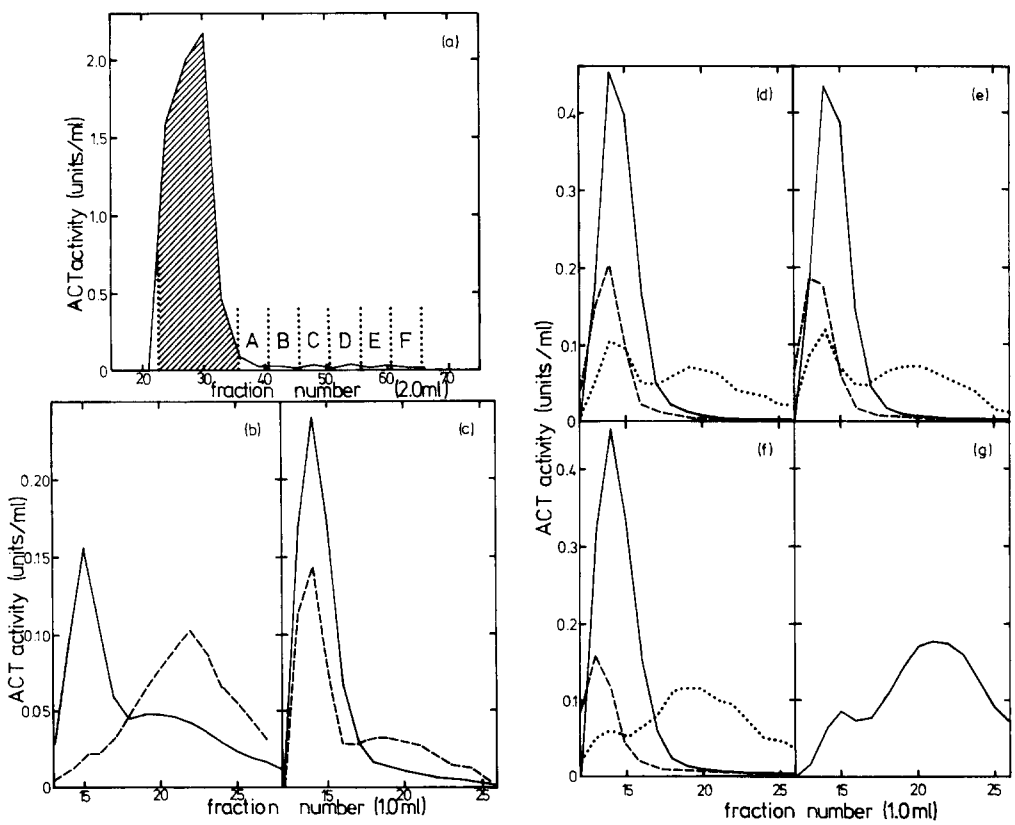


Fig. 6. Separation of the putative stabiliser from aspartate carbamoyltransferase-N by gel filtration on Sephadex G150. (a) aspartate carbamoyltransferase elution profile of an $(NH_4)_2SO_4$-fractionated extract on a Sephadex G150 column (77 × 1.5 cm). The void volume pool is indicated by the shaded area, and pools A—F by the dotted lines. (b) aspartate carbamoyltransferase elution profiles of a mixture of samples of the void volume pool and of pool A on a Sephadex G150 column (20 × 1.5 cm). (c) As with (b) except that pool A was replaced by pool B. (d) Pool A replaced by pool C. (e) Pool A replaced by pool D. (f) Pool A replaced by pool E. (g) Pool A replaced by pool F. (———) 3—4 days storage at 0—5°C, (- - - - -) 7—8 days, (· · · · ·) 14 days. ACT, aspartate carbamoyltransferase.

B—E led to no dissociation of aspartate carbamoyltransferase (Figs. 6c—f). After 7—8 days, aspartate carbamoyltransferase-L had increased in fraction A, was present in B, but still absent from C, D, and E. After 14 days, some aspartate carbamoyltransferase-L was present in fractions C and D, considerably more than that in fraction E.

These data strongly suggest that the stabiliser is eluted mainly in fractions B—E, with highest concentrations in C and D. Its peak is at a position similar to that shown by cytochrome *c*. Therefore, the molecular weight of the stabiliser is approx. $1.2 \cdot 10^4$, consistent with the limits estimated from the earlier experiment. The very rapid dissociation of aspartate carbamoyltransferase observed when mixed with the very low molecular weight fraction (F) is consistent with the earlier finding that elution on Sephadex G25 gave a slower dissociation of aspartate carbamoyltransferase than did the control. This again is suggestive of a very small activator, possibly an ion.

One possible explanation for the dissociation of aspartate carbamoyltransferase and for the function of the stabiliser was thought to be that of an equilibrium between an aspartate carbamoyltransferase-stabiliser complex and the two free molecules. The free aspartate carbamoyltransferase-N molecules might then dissociate. Re-association might be achieved by adding more free stabiliser to the dissociated aspartate carbamoyltransferase-N components. This attempted re-association was carried out, but with negative results.

Other means were sought to influence the dissociation of aspartate carbamoyltransferase. The effect of pH was investigated first. It was found that by raising the pH to 8.1, the dissociation of aspartate carbamoyltransferase was prevented, or at least greatly reduced, while acceleration of the dissociation could be achieved by lowering the pH to 6.1, albeit with some loss of activity.

The effect of pre-heating was then examined. A sample of extract was eluted through Sephadex G150 (79 × 1.5 cm); the void volume peak was pooled and divided into two batches. One of these was heated to 45° C for 20 min and this treatment greatly reduced the dissociation of aspartate carbamoyltransferase. This result would not have been predicted from a model of passive, slow dissociation of aspartate carbamoyltransferase. On the contrary, if heat treatment had any effect, such a model would predict an accelerated dissociation as a result. The observed effect, showing that dissociation is a heat-labile process, suggests the possible involvement of another enzyme.

In view of the number of reports of active proteases being purified along with different enzymes, and the recent demonstration that some proteases from *Neurospora* are sensitive to phenylmethylsulphonylfluoride and *p*-chloromercuribenzoate, the effect of these inhibitors on the dissociation of aspartate carbamoyltransferase was studied. Unfortunately, *p*-chloromercuribenzoate inhibits pyrimidine-specific carbamoyl-phosphate synthase activity very strongly, and at higher concentrations also inhibits aspartate carbamoyltransferase. Furthermore, although low concentrations of *p*-chloromercuribenzoate (0.2 mM) had no noticeable effect on aspartate carbamoyltransferase activity after 1 h, this was inhibitory after overnight incubation.

Because of the low solubility of phenylmethylsulphonylfluoride in water, it was found necessary to dissolve it in 10% ethanol. This caused certain problems, as 10% ethanol inactivated pyrimidine-specific carbamoyl-phosphate synthase

and stimulated aspartate carbamoyltransferase, as shown in Table I. Table II shows the effect of ethanol and phenylmethylsulphonylfluoride on aspartate carbamoyltransferase activity, and the implication is that phenylmethylsulphonylfluoride in itself has no effect on aspartate carbamoyltransferase. Its effect on the rate of dissociation of aspartate carbamoyltransferase was tested by dialysis of two Sephadex G150-eluted batches, one against buffer, pH 6.1, in the presence of 10% ethanol, and the other against a 10% ethanol solution of 2 mM phenylmethylsulphonylfluoride in buffer, pH 6.1. Both were analysed on a Sephadex G150 column (22 × 1.5 cm), and the results are shown in Fig. 7. These two elution profiles clearly show that phenylmethylsulphonylfluoride prevents the dissociation of aspartate carbamoyltransferase.

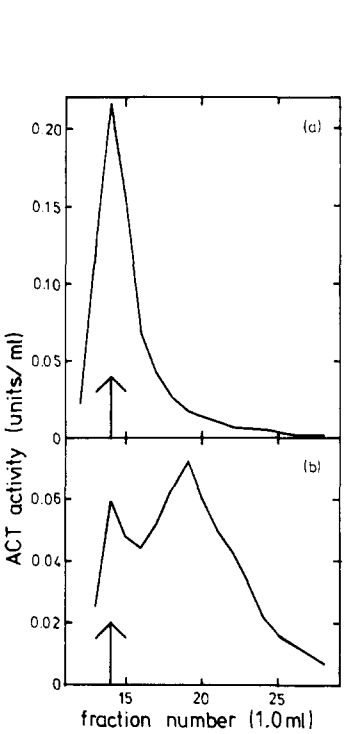
If the dissociation of aspartate carbamoyltransferase were protease-mediated, as seemed very likely, then the addition of proteases from other sources could possibly mimic the effect. Furthermore, by comparing the actions of a wide variety of proteases, it might be possible to determine whether the proteolysis was brought about by an endopeptidase or an exopeptidase. The added proteases were tested on this system under conditions in which the *Neurospora* protease was thought to be inactive (i.e. on fresh extracts, at pH 7.3, after elution through Sephadex G25). Proteases tested were carboxypeptidase A, carboxypeptidase B, chymotrypsin, elastase, imidopeptidase, thermolysin, and trypsin. They were added, at 100 μg/ml, to the extracts, incubated at 25°C for 2.5 h, and then assayed for pyrimidine-specific carbamoyl-phosphate synthase, aspartate carbamoyltransferase, and ornithine carbamoyltransferase. The results of these experiments are given in Table III. All four endopeptidases activated aspartate carbamoyltransferase while destroying pyrimidine-specific carbamoylphosphate synthase activity, whereas the three exopeptidases had no significant effect on either activity.

Samples from extracts treated with carboxypeptidase A, chymotrypsin, and elastase were compared with an untreated extract, by elution through Sephadex G150 (23 × 1.5 cm), and the results are shown in Fig. 8. These show that elastase and chymotrypsin both attacked the pyrimidine-specific carbamoylphosphate synthase · aspartate carbamoyltransferase complex, generating aspartate carbamoyltransferase-L, whereas carboxypeptidase A did not. Subsequently, it was found that thermolysin and trypsin would also induce the dissociation.

TABLE II

THE EFFECT OF PHENYLMETHYLSULPHONYLFLUORIDE IN 10% ETHANOL ON ASPARTATE CARBAMOYLTRANSFERASE ACTIVITY AFTER 1 h AT 25°C

Ethanol conc. (%)	Phenylmethylsulphonylfluoride conc. (mM)	Aspartate carbamoyltransferase activity (%)
0	0	100
10	0	133
10	0.2	118
10	0.4	143
10	1.0	137
10	2.0	128



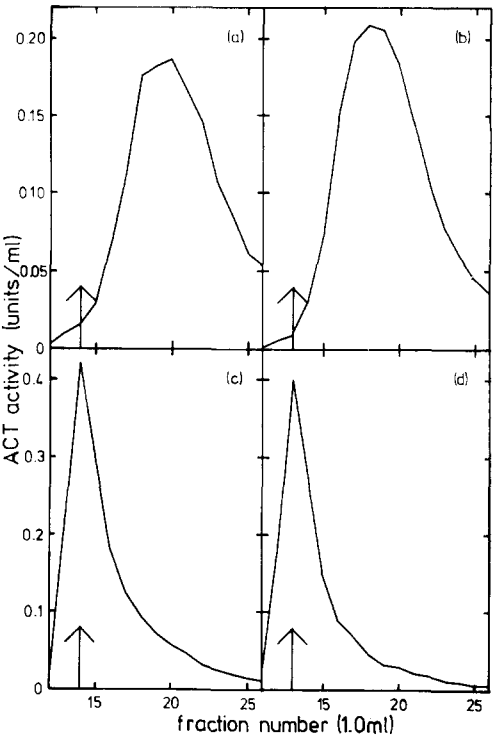


Fig. 7. Effect of phenylmethylsulphonylfluoride on the rate of dissociation of aspartate carbamoyltransferase (ACT). Elution profiles of extracts, after filtration through Sephadex G150: (a) in the presence of 10% ethanol and 2 mM phenylmethylsulphonylfluoride during dialysis. (b) in the presence of 10% ethanol during dialysis. The elution profiles were obtained on a Sephadex G150 column (22 X 1.5 cm).

Fig. 8. The effect of various proteases on aspartate carbamoyltransferase (ACT). Adpartate carbamoyl transferase elution profiles of desalted crude extracts after incubation with various proteases at 100 μg/ml. (a) chymotrypsin, (b) elastase, (c) carboxypeptidase A, (d) none. Elution profiles were obtained on a Sephadex G150 column (23 X 1.5 cm).

TABLE III

THE EFFECT OF VARIOUS PROTEASES ON PYRIMIDINE-SPECIFIC CARBAMOYL-PHOSPHATE SYNTHASE, ASPARTATE CARBAMOYLTRANSFERASE AND ORNITHINE CARBAMOYLTRANSFERASE ACTIVITIES

Protease (μg/ml)	Pyrimidine-specific carbamoyl-phosphate synthase activity (%)	Aspartate carbamoyltransferase activity (%)	Ornithine carbamoyltransferase activity (%)
None	100	100	100
Carboxypeptidase A	79	94	90
Carboxypeptidase B	74	92	97
Chymotrypsin	0	141	72
Elastase	0	147	—
Imidopeptidase	74	99	93
Thermolysin	0	143	83
Trypsin	0	140	23

Discussion

The results show that both aspartate carbamoyltransferase-N and aspartate carbamoyltransferase-H are capable of undergoing apparently irreversible dissociation to a low molecular weight form, aspartate carbamoyltransferase-L. Possibly aspartate carbamoyltransferase-N does not directly dissociate to form aspartate carbamoyltransferase-L, but is converted indirectly via aspartate carbamoyltransferase-H.

The investigation into the dissociation of aspartate carbamoyltransferase showed that its rate could be increased by the removal of a molecule of approx. $1.2 \cdot 10^4$ daltons, or by lowering the pH. The rate of dissociation could be decreased by replacing the $1.2 \cdot 10^4$-dalton molecule, raising the pH, heating to 45°C, or by adding the serine protease inhibitor, phenylmethylsulphonylfluoride. Dissociation could be induced, in the presence of the physiological inhibitor, by the addition of chymotrypsin, elastase, thermolysin, or trypsin, but not by carboxypeptidase A, carboxypeptidase B, or imidopeptidase. This evidence points to proteolysis being responsible for the dissociation of aspartate carbamoyltransferase. Of the proteases isolated by Siepen et al. [6], this protease appears to be the same as their phenylmethylsulphonylfluoride-sensitive endopeptidase in view of the large number of properties in common: heat-lability, inhibition by phenylmethylsulphonylfluoride, molecular weight of its specific inhibitor (originally referred to as the stabiliser of aspartate carbamoyltransferase-N), and by the ability to mimic its effect on aspartate carbamoyltransferase by purified endopeptidases, but not exopeptidases, from other sources. Furthermore, the protease of Siepen's group had a pH-dependence such that activity was higher at pH 6—7 than at pH 7—8. This is consistent with the observations made on aspartate carbamoyltransferase dissociation.

The pH-dependence can explain an initially puzzling observation, that of the low molecular weight material which appeared to activate the dissociation of aspartate carbamoyltransferase. The buffering system of *Neurospora* is such that crude extracts in phosphate buffer at 50 mM and pH 7.3 frequently have a final pH lower than this, usually about pH 6.8. Passage of such crude extracts through any Sephadex column at pH 7.3 inevitably leads to a low molecular weight peak of ions corresponding to this lower pH. As lowering the pH has been shown to activate the protease, this ionic peak would therefore exhibit the properties of an activator of the protease.

The results indicate the existence of a protease-inhibitor association-dissociation equilibrium. Passage through Sephadex G150 would separate any free inhibitor from the complex, yielding more free protease by mass action. As the molecular weight of the complex is $3.5 \cdot 10^4$, and of the free protease is $2.4 \cdot 10^4$, it might have been expected that gel filtration would separate such small molecules from aspartate carbamoyltransferase-N, with its molecular weight of $6.5 \cdot 10^5$. Yu et al. [4] found most of the protease activity eluting from Sephadex G200 ahead of tryptophan synthase ($1.5 \cdot 10^5$). A possible explanation for these findings is that the protease forms complexes with its substrates when not in association with its inhibitor. The overall effect of gel filtration, or indeed other purification procedures, would therefore be to increase the concentration of free protease.

Loss of pyrimidine-specific carbamoyl-phosphate synthase activity, whether brought about by incubation at cold-room temperatures, by the action of ethanol, or the sulphydryl inhibitor *p*-chloromercuribenzoate, is always accompanied by a rise in aspartate carbamoyltransferase activity. This is true also of pyrimidine-specific carbamoyl-phosphate synthase inactivation by the proteolytic action of chymotrypsin, elastase, thermolysin, or trypsin. Two molecular weight changes take place in aspartate carbamoyltransferase following loss of pyrimidine-specific carbamoyl-phosphate synthase activity, but neither change parallels the loss. Despite almost complete loss of pyrimidine-specific carbamoyl-phosphate synthase activity, and a 50% increase in aspartate carbamoyltransferase activity, the aspartate carbamoyltransferase is almost all in the aspartate carbamoyltransferase-N form. It, therefore, appears that the loss of pyrimidine-specific carbamoyl-phosphate synthase activity is one consequence of a conformational change, of which the other is activation of aspartate carbamoyltransferase. In the light of the appearance of a very small peak of aspartate carbamoyltransferase-H after pyrimidine-specific carbamoyl-phosphate synthase inactivation (Fig. 3a) and subsequent observations, it seems that this conformational change is a necessary pre-requisite for the molecular weight changes which follow.

In view of the established role of a protease in dissociating aspartate carbamoyltransferase, the possibility that this conformational change is also protease-mediated must be considered. Table III shows that this change can be brought about by the action of endopeptidases, so clearly pyrimidine-specific carbamoyl-phosphate synthase in a native conformation is susceptible to attack by proteases. Williams and Davis [19] reported lability of pyrimidine-specific carbamoyl-phosphate synthase at 0 and 25°C. Cold-inactivation could be considerably retarded by the feedback-inhibitor UTP, while L-glutamine protected pyrimidine-specific carbamoyl-phosphate synthase against moderate temperature-inactivation. Our failure to observe these stabilisations could be due to the presence of higher levels of active protease or the release or activation of different proteases. However, the stabilisation observed with UTP and L-glutamine can equally well be explained by an inherent instability of the molecule. That it is unstable is evident from its inactivation by ethanol and *p*-chloromercuribenzoate, both of which are unlikely to involve a protease.

The appearance of a peak of aspartate carbamoyltransferase-H ($>2 \cdot 10^6$ daltons) after the loss of pyrimidine-specific carbamoyl-phosphate synthase activity has already been mentioned. Aspartate carbamoyltransferase-H frequently occurs, but its amount is variable. It has been found in the presence of pyrimidine-specific carbamoyl-phosphate synthase activity, but pyrimidine-specific carbamoyl-phosphate synthase co-elutes with aspartate carbamoyltransferase-N, and not aspartate carbamoyltransferase-H. The explanation for the occurrence of aspartate carbamoyltransferase-H is not clear. In mammalian systems, lactate dehydrogenase has been shown to form aggregates which are incapable of yielding the active tetramer [23], and in *Escherichia coli*, abnormal proteins have been found aggregated together prior to proteolytic digestion [24].

From the results of the present work, it is impossible to say whether aspartate carbamoyltransferase-H is the substrate for proteolytic production of aspartate carbamoyltransferase-L, or whether this is merely an alternative route

to direct proteolysis of aspartate carbamoyltransferase-N. One possibility is that partially-inactivated pyrimidine-specific carbamoyl-phosphate synthase—aspartate carbamoyltransferase complexes tend to aggregate, and that proteolysis of such aggregates is a physiological process for freeing the cell of inactive proteins.

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