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THE INVOLVEMENT OF PROTEOLYSIS IN CONFORMATIONAL STABILITY OF THE CARBAMOYL-PHOSPHATE SYNTHETASE/ASPARTATE CARBAMOYLTRANSFERASE ENZYME OF *NEUROSPORA CRASSA*

DAVID J. RIGBY and ALAN RADFORD

Department of Genetics, The University of Leeds, Leeds LS2 9JT (U.K.)

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The *pyrimidine-3* gene of *Neurospora crassa* codes for a bifunctional enzyme catalysing the first two steps of the pyrimidine biosynthetic pathway. Difficulties have been experienced in purification due to the lability of the enzyme. The enzyme loses carbamoyl-phosphate synthetase (carbon-dioxide: ammonia ligase (ADP-forming, carbamate-phosphorylating), EC 6.3.4.16) activity and undergoes a change in apparent molecular weight from the native 650 000 to 100 000 of the only detectable fragment. Attempts have been made therefore to stabilize the enzyme so as to minimise these effects. Elastinal, a protease inhibitor, reduces the effects, as do certain ultraviolet-sensitive mutant strains which lack a minor protease. The nature of the loss of carbamoyl-phosphate synthetase suggests an instability in the tertiary structure of the enzyme which can be reduced by the use of glycerol. Glycerol also exhibits a protease-inhibiting effect in this system. Although a range of protease inhibitors and use of *uvs* mutants can reduce the rate of decay of carbamoyl-phosphate synthetase activity, only glycerol can stabilize the native molecular weight. Our results support the hypothesis that the loss of carbamoyl-phosphate synthetase activity and change in molecular weight of the enzyme is a three-step sequence of proteolysis, conformational shift and cleavage of a further non-covalent bond.

Introduction

It has been reported on numerous occasions that intracellular proteases of *Neurospora* have degraded enzymes when attempts have been made to purify them [1–4]. The proteolytic digestion can be of a limited nature, and may have two major effects on the enzyme being studied. The enzyme may lose its specific catalytic activity, and may also undergo a change in molecular weight.

Yu and Kula [5] have shown that *Neurospora* contains at least five intracellular proteases, several having specific inhibitors, suggesting a method of protease regulation at the enzyme level. The bifunctional enzyme carbamoyl-phosphate synthetase/aspartate carbamoyltransferase (carbon-dioxide: ammonia ligase (ADP-forming, carbamate-phosphorylating/carbamoylphosphate: L-aspartate carbamoyltransferase, EC 6.3.4.16/EC 2.1.3.2.) *, of *Neurospora crassa* catalyses the first two steps of the pyrimidine biosynthetic pathway, the synthesis of carbamoyl phosphate and its subsequent coupling with aspartate to form carbamoyl aspartate. The enzyme has been shown to be the target of an intracellular protease which destroys the carbamoyl-phosphate synthetase activity and produces a fragment with only the aspartate carbamoyltransferase activity. The size of the native enzyme and the aspartate carbamoyltransferase fragment are 650 000 and 100 000, respectively [3].

This paper reports the results of investigations into the instability of the 'synthetase/transferase' enzyme, with a view to minimising the above effect, and stabilizing the enzyme in order that it may be purified.

* The enzyme will hereafter be designated the "synthetase/transferase".

Materials and Methods

Neurospora crassa strains used in this investigation contained the *arg-3* (30300) mutant and/or one or other of the two ultraviolet-sensitive mutants *uvs-3* (ALS 11) and *uvs-6* (ALS 35).

Mycelium was grown overnight in a liquid medium at 25°C, in an orbital incubator. The medium contained Vogel's medium 'N' [6], 1.5% sucrose, and arginine at 100 mg/ml when required. After incubation, mycelium was harvested, rinsed, freeze-dried, and stored at −20°C prior to extraction and assay.

Mycelium was extracted in 0.05 M phosphate buffer, pH 7.3, using 1 g freeze-dried mycelium in 10 ml buffer, adding glycerol where stated at 20% (v/v). Cell debris was removed by centrifuging twice at 40 000 × *g* for 15 min at 15°C. The pellicle of lipid was removed after the first centrifugation. The extract at this stage was referred to as the 'crude extract'. For further preparation of the extract, nucleic acids were removed by addition of 1 mg protamine sulphate/ml extract. The precipitate was removed immediately by centrifugation at 40 000 × *g* for 20 min at 15°C. The sample was then concentrated by adding solid $(NH_4)_2SO_4$ to a final concentration of 40% saturation, and the precipitate pelleted immediately by centrifugation at 40 000 × *g* for 15 min at 15°C. The pellet was resuspended in a minimum amount of extraction buffer. The extract at this stage is referred to as the 'semi-crude extract', and had a specific activity of 0.401 (aspartate carbamoyltransferase) and 0.076 (carbamoyl-phosphate synthetase) compared with 0.141 and 0.014, respectively, for the crude extract.

Protein concentration was estimated by the method of Lowry et al. [7].

The carbamoyl-phosphate synthetase assay was carried out by converting carbamoyl phosphate formed by the reaction into carbamoyl aspartate via the endogenous aspartate carbamoyltransferase present in excess in the extract. The final volume of reaction mixture in each tube was 1 ml, composed of $MgCl_2$ (12 μmol)/$KHCO_3$ (30 μmol)/L-glutamine (6 μmol)/L-aspartate (10 μmol)/Tris-acetate buffer, pH 7.5 (100 μmol)/0.4 ml mycelial extract. The reaction was carried out at 25°C for 30 min. It was stopped by the addition of 1 ml of the assay colour reagent, which was composed of 0.5% antipyrene in 50% sulphuric acid and 0.8% diacetyl monoxime in 5% acetic acid in a 2 : 1 ratio [8]. The colour was developed at 75°C for 2 h, and the absorbance measured at 466 nm. The rate of reaction was constant over the 30-min reaction period.

The aspartate carbamoyltransferase assay was carried out using 0.2 ml extract in a mixture containing L-aspartic acid (10 μmol)/carbamoyl phosphate (5 μmol)/glycine-NaOH buffer, pH 9.0 (150 μmol), to a final volume of 1 ml. The reaction was allowed to proceed for 10 min at 25°C, and then stopped and the carbamoyl aspartate concentration was determined as in the carbamoyl-phosphate synthetase assay method above.

Activities of carbamoyl-phosphate synthetase and aspartate carbamoyltransferase are expressed as units/ml or units/mg protein, 1 unit = 1 μg/mol carbamoyl phosphate (for carbamoyl-phosphate synthetase) or carbamoyl aspartate (for aspartate carbamoyltransferase) synthesized/min.

Protease activity was estimated by the method of Rigby et al. [9]. The basis of the assay is the proteolytic digestion of the gelatin layer of undeveloped photographic film. Digestion of the gelatin released the silver grains contained, resulting in a progressive clearing of the film. Time taken for clearing was found to be proportional to protease activity, with dilution series of both standard commercial pure proteolytic enzymes and the proteases in mycelial extracts. Samples to be assayed (0.4 ml) were dispensed into 10 × 75 mm tubes and the temperature equilibrated to 37°C. A strip of undeveloped photographic film (2.5 × 35 mm) was placed into each tube and the time taken for complete clearing of the immersed part of the film determined. The film gelatin method of protease estimation was calibrated against trypsin, and found to be linear between enzyme concentrations of 0.5% and $5 \cdot 10^{-3}$%. Errors arose outside that range due to difficulties in precise end point determination. In this paper, the results of protease assays are left in units of time, and all such data are directly comparable.

Dithiothreitol, chymostatin, and pepstatin were obtained from Sigma Chemical Co., leupeptin and elastatinal from the Peptide Institute, Japan, and antipain was a gift from Dr. D.J. Etherington.

Results

Separation of native synthetase/transferase enzyme from proteases by gel filtration

In order to separate the proteases from the native enzyme, a gel matrix of the Sephadex type, capable of withstanding the high flow rates necessary for rapid separation, was required. For this reason, Sephacryl S-200 was chosen. Sephacryl S-200 has a separation range of 5000–250 000 daltons for globular proteins. Hence, in using this gel, the native enzyme should be eluted in the void volume while the proteases, which are of much lower molecular weight, would be retarded.

The protease activity is eluted continuously, with a profile matching that of the total protein concentration. This indicates that the proteases must be bound to their substrates, that is the cellular proteins, and are eluted along with them.

The use of strains lacking specific proteases

It has been reported by Fraser [10] that several strains of *Neurospora* which show increased sensitivity to ultraviolet light are deficient in certain proteases. The proteases are required for post-translational modification and activation of nucleases involved in ultraviolet repair mechanisms, the absence of these particular proteases rendering such strains ultraviolet-sensitive.

Two of the strains, *uvs-3* and *uvs-6* were investigated. The rate of change of carbamoyl-phosphate synthetase and aspartate carbamoyltransferase activities was monitored, and the protease content compared by the film gelatin method. Crude extracts of each strain were prepared and filter sterilised. At intervals samples were taken, desalted through Sephadex G-25, and assayed for carbamoyl-phosphate synthetase and aspartate carbamoyltransferase. An *arg-3* strain, normal in its ultraviolet response, was used as a control. Fig. 1 shows the results. It can be seen that the rate of change of the enzyme activities is less in the *uvs-3* strain than in *uvs-6* or wild type. It is also apparent that the increase of aspartate carbamoyltransferase activity does not parallel the decrease of carbamoyl-phosphate synthetase activity, the former continuing to increase after the latter reaches zero activity.

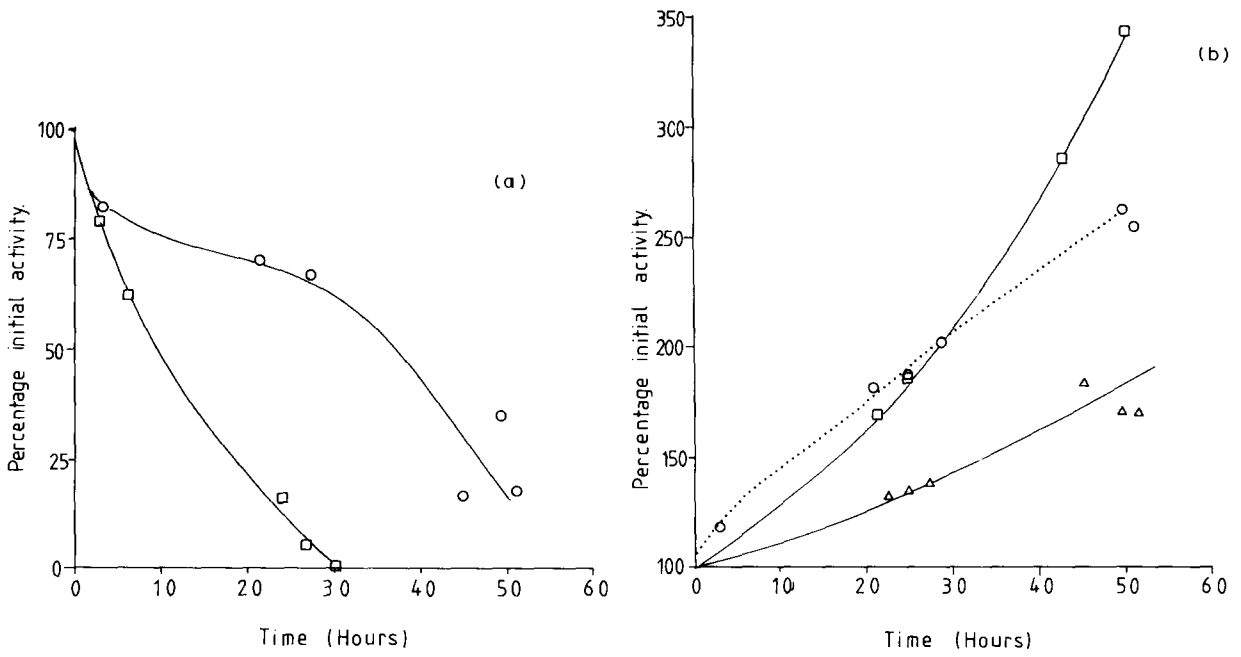

Fig. 1. Stability of carbamoyl-phosphate synthetase (a) and aspartate carbamoyltransferase (b) activities in the strains *arg-3* (□———□), *uvs-3* (△———△) and *uvs-6* (○· · · · ·○). Note that carbamoyl-phosphate synthetase decay was the same in the two *uvs* strains, so only the *uvs-6* curve is shown.

Direct assay of proteolytic activity in the three strains by the film gelatin method revealed no detectable differences between them, so the *uvs-3* mutant must only be defective in a comparatively minor protease, or inactive under the conditions of assay.

The molecular weight change of the synthetase/transferase enzyme was monitored in the following way. A semi-crude extract was passed down a Sephacryl S-200 column as above, and the position of the aspartate carbamoyltransferase peak in relation to the overall protein profile noted. After concentration of the sample by $(NH_4)_2SO_4$ precipitation, it was stored for 24 h, passed down a similar column, and any change of molecular weight identified by the appearance of a new aspartate carbamoyltransferase activity peak. Fig. 2 shows the results of such a second elution. It can be seen that the proportion of activity undergoing the molecular weight shift was, over the 24 h period of the experiment, far less in the *uvs* strain than in the control.

The effect of protease inhibitors on the synthetase/transferase shift

Further attempts to prevent proteolysis and changes in carbamoyl-phosphate synthetase and aspartate carbamoyltransferase activities were made by using a range of protease inhibitors.

p-Chloromercuribenzoate was found by Makoff and Radford [3] to destroy the enzyme activity of the synthetase/transferase overnight, and so was not suitable for use in experiments over the time span of the present ones. The use of PMSF (phenylmethylsulphonyl fluoride) was also found to increase the rate of change of carbamoyl-phosphate synthetase and aspartate carbamoyltransferase activities, but this effect was due to the presence of ethanol at 10% in the solvent used for the compound. This effect could readily be overcome by the addition of 10% ethanol to the control, and with this modification, PMSF was then found to decrease the rate of change below that in the control. PMSF did not, however, block the molecular weight change. Dithiothreitol, or Cleland's reagent was reported by Williams et al. [11] to stabilize the synthetase/transferase, but later work produced contradictory results [3]. The effect of dithiothreitol may be expressed under certain conditions not considered in these earlier reports. Therefore, the effect of dithiothreitol on proteases was

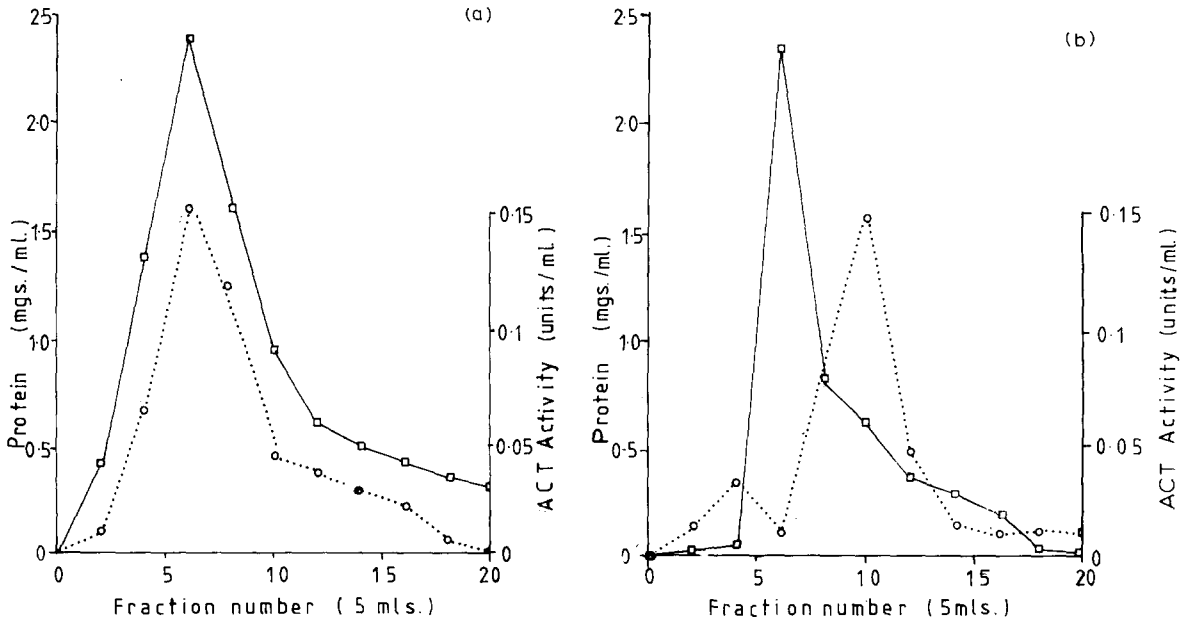

Fig. 2. Elution profile of a typical *uvs* strain (a) and the *arg-3* strain (b) semi-crude extracts after 24 h storage at room temperature, showing protein concentration (□———□) and aspartate carbamoyltransferase activity (○· · · · · ○).

investigated, and the results are summarized in Table I. As can be seen, dithiothreitol increased the proteolytic activity of a crude *Neurospora* extract by approx. 100%. When used against trypsin however, the proteolytic activity decreased. It is possible that major *Neurospora* proteases are more active in a reducing environment, with the dithiothreitol in this case rapidly re-reducing part of the active site and thus bringing about a higher rate of catalysis.

The specific protease inhibitors chymostatin, pepstatin and elastatinal were used at a concentration of 0.1 mM [12]. Chymostatin and elastatinal were soluble in water, and pepstatin was dissolved in one drop of acetic acid before being mixed with the extraction buffer [13]. From mycelium of the *arg-3* strain, four crude extracts were made, one with each inhibitor and one control. Samples from each were taken at intervals and assayed for carbamoyl-phosphate synthetase and aspartate carbamoyltransferase activities, and the results are presented in Fig. 3. It can be seen that only elastatinal decreased the rate of change of activity. Direct assay of the proteases by the film gelatin method showed no detectable difference between the experimental and control samples.

The effect of glycerol on the synthetase/transferase enzyme activities

The aspartate carbamoyltransferase activity continues to increase with time beyond the point where carbamoyl-phosphate synthetase activity has reached zero. This suggests that conformational changes are a further factor contributing to the change of specific activities with time. Attempts were therefore made to stabilize the enzyme with glycerol.

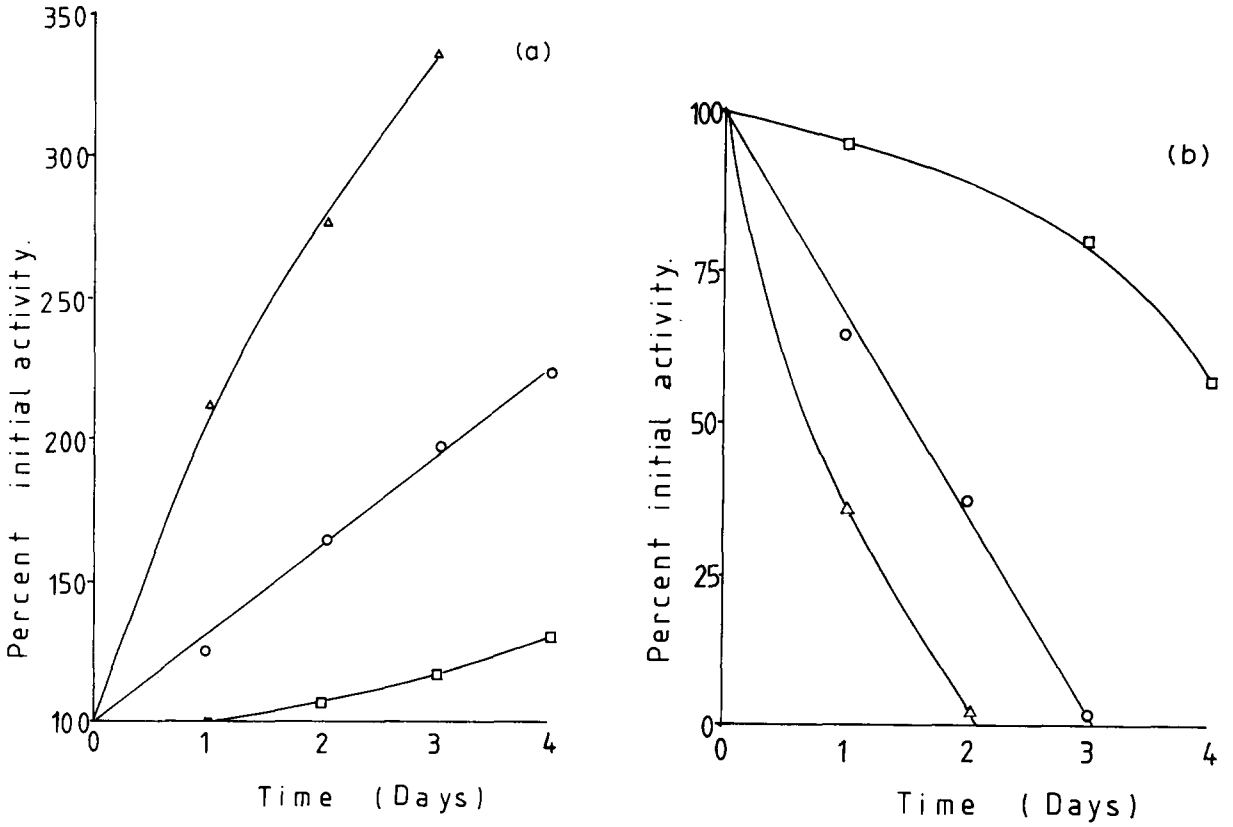

Fig. 3. Stability of aspartate carbamoyltransferase (a) and carbamoyl-phosphate synthetase (b) of an *arg-3* crude extract with addition of elastatinal (□——□), leupeptin (△——△) and control (○——○). Pepstatin was similar to the control and so has been omitted for clarity.

Extracts of the three strains, *arg-3*, *uvs-3* and *uvs-6* were made in 20% glycerol (an arbitrary concentration), and the change of the enzyme activities monitored. Fig. 4 shows the results of that experiment. The addition of 20% glycerol to the buffer clearly slows the rate of change of the two activities, and no change of molecular weight of the enzyme could be detected over the period of the experiment.

There are two possibilities as to the nature of the glycerol effect described above. The glycerol could be stabilising the synthetase/transferase enzyme by preventing a conformational shift which would reveal a proteolytic cleavage site, cleavage of which would result in the loss of the carbamoyl-phosphate synthetase activity. Alternatively, the glycerol could be acting directly on the proteases, reducing proteolytic activity. Possibly these two mechanisms may occur together. A third possibility is that glycerol may stabilize the aggregation state of the oligomer.

The nature of the glycerol effect on the enzyme

Makoff and Radford [3] found a low molecular weight inhibitor was involved in the activation and inactivation of *Neurospora* proteolytic enzymes. It is possible that the protease/inhibitor complex is being stabilized by glycerol, thus keeping the protease in an inactive form. Experiments were therefore designed to investigate these possibilities.

The low molecular weight inhibitor of the protease may be separable from the protease by gel filtration through the appropriate grade of Sepharose, i.e., G-75. However, if the complex is stabilized in the inactive form by glycerol, then gel filtration would not cause dissociation of the complex and the protease would remain in the inactive form.

Extracts of the *arg-3* strain were made, held with and without immediate filtration through Sephadex G-75 (the latter being desalted through G-75 only immediately prior to assay), with and without 20% glycerol during extraction and holding. From comparisons between the results when the time of desalting was varied, there is no evidence for glycerol stabilizing the postulated protease + inhibitor complex in the inactive form. However, all extracts with glycerol were more stable than parallel ones without, suggesting that glycerol stabilizes the synthetase/transferase tertiary structure, and that any effect of glycerol on the protease is a minor one.

The proteolytic activity was estimated, by the film gelatin method, for extracts 1 to 5 above, and also for

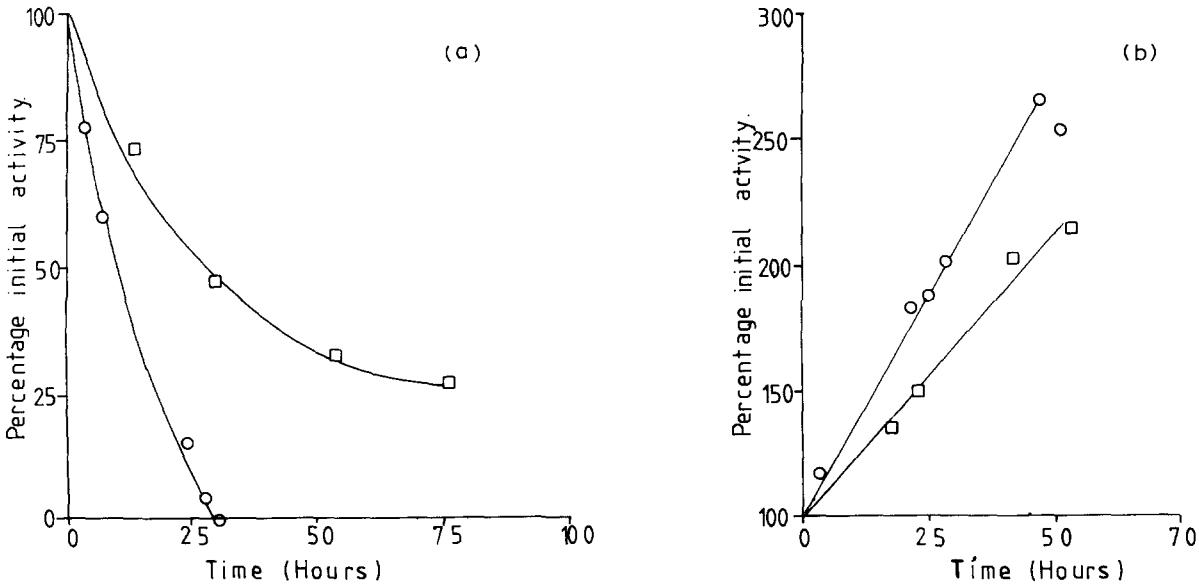

Fig. 4. Stability of carbamoyl-phosphate synthetase (a) and aspartate carbamoyltransferase (b) of a crude extract of *arg-3* in extraction buffer alone (o——o) and buffer plus 20% glycerol (□——□).

crude extracts of *uvs-3* and *uvs-6*, both with and without 20% glycerol. The results of these assays are shown in Table I, and indicate that the glycerol acts directly upon the proteases, inhibiting them although not stabilizing the protease/inhibitor complex. As a further control, purified trypsin was diluted in buffer, both with and without glycerol, to a concentration of 0.05%. This enzyme gave similar results.

The possibility of increasing the stability of the synthetase/transferase enzyme by the combined use of glycerol and protease inhibitors was also examined. Using antipain, leupeptin, chymostatin and elastatinal (0.1 M) in extracts of *arg-3* made in phosphate buffer plus 20% glycerol, the rate of change of carbamoyl-phosphate synthetase activity with time did not differ from that of the control extract in glycerol but without using specific protease inhibitors.

The relationship between enzyme stability and glycerol concentration

In the experiments so far, an arbitrary glycerol concentration of 20% has been used. Finally, the optimum concentration of glycerol for stabilization was estimated. Extracts of *Neurospora* were made in increasing concentrations of glycerol from 0 to 35%, and the carbamoyl-phosphate synthetase activity measured. The extracts were then stored at room temperature for five days, and re-assayed. No detectable stabilizing effect was found below 10–15% glycerol, increasing to a maximum at 20% and remaining at this plateau at higher concentrations.

Discussion

The most unusual observation in the results described is the simultaneous loss of carbamoyl-phosphate synthetase activity and rise in aspartate carbamoyltransferase activity, occurring at different rates. This is most easily explained on a conformational change hypothesis. If the synthetase/transferase enzyme is unstable at a position near the carbamoyl-phosphate synthetase active site, and the carbamoyl-phosphate synthetase and aspartate carbamoyltransferase active sites are also close together, this would explain the efficient channelling of carbamoyl phosphate in the pyrimidine pathway, with no interchange with the equivalent pool in the arginine biosynthetic pathway. Further support comes from the observation that carbamoyl-phosphate synthetase specific activity is much lower than that of aspartate carbamoyltransferase. A conformational change at an unstable site would destroy carbamoyl-phosphate synthetase activity, and also begin to increase the accessibility for substrates to the aspartate carbamoyltransferase active site. This process would continue for a time after the loss of carbamoyl-phosphate synthetase activity by further conformational change. This pattern of activity change of carbamoyl-phosphate synthetase and aspartate carbamoyltransferase is however stopped by both protease inhibitors and glycerol, suggesting that the process is initiated by a proteolytic cleavage. The action of glycerol in this case, therefore, may not be in stabilization but as a protease inhibitor itself. The possibility that glycerol is having this effect instead by stabilizing the oligomeric state of the synthetase/transferase enzyme does not fit the observation that the molecular weight of the enzyme can remain at the native value after the carbamoyl-phosphate synthetase activity has been lost, when glycerol has been included in the extraction buffer. This suggests instead that a further cleavage of some kind is necessary before physical breakdown of the native enzyme to give rise to the 100 000 dalton aspartate carbamoyltransferase fragment. This second cleavage, at a site revealed by the conformational change associated with loss of carbamoyl-phosphate synthetase and increase in aspartate carbamoyltransferase activities, could be either a proteolytic cleavage or the breakage of a non-covalent link, depending on whether glycerol is acting in this case as a protease inhibitor or as a conformational stabilizing agent.

From the results with PMSF, the presence of which did not prevent the molecular weight change, it seems that the final cleavage would be of the non-covalent variety, stabilized conformationally by the presence of glycerol. There is a further possibility however. If the final cleavage were proteolytic, it may be that PMSF is not inhibitory to the specific enzyme responsible. In this case, glycerol could be acting again as either a protease inhibitor, capable of inhibiting a specific enzyme which is insensitive to PMSF, or by stabilizing and protecting the potential proteolysis site such that it never becomes exposed.

On the basis of the results of experiments with PMSF, the former hypothesis is preferred, involving

the breakage of a non-covalent bond, and resulting in the release of the aspartate carbamoyltransferase fragment at 100 000 daltons.

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