Isolation of the aspartokinase domain of bifunctional aspartokinase I-homoserine dehydrogenase I from *E.coli* K12

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A proteolytic fragment ($M_r \sim 25\,000$) carrying only the aspartokinase activity has been purified by chromatofocusing after limited proteolysis of aspartokinase I-homoserine dehydrogenase I from *E.coli* K12. The NH_2-terminal sequence shows that it corresponds to the amino terminal peptide of the native enzyme. The results confirm a previous hypothesis about the organization of native aspartokinase I-homoserine dehydrogenase I.

Aspartokinase I-homoserine dehydrogenase I *Limited proteolysis* *Gene fusion*

1. INTRODUCTION

Aspartokinase I-homoserine dehydrogenase I (AK I-HDH I) from *E. coli* K12 is a tetrameric protein which catalyses two non-consecutive reactions in the biosynthetic pathway leading from aspartate to threonine and isoleucine [1]. The four subunits are identical [2,3] (M_r 89 000); the amino acid polypeptide sequence has been established [4]. Thus, each subunit carries the two catalytic activities which are both subject to feedback inhibition by L-threonine [5,6].

Mild proteolysis has been used to probe the structure of this complex protein [7,8], and it has been shown that a fragment of M_r 59 000 carrying only the dehydrogenase activity corresponds to the C-terminal half of the complete polypeptide, whereas an N-terminal fragment of M_r 45 000 carrying the kinase activity has been extracted from a nonsense mutant [9]. Recently, the detailed analysis of the various fragments produced upon proteolysis under very mild conditions, has shown that smaller fragments could be isolated that had retained one or the other activities, although at a lower level [10]. Here, a monomeric aspartokinase fragment of M_r 25 000 was detected which was tentatively assigned to the very N-terminal part of the subunit. We describe here the isolation of this fragment and establish its N-terminal localisation.

Abbreviations: AK, aspartokinase; HDH, homoserine dehydrogenase; AK I-HDH I, aspartokinase I-homoserine dehydrogenase I; PMSF, phenylmethanesulfonyl fluoride

2. MATERIALS AND METHODS

AK I-HDH I strain Tir8 was purified and stored as described [10]. Buffers A and P and chemicals as well as measurements of activities and gel electrophoresis conditions have also been described in [10]. The chromatofocusing gel was PBE^{TM}94 from Pharmacia. YM10 membranes were from Amicon.

2.1. *Conditions for proteolysis*

The ammonium sulfate precipitate of AK I-HDH I was centrifuged and the pellet resuspended in the appropriate volume of buffer P to yield an enzyme solution at 0.5 mg/ml which was dialyzed

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overnight against the same buffer. Proteolysis was carried out in buffer P at 27°C in the presence of 2% (w/w) pronase type VI from *Streptomyces griseus* (Sigma) as described in [8,11]. The progress of proteolysis was monitored by measuring the decrease in aspartokinase activity [7]. The reaction was terminated by addition of 2 mM PMSF to inhibit the protease. 2 mM L-threonine and 0.15 M KCl were then added to protect unproteolyzed AK I-HDH I from possible proteolysis by residual protease activity.

2.2. *Purification of AK fragment*

About 100 mg of AK I-HDH I equilibrated in buffer P were incubated for 60 min at 27°C with 2 mg of protease in a total volume of 200 ml. After the proteolysis was arrested as described above, the mixture of proteolytic fragments was filtered at 10 ml/h through a column (1 × 14 cm) filled with chromatofocusing gel (PBE^{TM}94) and the eluate was concentrated 100 times to 2.2 ml on YM10 amicon membranes. The protein concentration in the eluate was measured by the method of Bradford [12] using bovine-γ-globulin as standard.

2.3. *NH_2 terminal sequence determination*

Approx. 2 nmol of the concentrated AK fragment (based on a molecular mass of 25 kDa (see section 3) were dialyzed twice against 2 l of 100 mM ammonium bicarbonate, then against the same volume of 50 mM ammonium bicarbonate. The dialysate was lyophilized and the NH_2-terminal sequence was determined in a modified 890C Beckman sequencer. An HPLC system was used to identify the phenylthiohydantoin amino acid derivatives as in [12].

3. RESULTS AND DISCUSSION

Fig.1 shows an analysis on native polycrylamide gels of the proteolytic fragments produced upon incubation for various time of samples of native AK I-HDH I with 2% (w/w) protease. We have focused our interest on the intermediate fragments which appear as a doublet (arrow). The major bands representing respectively native AK I-HDH I (A) and the fragments H (B) and HDH_D (C) have been purified and described in detail elsewhere [7,10]. In the preparative experiment (see section 2), the mixture of fragments obtained after stopping the proteolytic reaction was analogous to that analyzed on gel 4 in fig.1. This mixture of native and proteolysed AK I-HDH I (fig.2A,3A) was poured onto the chromatofocusing column and the eluate was collected. This resulted in the total purification of the material appearing as a doublet on non-denaturing gels, which was recovered in the flow through of the column (fig.2B). All of the other fragments were retained on the column and we did not attempt to elute them with the polybuffer usually used in chromatofocusing.

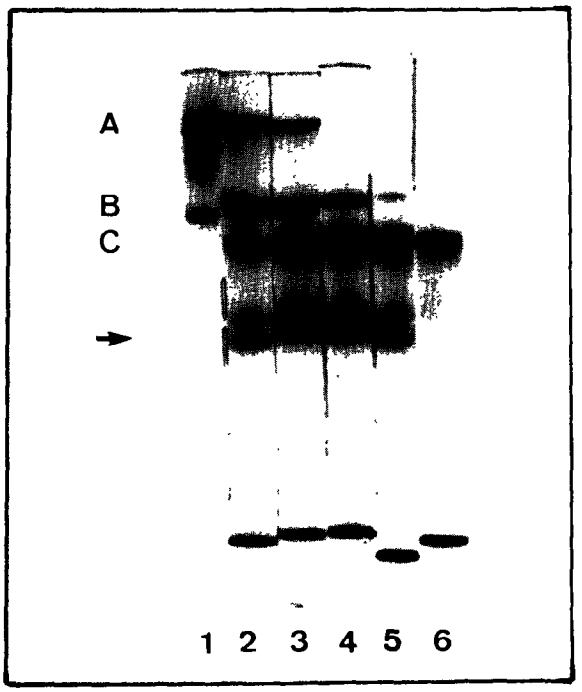

Fig.1. Kinetics of proteolysis of AK I-HDH I analyzed on native polyacrylamide gels. Proteolysis was carried out as described under section 2; aliquots were removed at various time intervals and layered on the top of tube polyacrylamide gels after stopping the proteolytic reaction. Gels 1–6, respectively, correspond to 0, 15, 30, 60, 120 and 180 min of protease treatment.

The material collected in the flow through of the column was analyzed on native and SDS gels, respectively. SDS gel electrophoresis showed only one band of M_r 25 000 with no evidence for a doublet (fig.3B). Filtration on Sephadex G100 showed that the M_r of the native fragment was in the 25 000–30 000 range (not shown), indicating that the fragment is a monomer. However, when

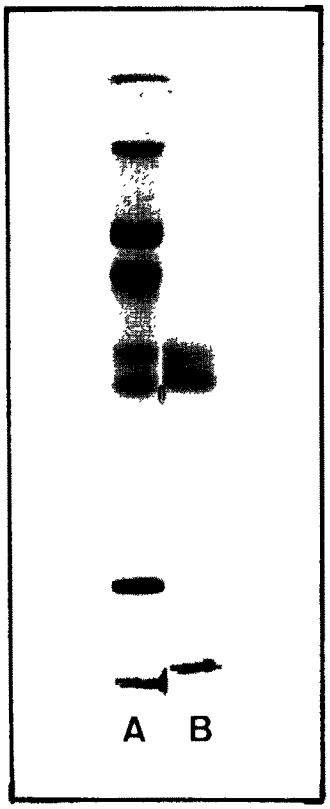

Fig.2. Native polyacrylamide gels of the mixture of proteolytic fragments and purified AK fragment. A, analysis of the mixture of proteolytic fragments before chromatofocusing. B, analysis of flow through fractions of chromatofocusing.

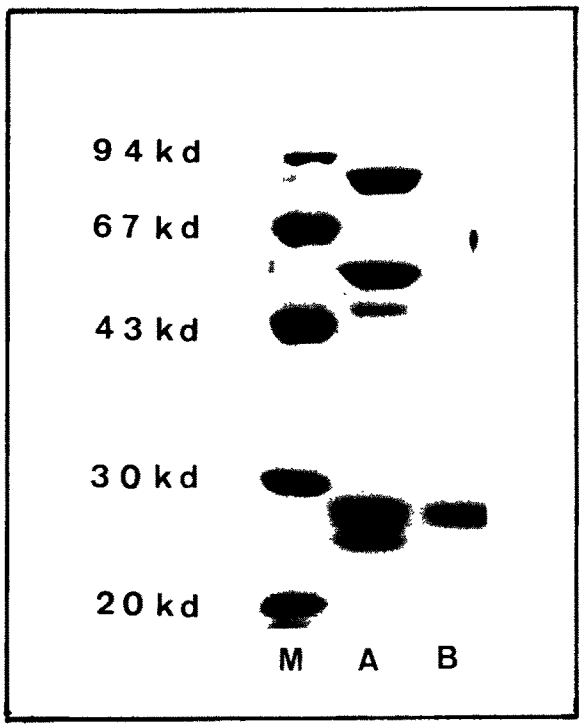

Fig.3. SDS gel electrophoresis of mixture of proteolytic fragments and purified AK fragment. M, marker proteins were phosphorylase *a*; bovine serum albumin; ovalbumin; carbonic anhydrase; Soybean trypsin inhibitor. Samples A and B are identical to those in lanes A and B in fig.2.

concentrated, the purified AK fragment has a tendency to form large aggregates.

The AK fragment is devoid of any homoserine dehydrogenase activity but carries aspartokinase activity, although at a low specific activity compared to that of the native enzyme (0.5%). This activity is totally insensitive to inhibition by 2 mM of the allosteric inhibitor of the native enzyme. Likewise, the migration of the doublet on native polyacrylamide gels was not affected if threonine was omitted in both the gel and the electrophoresis buffer, contrary to what is observed with native AK I-HDH I. These results suggest that threonine is not able to bind to the purified aspartokinase fragment.

The UV absorption spectrum of the purified fragment was recorded (not shown) and a molecular extinction coefficient of the AK fragment at 280 nm of 0.51 cm^2/mg was calculated.

The amino-terminal sequence of the AK fragment purified by chromatofocusing as above was analyzed in an automatic peptide sequencer as described in section 2. Inspection of the HPLC profile showing the phenylthiohydantoin amino acid derivatives formed allowed the identification of the sequence Met_1-Arg-Val-Leu-Lys-Phe-Gly-Gly-Thr... Other amino acid derivatives were also detected at each run of Edman degradation showing the presence of contaminants in the sample subjected to the sequence procedure. However, each of these contaminants, probably reflecting the presence of minor proteolytic products also eluted in the flow through of the chromatofocusing column, was too dilute to be detected on polyacrylamide gels. The sequence determined above is identical to the NH_2-terminal sequence of

the complete polypeptide chain of AK I-HDH I [4].

The reason why the AK fragment migrates as a doublet on native polyacrylamide gels is not clear. It is possible that the proteolysis can occur at two sites close to one another producing fragments with different C-terminal ends leading to different net electric charges under the conditions of non-denaturing electrophoresis, but indistinguishable upon SDS-electrophoresis. The complete amino acid sequence of AK I-HDHI being known [4], the exact number of residues corresponding to a given fragment of known molecular mass is easy to calculate. Thus, an N-terminal fragment of M_r 24969 would have a sequence corresponding to residues 1–235. If one assume that SDS gels would not discriminate between two polypeptides differing in M_r by less than 1000, the AK fragment that we analyse could have any size between 230 and 245 residues. The presence of several charged amino acids in the sequence comprised between residues 230 and 245 is in agreement with the above hypothesis for the presence of a doublet on non-denaturing gels. Alternatively, another possibility could be that some of the asparagine and/or glutamine residues could be deamidated.

In conclusion, we have directly demonstrated that the fragment carrying the aspartokinase activity is located at the NH_2 extremity of the total polypeptide, in agreement with the previously proposed model for the tetrameric molecule of AK I-HDH I [10,13].

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