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CHARACTERIZATION OF THE ASPARTATE CARBAMOYLTRANSFERASE FRAGMENT GENERATED BY PROTEASE ACTION ON THE *PYRIMIDINE–3* GENE PRODUCT OF *NEUROSPORA CRASSA*

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Summary

The molecular weight of the fragment of aspartate carbamoyltransferase (carbamoylphosphate: L-aspartate carbamoyltransferase, EC 2.1.3.2) of *Neurospora crassa* following proteolysis was found to be $1.0 \cdot 10^5$ (aspartate carbamoyltransferase-L). It differs from the native form of the enzyme (aspartate carbamoyltransferase-N, $6.5 \cdot 10^5$) in several respects. It has a lower V, has a much greater affinity (approx. 3-fold) for L-aspartate, and is strongly activated by glycine. Both forms of aspartate carbamoyltransferase have a pH optimum of approx. 9.5, and they exhibit similar affinities for carbamoyl phosphate.

Introduction

In the preceeding paper, the evidence for an endopeptidase, and its effects on the complex enzyme, pyrimidine-specific carbamoyl-phosphate synthase · aspartate carbamoyltransferase (ATP:carbamate phosphotransferase (dephosphorylating), EC 2.7.2.5 · carbamoylphosphate:L-aspartate carbamoyltransferase, EC 2.1.3.2) were presented [1]. One product of the action of endopeptidase on the initial gene product, the native pyrimidine-specific carbamoyl-phosphate synthase · aspartate carbamoyltransferase enzyme, was a fragment which had lost the pyrimidine-specific carbamoyl-phosphate synthase activity but still retained the aspartate carbamoyltransferase activity (aspartate carbamoyltransferase-L). This paper describes the determination of the molecular weight of the aspartate carbamoyltransferase-L fragment, and the establishment of its kinetic and other characteristics, and compares these with the properties of the native, $6.5 \cdot 10^5$-dalton, pyrimidine-specific carbamoyl-phosphate synthase · aspartate carbamoyltransferase enzyme.

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Materials and Methods

The strain of *Neurospora crassa* used in this work was the double mutant *arginine—3* (30300), *pyrimidine—1* (KS12p1). The culture method, method for enzyme extraction, and assay proceedures are published elsewhere [2].

The method for purifying aspartate carbamoyltransferase-L was as follows: To a crude extract was added a 20 mg/ml solution of protamine sulphate, to give a final ratio of 10 : 1 [3]. The precipitate was removed by centrifugation at 40 000 × *g* and 5°C. The supernatant was then fractionated with $(NH_4)_2SO_4$ and the 25—40% fraction retained. This fraction was redissolved and then, in rapid succession, two Sephadex G150 elutions were carried out, pooling and concentrating the void volume after each elution. The runs were carried out at pH 8.1, on an 80 × 1.5 cm column. Any aspartate carbamoyltransferase-L, together with low molecular weight contaminants, were removed by these runs. Aliquots of the void volume after two runs were then analysed at various times by elution through a small Sephadex G150 column, and after approx. 2 weeks it was apparent that a substantial amount of dissociation had occurred. (During this period, the extract was stored in 20% $(NH_4)_2SO_4$, with chloramphenicol (25 μg/ml) added to inhibit bacterial growth). A further Sephadex G150 run was then carried out, this time discarding the void volume, but retaining the large peak of aspartate carbamoyltransferase-L activity. The final specific activity of the pooled aspartate carbamoyltransferase-L peak was 9 units/mg.

The method described above was subsequently modified for the preparation of large amounts of aspartate carbamoyltransferase-L generated by elastase rather than the physiological protease. The gel filtrations were carried out on 92 × 4.5 cm columns. After the second elution, the aspartate carbamoyltransferase-N fractions were pooled, and incubated overnight with elastase (30 μg/ml). The treated extract was then fractionated between 35—70% saturated $(NH_4)_2SO_4$. The extract was then again eluted through the large Sephadex G150 column, and fractions containing aspartate carbamoyltransferase-L pooled. Comparable specific activities (approx. 10 units/mg) were obtained in this way.

The marker proteins used to calibrate the Sephadex G150 column for determination of the molecular weight of aspartate carbamoyltransferase-L were lactate dehydrogenase (Boehringer-Mannheim), cytochrome *C* (Miles-Seravac), myoglobin, catalase, malate dehydrogenase and thyroglobulin (Sigma). Catalase was assayed according to the method of Beers and Sizer [4]. Lactate dehydrogenase was assayed by measuring the change in A_{340nm} of a solution containing 50 mM phosphate buffer at pH 7.3/0.1 mg/ml NADH/3 mM pyruvate at 37°C. Malate dehydrogenase was assayed in the same way, except that pyruvate was replaced by oxaloacetate. However, oxaloacetate is unstable, and always contains some contaminating pyruvate. Because of this, lactate and malate dehydrogenases were never run together on the same column. Cytochrome *C* and myoglobin were estimated by A_{410nm} measurement. Again, these two markers were never run together on the same column. Thyroglobulin was estimated by A_{280nm} measurement, and buffer ionic strength by its conductivity.

Results

The molecular weight of aspartate carbamoyltransferase-L was estimated by gel filtration using Sephadex G150 (80 × 1.5 cm). Markers which are known to behave as globular proteins on gel filtration were used: catalase (M_r 2.5 · 10^5), lactate dehydrogenase (M_r 1.4 · 10^5), malate dehydrogenase (M_r 7 · 10^4), myoglobin (M_r 1.7 · 10^4), and cytochrome *C* (M_r 1.2 · 10^4). Because of the mutual interference of the assays of cytochrome *C* with myoglobin, and of lactate dehydrogenase with malate dehydrogenase, two elutions were performed. One comprised thyroglobulin (void volume marker), catalase, lactate dehydrogenase, myoglobin, sodium chloride, and aspartate carbamoyltransferase-L (produced by the *Neurospora* protease, and at 3 units/mt). In the second, malate dehydrogenase replaced lactate dehydrogenase, and cytochrome *C* replaced myoglobin. By means of the markers common to both elutions, the second filtration was normalised with respect to the first, and the semi-log plot of elution volume against molecular weight was derived (Fig. 1). As can be seen, the five markers are clearly on a straight line, and using this calibration, the molecular weight of the aspartate carbamoyltransferase-L appears to be 1.0 · 10^5.

This then is the molecular weight of the aspartate carbamoyltransferase-L as produced by the *Neurospora* protease. The molecular weight of the aspartate carbamoyltransferase-L produced by exogenous proteases was not directly estimated by the above method. However, consecutive elutions of natural and artificially produced aspartate carbamoyltransferase-L (produced by *Neurospora* protease and elastase, respectively) on a smaller Sephadex G150 column

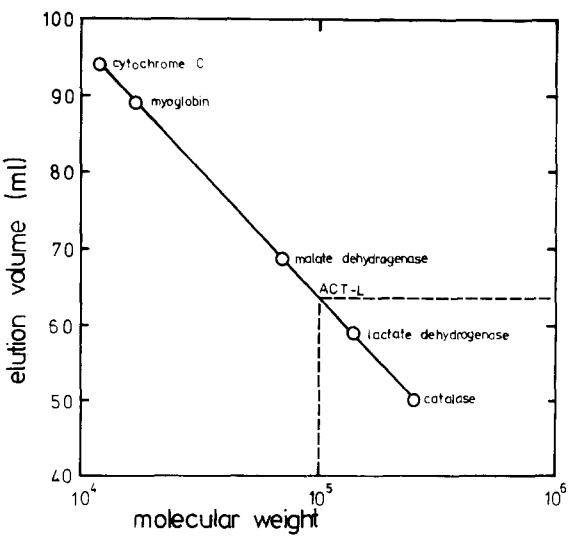

Fig. 1. Calibration of a Sephadex G150 column and determination of the molecular weight of aspartate carbamoyltransferase-L. The calibration was obtained by two consecutive elutions through a Sephadex G150 column (80 × 1.5 cm). The elutions were (i) catalase, lactate dehydrogenase, aspartate carbamoyltransferase-L, myoglobin (+ thyroglobulin and NaCl) and (ii) catalase, aspartate carbamolytransferase-L, malate dehydrogenase, cytochrome *C* (+ thyroglobulin and NaCl). ACT, aspartate carbamoyltransferase.

did not reveal any difference in elution volume. Therefore any difference must be slight (certainly within the range ±20%).

The pH-dependence of the various forms of aspartate carbamoyltransferase was then investigated. Aspartate carbamoyltransferase-L produced by the *Neurospora* protease, aspartate carbamoyltransferase-L produced by elastase, and aspartate carbamoyltransferase-N were directly compared, in partially purified preparations, and their pH dependence profiles are shown in Fig. 2. As can be seen, all three had a similar pH optimum, at approx. 9.5. However, glycine/NaOH buffer appeared to give rise to a greater aspartate carbamoyltransferase activity than was observed in Tris/acetate buffer at the same pH. This was probably due to activation by glycine, first described by Donachie [5]. This effect was clearly much more marked with aspartate carbamoyltransferase-L prepared by either method than it was with aspartate carbamoyltransferase-N. This provided more evidence for the very close similarity, if not identity, between the natural aspartate carbamoyltransferase-L and that produced by elastase digestion. These experiments also demonstrated the higher activity of elastase-produced aspartate carbamoyltransferase-L over aspartate carbamoyltransferase-N, activation which had previously been observed for the natural aspartate carbamoyltransferase-L [1]. On some occasions, the dissociation did lead to a lowering of aspartate carbamoyltransferase activity, but this only occurred when the dissociation was slow. This apparent contradiction is presumbly due to slow inactivation of aspartate carbamoyltransferase-L under the storage conditions used.

The activation normally caused by dissociation was then examined further.

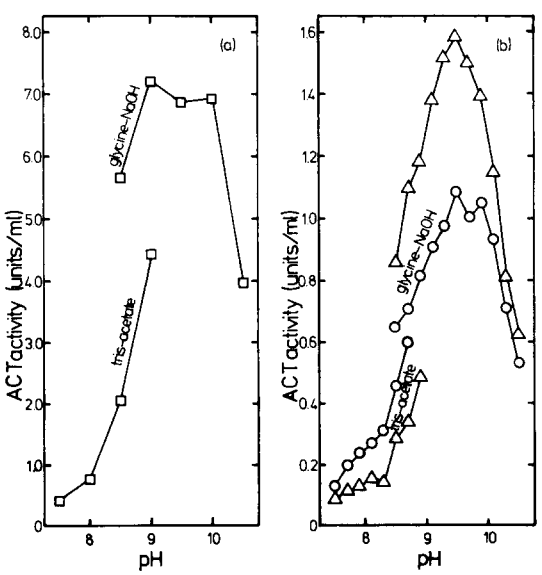

Fig. 2. Comparison of pH profiles of aspartate carbamoyltransferase-N and aspartate carbamoyltransferase-L prepared by the action of elastase and of *Neurospora* protease. (a) pH profile of aspartate carbamoyltransferase-L produced by the action of *Neurospora* protease. (b) pH profiles of aspartate carbamoyltransferase-N and of aspartate carbamoyltransferase-L produced by elastase action. ○ aspartate carbamoyltransferase-N, △ aspartate carbamoyltransferase-L from elastase, □ aspartate carbamoyltransferase-L from *Neurospora* protease. ACT, aspartate carbamoyltransferase.

The substrate-dependence curves of aspartate carbamoyltransferase activity were determined for both L-aspartate and carbamoyl phosphate before and after elastase treatment. The extracts used were prepared in the same way as for the determination of pH-dependence (protamine sulphate precipitation, $(NH_4)_2SO_4$ fractionation, and de-salting on Sephadex G25). Preliminary experiments had shown that aspartate carbamoyltransferase-N is still unsaturated at 30 mM L-aspartate under the experimental conditions. Furthermore, above 3 mM carbamoyl phosphate, substrate inhibition was observed. Because of these findings, the determinations were carried out at 50 mM L-aspartate while the effect of carbamoyl phosphate was investigated, and at 3 mM carbamoyl phosphate while that of L-aspartate was studied. The experiments were carried out at pH 9.5, the optimum for both aspartate carbamoyltransferase-L and aspartate carbamoyltransferase-N. Figs. 3 and 4 show the two plots. The V was higher for aspartate carbamoyltransferase-N than aspartate carbamoyltransferase-L, but the affinity for L-aspartate was approximately 3-fold higher with aspartate carbamoyltransferase-L than aspartate carbamoyltransferase-N. The affinity for carbamoyl phosphate was approx. the same for both forms of aspartate carbamoyltransferase. The K_m values for the two substrates were not determined because of the large deviations from linearity of the Lineweaver-Burk double-reciprocal plots.

During other purification procedures attempted for aspartate carbamoyltransferase-L, it was discovered that aspartate carbamoyltransferase-L in 50 mM phosphate buffer does not bind DEAE-cellulose or DEAE-Sephadex A50, whereas aspartate carbamoyltransferase-N binds to both. Unlike aspartate carbamoyltransferase-N, aspartate carbamoyltransferase-L is also incapable of

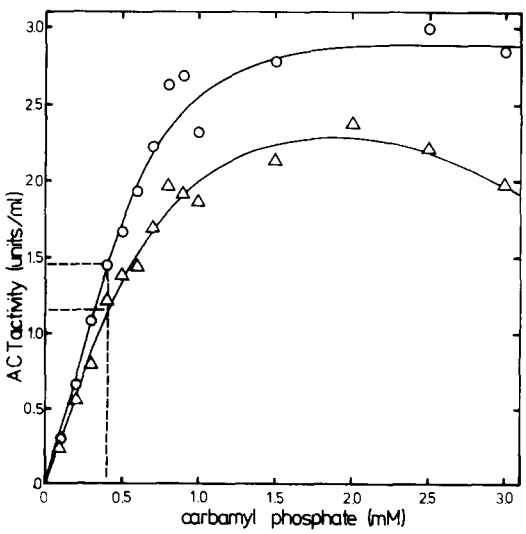

Fig. 3. Carbamoyl phosphate dependence of aspartate carbamoyltransferase-N and aspartate carbamoyltransferase-L. Symbols as for Fig. 2.

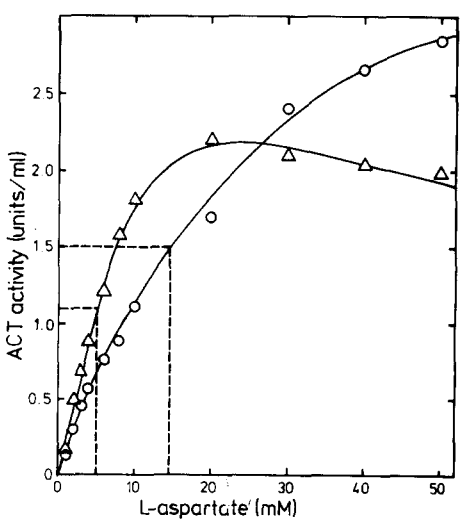

Fig. 4. L-aspartate dependence of aspartate carbamoyltransferase-N and aspartate carbamoyltransferase-L. Symbols as for Fig. 2.

being adsorbed by calcium phosphate gel. Aspartate carbamoyltransferase-L appears, therefore, to differ from aspartate carbamoyltransferase-N in at least six properties, namely molecular weight, activation by glycine, V, affinity for L-aspartate, binding to DEAE-carbohydrate matrices, and adsorption by calcium phosphate gel.

Discussion

Comparison of the pH profiles of aspartate carbamoyltransferase-L (prepared by either method) and aspartate carbamoyltransferase-N showed similar optima. One striking difference, however, is the enormous activation, presumably brought about by glycine, which was observed for aspartate carbamoyltransferase-L prepared by either method. Aspartate carbamoyltransferase-N underwent a much smaller activation (Fig. 2). When investigating the properties of aspartate carbamoyltransferase, Donachie [5] published an L-aspartate-dependence curve for aspartate carbamoyltransferase very similar to that shown by aspartate carbamoyltransferase-L. There are, therefore, two properties which the aspartate carbamoyltransferase studied by Donachie has in common with aspartate carbamoyltransferase-L. As his procedure included de-salting on Sephadex G50, which has now been shown to remove the protease inhibitor, it is very probable that he was dealing with aspartate carbamoyltransferase-L [1]. This may explain some of the discrepancies between his data and those of other workers [6,7].

The demonstration of fragments with aspartate carbamoyltransferase activity does not distinguish between the two models for the pyrimidine-specific carbamoyl-phosphate synthase/aspartate carbamoyltransferase complex discussed in the previous paper [1]. Aspartate carbamoyltransferase-L could be subunits of aspartate carbamoyltransferase which arose as a result of proteolytic cleavage of critical residues in pyrimidine-specific carbamoyl-phosphate synthase subunits, such that the whole complex fell apart to liberate aspartate carbamoyltransferase subunits. Alternatively, it is still possible that they could be domains of aspartate carbamoyltransferase activity freed from bifunctional polypeptides in the enzyme complex by proteolytic digestion of the connecting peptide sequences. Differentiation between these two models awaits purification of the native enzyme and its dissociation in the total absence of any possible proteolysis.

References

1 Makoff, A.J. and Radford, A. (1977) Biochim. Biophys. Acta 485, 314—329
2 Makoff, A.J. and Radford, A. (1976) Mol. Gen. Genet. 149, 175—178
3 Rhodes, C., Germershausen, J. and Suskind, S.R. (1971) Methods Enzymol. 22, 80—86
4 Beers, R.F. and Sizer, I.W. (1952) J. Biol. Chem. 195, 133—140
5 Donachie, W.D. (1964) Biochim. Biophys. Acta 82, 284—292
6 Davis, R.H. (1960) Proc. Natl. Acad. Sci. U.S. 46, 667—682
7 Hill, J.M. and Woodward, V.W. (1968) Arch. Biochem. Biophys. 125, 1—12