Escherichia coli L-aspartate-α-decarboxylase: preprotein processing and observation of reaction intermediates by electrospray mass spectrometry

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The *Escherichia coli panD* gene, encoding L-aspartate-α-decarboxylase, was cloned by PCR, and shown to complement a *panD* mutant defective in β-alanine biosynthesis. Aspartate decarboxylase is a pyruvoyl-dependent enzyme, and is synthesized initially as an inactive proenzyme (the π-protein), which is proteolytically cleaved at a specific X–Ser bond to produce a β-subunit with XOH at its C-terminus and an α-subunit with a pyruvoyl group at its N-terminus, derived from the serine. The recombinant enzyme, as purified, is a tetramer, and comprises principally the unprocessed π-subunit (of 13.8 kDa), with a small proportion of the α- and β-subunits (11 kDa and 2.8 kDa respectively). Incubation of the purified enzyme at elevated temperatures for several hours results in further processing. Using fluorescein thiosemicarbazide, the completely processed enzyme was shown to contain three pyruvoyl groups per tetrameric enzyme. The presence of unchanged serine at the N-terminus of some of the α-subunits was confirmed by electrospray mass spectrometry (ESMS) and N-terminal amino acid sequencing. A novel HPLC assay for aspartate decarboxylase was established and used to determine the K_m and k_{cat} for L-aspartate as $151 \pm 16\ \mu$M and $0.57\ s^{-1}$ respectively. ESMS was also used to observe substrate and product adducts trapped on the pyruvoyl group by sodium cyanoborohydride treatment.

INTRODUCTION

Pantothenate is the essential precursor for phosphopantotheine, the acyl carrier found in CoA and acyl carrier protein. Pantothenate is the product of the condensation reaction between D-pantoate and β-alanine (3-aminopropionic acid) (see Figure 1 in [1]). The source of β-alanine depends on the organism. In plants and fungi it is derived from the degradation of uracil (see references in [2]) and although a similar pathway was implicated to be present in bacteria [3], later studies indicate that this may not be the case [4]. Instead, the major route of β-alanine production for pantothenate in bacteria is by the α-decarboxylation of L-aspartate, catalysed by the enzyme L-aspartate-α-decarboxylase (EC 4.1.1.15). This enzyme is unusual in that it contains a covalently bound pyruvoyl group involved in catalysis [5]. It is thus a member of a small group of mechanistically related enzymes that includes *S*-adenosylmethionine decarboxylase (involved in spermine and spermidine biosynthesis), phosphatidylserine decarboxylase (involved in membrane formation), proline reductase (involved in the use of amino acids for anaerobic respiration), and the bacterial histidine decarboxylase (EC 4.1.1.22), which forms histamine (for reviews see [6,7]). The most extensively studied is histidine decarboxylase from *Lactobacillus* 30a, for which there is high resolution crystal structure [7a]. For most if not all of these enzymes, the protein is initially translated as an inactive proenzyme (designated the π-protein), which is proteolytically cleaved at a specific X–Ser bond (Gly^{24}–Ser^{25} for aspartate decarboxylase) to produce a β-subunit with XOH at its C-terminus and an α-subunit with a pyruvoyl group at its N-terminus. The generation of the pyruvoyl group from the serine residue is proposed to occur via an unusual mechanism that is shown in path (a) of Figure 1. Despite this similarity in both protein structure and enzyme mechanism, the only sequence conservation in those pyruvoyl-dependent enzymes whose genes have been sequenced is the serine at the cleavage site.

In conjunction with the biochemical work, genetic studies enabled the isolation of *Escherichia coli panD* mutants [4,8] that grow only in the presence of exogenously added β-alanine or pantothenate and not in the presence of exogenously added L-aspartate, and are defective in L-aspartate-α-decarboxylase [4]. The *panD* gene was mapped at 3.1 min on the *E. coli* chromosome, closely linked to the *panB* and *panC* genes that encode other pantothenate biosynthesis enzymes [4]. The gene for *panD* was isolated and sequenced in [9] and found to encode a protein of 13834 Da. The sequence of *panD*, as part of the region containing *panB* and *panC*, was subsequently deposited in the GenBank database [W. K. Merkel and B. P. Nichols (1993) EMBL sequence database accession number L17086].

In this paper we describe the amplification of the *E. coli panD* gene using PCR, overexpression of the gene, and purification to homogeneity of large quantities of recombinant aspartate decarboxylase. This has been used to investigate a number of its properties, including the processing of the π-subunit to the active enzyme. Evidence has been obtained for the two routes shown in Figure 1.

MATERIALS AND METHODS

All reagents were of the highest available grade and, unless otherwise indicated, were purchased from the Sigma Chemical Co. (Poole, Dorset, U.K.).

General methods

The molecular biology protocols, unless otherwise stated, were as described in Sambrook et al. [10]. Oligonucleotide synthesis, DNA sequencing and amino acid sequencing were carried out as

Abbreviations used: DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); DTT, dithiothreitol; ESMS, electrospray mass spectrometry; IPTG, isopropyl-β-D-thiogalactoside; PLP, pyridoxal phosphate; PNACF, Protein and Nucleic Acid Chemistry Facility.

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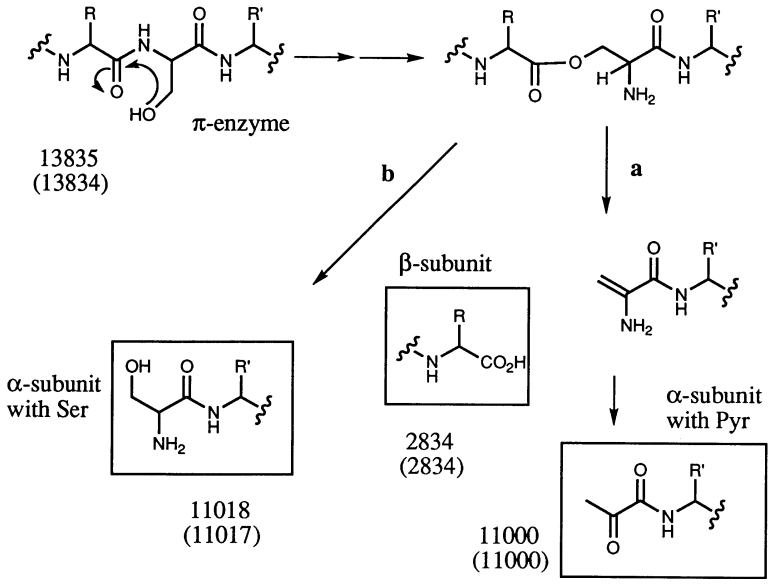

Figure 1 Proposed mechanism of processing of L-aspartate-α-decarboxylase

The proposed mechanism for the processing of the proenzyme (π-subunit) to form the α-subunit with pyruvoyl, α-subunit with serine and β-subunit. The numbers shown below the structures are the observed molecular masses in daltons determined by ESMS. The values shown in parentheses are the corresponding predicted values based on the amino acid sequence.

a service provided by the Protein and Nucleic Acid Chemistry Facility (PNACF), Cambridge Centre for Molecular Recognition, Department of Biochemistry, University of Cambridge. Computer analysis of the DNA and protein sequences were carried out using the GCG version 8.0 computing package [11].

E. coli DH5α [10] was the general host for subcloning and plasmid propagation. Functional complementation experiments and subsequent overexpression of recombinant aspartate decarboxylase were carried out in *E. coli* SJ16 (*pan*D2 *met*B1 *rel*A1 *spo*T1 *gyr*A216 *zad*220 Tn10 λ^- λ^r F^-; [8]). Selection of plasmid-containing *E. coli* strains was carried out by plating cells on media plates containing ampicillin (100 μg/ml). The QIAprep-spin columns kit (Qiagen Inc., Chatsworth, CA, U.S.A.) was used to purify plasmid DNA from *E. coli* strains. All bacterial cultures were grown in baffled culture flasks and incubated at 37 °C in a rotary incubator shaking at 150 rev./min.

Protein concentrations were calculated by the method of Lowry et al. [12] or Bradford [12a] (Bio-Rad protein assay kit), and standardized to an amino acid composition determined by the PNACF, as described in the Results section. Routine SDS/PAGE was carried out using the method of Laemmli [13] and proteins were stained with an equal mixture of Coomassie R250/G250 stain. High resolution, broad range SDS/PAGE was carried out according to the method described by Schägger and von Jagow [14], including protein visualization.

Isolation, cloning and functional complementation of the *panD* gene

The *E. coli panD* gene was cloned using the expression cassette method described by MacFerrin et al. [15]. Forward and reverse primers were designed using the *panD* sequence [W. K. Merkel and B. P. Nichols (1993) EMBL sequence database accession number L17086], and incorporated restriction sites (in bold) at the 5′ ends. The oligonucleotide sequences were CGCGC**AAGCTT**AGGAGGATTTAAAATGATTCGCACGATGCTGCAGGGC and GCGC**TCTAGA**TCAAGCAACCTGTACCGGAATCGC, with the start and stop codons underlined.

PCR amplification was carried out in reaction buffer (Bioline U.K. Ltd., London, U.K.) containing 200 μM dNTPs (Pharmacia, Milton Keynes, U.K.), 2 mM $MgCl_2$, 200 nM of each forward and reverse primer, 100 ng total *E. coli* DH5α genomic DNA and 2.5 units of *Taq* polymerase (Bioline) per 50 μl reaction. After an initial denaturation cycle (96 °C for 6 min), DNA amplification was carried out in 30 cycles of 96 °C for 2 min, 55 °C for 2 min and 72 °C for 2 min (PHC-3 thermo cycler; Techne). The PCR product was purified from an agarose gel using the USBioClean MP Kit (United States Biochemical, Cleveland, OH, U.S.A.) and subsequently cloned into the plasmid vector pBluescript KS (Stratagene, San Diego, U.S.A.) using the *Hin*dIII and *Xba*I restriction enzyme cloning sites incorporated at the ends of the primers, to form plasmid pDKS1.

E. coli SJ16 cells harbouring pDKS1 or pBluescript alone, were grown in Luria–Bertani broth in the presence of ampicillin. These cultures were subsequently diluted 1/100 in GB1 minimal medium (13.6 g/litre KH_2PO_4, 2 g/litre $(NH_4)_2SO_4$, 4 g/litre glucose, 0.25 g/litre $MgSO_4,7H_2O$, 0.25 mg/litre $FeSO_4,7H_2O$, 5 mg/litre vitamin B_1, 100 mg/litre D,L-methionine, pH 7.0), with or without β-alanine (100 μg/ml). Aliquots were removed at various time points after inoculation and growth was monitored by measuring the absorbance at 630 nm. *E. coli* K12 was used as the positive control for the complementation experiments.

Purification of recombinant aspartate decarboxylase

A standing culture of *E. coli* SJ16::pDKS1 cells was used to inoculate 500 ml of Terrific Broth [10] containing 60 μg/ml ampicillin and 80 μg/ml isopropyl-β-D-thiogalactoside (IPTG). After overnight incubation, cells were harvested by centrifugation at 4000 rev./min for 10 min. The cells were frozen in liquid nitrogen and stored at −70 °C until required.

Purification of recombinant aspartate decarboxylase was based on previously reported methods [5,9] using the following modified scheme. Frozen cells (2.5 g wet weight) were resuspended in 5 ml of extraction buffer [50 mM potassium phosphate, pH 7.0/1 mM EDTA/1 mM dithiothreitol (DTT)/1 mM PMSF] and the cells ruptured by two passages through a French press. A further 15 ml of extraction buffer was added to the sample followed by

0.5 mg of DNase I (Boehringer, Lewes, Sussex, U.K.). This was incubated at 4 °C for 30 min with gentle shaking and the sample subsequently centrifuged at 15000 rev./min (JA-20 rotor; Beckman Instruments, Beckenham, Kent, U.K.) for 30 min. The supernatant (crude extract) was recovered and loaded at 1 ml/min onto a DEAE–Sephacel column (1 cm diameter, 20 ml bed volume; Pharmacia), previously equilibrated in extraction buffer. The column was washed with two volumes of the same buffer and developed with an increasing linear gradient of KCl (0 to 0.5 M) in extraction buffer (without PMSF). Fractions (5 ml) containing aspartate decarboxylase (detected by SDS/PAGE) were pooled and the sample dialysed for 3 h against 4 litres of Buffer A (25 mM Tris/HCl, pH 7.0/1 mM EDTA). The sample was recovered and passed twice through a hydroxyapatite column (1 cm diameter, 20 ml bed volume; Bio-Rad, Richmond, CA, U.S.A.), eluting with Buffer A at 1 ml/min. The loading peak, which contained unbound aspartate decarboxylase, was collected and concentrated to approx. 5 ml by ultrafiltration (Amicon Model 8050 stirred cell equipped with a PM10 membrane).

Thiol group estimation of aspartate decarboxylase

Thiol estimation was carried out on either native aspartate decarboxylase, or enzyme denatured by boiling for 5 min in 5% (w/v) SDS, which was then diluted to 2.5% SDS. Samples of approx. 200 μg of protein in 1 ml of 50 mM potassium phosphate (pH 7.3)/1 mM EDTA (Buffer B) were adjusted to a final concentration of 5 mM DTT and incubated at 37 °C for 2 h. Separation of aspartate decarboxylase from excess DTT was carried out using two PD-10 (Pharmacia) columns connected in series and eluted with Buffer B. For each fraction the protein concentration was determined by the method of Lowry, and thiol estimation was carried out using 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) [16].

Electrospray mass spectrometry (ESMS) of aspartate decarboxylase

Aliquots (100 μl; 200 μg) of aspartate decarboxylase were dialysed extensively against four changes of 4 litres of water using 1000 Da molecular mass cut-off dialysis membrane (Spectra/Por; Medicell International Ltd., London, U.K.). The first two rounds of dialysis were carried out for 3 h each followed by an overnight dialysis and finally completed with a 3 h dialysis. The protein sample was recovered, diluted to 2 ml with water and then concentrated to 100 μl by ultrafiltration (Centricon 10; Amicon). Before ESMS the sample was diluted with one volume of acetonitrile containing 2% (v/v) formic acid and 10 to 20 μl of this was injected into the spectrometer at a flow rate of 4 μl/min.

For the substrate/product trapping experiment, 50 μl of aspartate decarboxylase (100 μg of fully processed enzyme) protein was diluted in 420 μl of 0.1 M potassium phosphate (pH 8.0). To this 17 μl of 35 mM L-aspartate in 0.1 M potassium phosphate (pH 8.0), and 12.5 μl of 40 mM sodium cyanoborohydride (in the same buffer) were added in succession. This was incubated at room temperature (22 °C) overnight. The sample was extensively dialysed, as described above, and the protein concentrated to approx. 150 μl (Centricon 10; Amicon). This was again diluted with one volume of acetonitrile containing 2% (v/v) formic acid, and 10 to 20 μl of this was injected into the machine as described above. The control enzyme sample consisted of aspartate decarboxylase incubated overnight in the absence of either L-aspartate or sodium cyanoborohydride.

Mass determination was carried out on an electrospray ionization (positive-ion mode) quadrapole mass spectrometer (BioQ; VG, Manchester, U.K.) using software supplied by the manufacturer. The mass/charge ratio (m/z) was 600–1600 and typically 30 scans were averaged to give a result ± S.D. Each determination was carried out at least three times. Mass calibration was done with horse myoglobin (100 pmol).

Pyruvoyl group determination of aspartate decarboxylase

The amount of pyruvoyl group present in purified aspartate decarboxylase was determined by the method of Ann et al. [17]. Briefly, 1 mg of fluorescein thiosemicarbazide in 950 μl of 0.2 M sodium carbonate (pH 11.1)/0.5% SDS, was placed in a light-protected vial capped with a Subaseal (William Freemans, Barnsley, Yorkshire, U.K.). This was flushed with a constant gentle nitrogen gas stream for 5–10 min. To this 50 μl of aspartate decarboxylase (114 μg) was added, the sample heated to 100 °C for 5 min followed by gentle stirring under nitrogen for 120 min. Separation of fluorescein-labelled protein and excess unreacted fluorescein thiosemicarbazide was carried out using Sephadex G-25 column (2.5 cm diameter; 20 ml bed volume) chromatography. Isocratic column elution was carried out using 0.2 M sodium carbonate (pH 11.1)/0.5% SDS. Fractions (1 ml) containing protein were pooled and concentrated to approx. 1 ml (Centricon 10; Amicon) before determination of the fluorescein ($\epsilon_{496\text{nm}}$ is 7.6×10^4 $M^{-1} \cdot cm^{-1}$; [18]) and protein concentrations.

A second method for the determination of the amount of pyruvoyl groups was based on the acid hydrolysis of the protein. A solution of aspartate decarboxylase (50 μl; 114 μg) was adjusted to 6 M HCl by addition of concentrated HCl (100 μl total reaction volume) and sealed in an evacuated boiling tube. This was incubated at 100 °C for 24 h, after which the sample was recovered. The amount of pyruvic acid present was determined by HPLC analysis using an organic acids column (Aminex HPX-87H; Bio-Rad) eluted at 0.6 ml/min with an isocratic gradient of 50 mM formic acid. Calibration was carried out using sodium pyruvate made up in running buffer (retention time 8.8 min).

Aspartate decarboxylase activity assay

Aspartate decarboxylase activity was measured by derivatization of L-aspartate and β-alanine with fluorescamine [19] followed by quantification on HPLC. Routine assays were performed at 25 ± 0.5 °C in 50 mM potassium phosphate (pH 7.0)/1 mM EDTA. All the assay components were incubated at 25 °C for at least 5 min before mixing, and assays were initiated by the addition of substrate. At given time intervals aliquots (100 μl) of the assay mixture were mixed with a one-tenth volume of 1 M sodium hydroxide to stop the enzyme reaction. Before HPLC analysis, 290 μl of 0.5 M borate (pH 10) and 100 μl of fluorescamine (Fluka; 0.3 mg/ml in 100% acetonitrile) were added in quick succession and the samples mixed thoroughly. The derivatized substrate and/or product(s) were quantified by reversed-phase HPLC using a C18 column (4.6 mm × 25 cm 5ODS2; Hewlett-Packard). Isocratic column elution was carried out at 0.75 ml/min using 20 mM sodium acetate (pH 5.9, adjusted with acetic acid) containing 15% (v/v) acetonitrile. HPLC analysis was carried out using a Hewlett-Packard (HP) Series 1050 workstation equipped with a HP1046A fluorescence detector, a Series 1050 diode array detector, an HP 35900E interface and an HP 486/66XM computer. The fluorescence (excitation at 390 nm and emission at 460 nm; photomultiplier tube gain of 11; 0.125 s sample rate) of the column eluant was monitored and the system calibrated with fluorescamine-derivatized L-aspartate (retention time 4.4 min) and β-alanine (retention time 6.0 min).

Data collection and analysis were carried out using software (HPLC 2D Chemstation Rev. A.03.01) supplied by the manufacturer.

RESULTS

Isolation, cloning and expression of the *E. coli panD* gene

From the nucleotide sequence of the *E. coli panD* gene lodged in the EMBL database (accession number L17086) we designed oligonucleotide primers corresponding to the 5′ and 3′ ends of the gene, and used PCR to amplify it up from *E. coli* DH5α chromosomal DNA. The primers included restriction sites at the 5′ ends to facilitate cloning, and the 5′ primer also had an optimal Shine–Dalgarno sequence [15]. The 416 bp PCR fragment was cloned into the vector pBluescript KS, to form plasmid pDKS1, so that the *panD* gene could be expressed from the *lacZ* promoter. The PCR-amplified insert was sequenced on both strands and found to be identical with the sequence for *panD* in the database (results not shown), predicted to encode a protein of 13834 Da. Furthermore, this clone was able to complement the *E. coli* strain SJ16 [8]. The exact genetic lesion of this strain is uncertain, but it has $< 0.3\,\%$ of the activity of wild-type aspartate decarboxylase. We found that it is unable to grow in liquid culture in the absence of β-alanine or pantothenate, although it is able to grow on unsupplemented solid media.

Overexpression of aspartate decarboxylase in *E. coli* strain DH5α containing pDKS1 was observed by the presence on SDS/PAGE (Figure 2, track 2) of extra protein bands (of approx. 14, 11 and 3 kDa), compared with cells containing vector alone (Figure 2, track 3). These extra bands correspond to the predicted sizes of the unprocessed π-subunit of the enzyme, and the α and β-subunits respectively. This result implies that, *in vivo*, the recombinant aspartate decarboxylase encoded by pDKS1 is not fully processed. This is also seen when recombinant enzyme is overproduced in the *panD E. coli* strain SJ16 (Figure 2, track 1). While it is possible that this incomplete processing is due to the overexpression and/or recombinant nature of the enzyme, the presence of the unprocessed π-subunit was also detected in non-recombinant aspartate decarboxylase purified from wild-type cells [5]. Overexpression was also confirmed by the fact that the activity of aspartate decarboxylase was 7 nmol·min^{-1}·mg^{-1} in cells containing pDKS1, compared with 0.17 nmol·min^{-1}·mg^{-1} in wild-type *E. coli* [5].

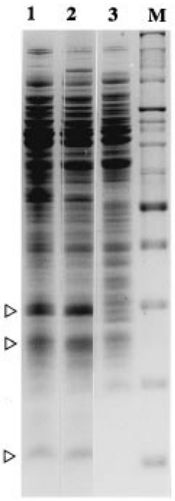

Figure 2 Overexpression of aspartate decarboxylase in *E. coli*

E. coli cells were grown overnight in the presence of IPTG as described in the Materials and methods section, and the cells disrupted by sonication. Soluble proteins were incubated with loading buffer containing 2% SDS and boiled for 10 min before loading onto a high-resolution SDS gel as described by Schägger and von Jagow [14]. Each sample contained approx. 25 μg of protein. Track 1, *E. coli* SJ16::pDKS1; track 2, *E. coli* DH5α::pDKS1; track 3, *E. coli* DH5α::pBluescript. M, broad range molecular mass markers from New England Biolabs (comprising 212, 158, 116, 97.2, 66.4, 55.6, 42.7, 36.5, 26.6, 20.0, 14.3, 6.5, 3.4/2.3 kDa; the last two markers are not resolved in this gel system). The three extra protein bands are arrowed.

Purification and characterization of recombinant aspartate decarboxylase

Cells of *E. coli* SJ16::pDKS1 were used for the purification of recombinant aspartate decarboxylase following the method described in the Materials and methods section. From 2.5 g of cells we could routinely prepare 2–2.5 mg of enzyme protein, which was more than 95% pure as judged by densitometry of non-denaturing PAGE. From the UV/visible spectrum, the purified enzyme preparation did not contain any pyridoxal phosphate (PLP; results not shown), confirming the previous report for the enzyme from wild-type cells [5]. To enable accurate determination of the protein concentration, amino acid analysis of purified aspartate decarboxylase was carried out and the protein concentration determined by the methods of Lowry et al. [12] and Bradford [11] (Bio-Rad protein assay kit). The latter two methods were shown to overestimate the concentration by 1.29 and 1.38 times respectively compared with the amino acid composition. The absorption coefficient of aspartate decarboxylase at 280 nm was calculated to be 1.9×10^4 M^{-1}·cm^{-1}.

The native molecular mass of recombinant aspartate decarboxylase was calculated to be 59 ± 4 kDa by gel filtration on Superose 12 FPLC (results not shown), which agrees with the determined molecular mass for the enzyme purified from wild-type cells [5]. Since the predicted size of the protein encoded by the *panD* gene is 13.8 kDa, this indicates that the native enzyme is a tetramer.

Analysis of the purified recombinant aspartate decarboxylase by SDS/PAGE (Figure 3, track 1) revealed that, as in crude lysate of bacterial cells, it contained three proteins of 13.8, 11 and 2.8 kDa, corresponding to the π-, α- and β-subunits respectively. Also visible is a minor contaminant of 42 kDa, but this appears unrelated to aspartate decarboxylase since the amounts varied between different enzyme preparations. In this track, the protein had been pre-treated before electrophoresis by boiling in loading buffer (containing 2% SDS) for 10 min. Less harsh denaturation (for example 10 min at 70 °C in 2% SDS) revealed the presence of a larger protein migrating at 55 kDa (Figure 3, track 2). If the protein were not boiled at all, but simply incubated at room temperature in loading buffer for 30 min (Figure 3, track 3), then virtually all the protein migrated at 55 kDa. Since this is likely to correspond to the tetrameric form of the enzyme, the anomalous behaviour indicates that the association between subunits is extremely strong.

Confirmation of this came from the observation that gel filtration of purified aspartate decarboxylase in the presence of alternative denaturing agents [0.5 M guanidinium hydrochloride, 6 M urea or 25% (v/v) acetonitrile in neutral pH buffer] or at a range of pH values from pH 4.5 to 9.0, did not result in dissociation of the tetramer, since the peak area and retention times remained the same as those for untreated protein (results not shown). To date, boiling the enzyme in the presence of 2% SDS for >1 min remains the only method that results in the

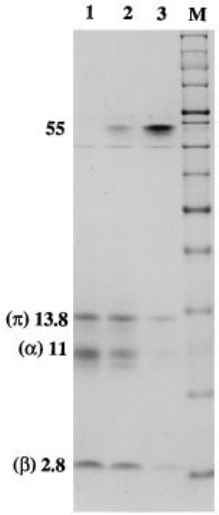

Figure 3 Gel electrophoresis of purified aspartate decarboxylase

Freshly purified aspartate decarboxylase (3 μg) was incubated in loading buffer containing 2% SDS before loading onto a high resolution SDS gel [14]. Track 1, boiled for 10 min; track 2, heated at 70 °C for 10 min; track 3, room temperature for 10 min; M, molecular mass markers (as in Figure 2). The sizes of the protein bands are indicated in kDa.

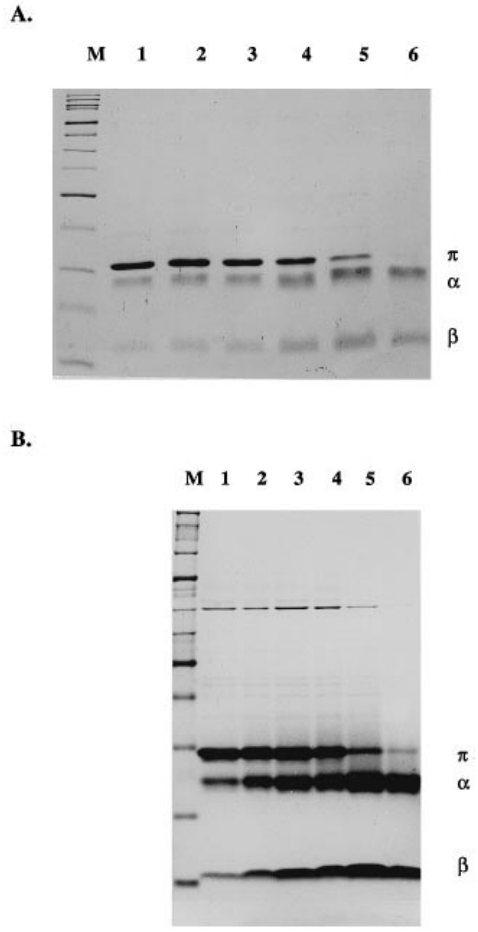

Figure 4 Processing of aspartate decarboxylase

(**A**) Freshly purified aspartate decarboxylase (10 μg) was incubated for 24 h at different temperatures, then boiled in 2% SDS loading buffer before electrophoresis on a 17% (w/v) polyacrylamide gel in Laemmli buffer. Track 1, liquid nitrogen; track 2, 4 °C; track 3, 20 °C; track 4, 37 °C; track 5, 50 °C; track 6, 70 °C. (**B**) Freshly purified aspartate decarboxylase (18 μg) was incubated for increasing lengths of time at 50 °C, then boiled in 2% SDS loading buffer before electrophoresis on a Schägger and von Jagow gel. Track 1, 0 h; track 2, 1 h; track 3, 4 h; track 4, 8 h; track 5, 24 h; track 6, 48 h. For (**A**) and (**B**) M = molecular mass markers (as in Figure 2) and the individual subunits are indicated as π, α and β.

complete dissociation of the tetramer. Attempts to allow re-formation of the tetramer by removal of SDS, such as exhaustive dialysis (in the presence and absence of potassium chloride) were unsuccessful. However, we did observe limited aggregation of the π- and β-subunits that had been electroeluted from an SDS gel, and then re-electrophoresed. In addition to either 13.8 kDa or 11 kDa bands, faint bands of 28 kDa or 22 kDa respectively were present (results not shown).

In order to determine if there are any disulphide bonds in the native enzyme that could, for example, bind the subunits, a thiol titration experiment with DTNB was carried out. The predicted amino acid sequence of the π-subunit contains two cysteine residues. For the native enzyme, we found one thiol group per 13.8 kDa subunit. In contrast, when aspartate decarboxylase was boiled with SDS before the addition of DTNB, two thiol groups were detected. These results indicate that aspartate decarboxylase does not have any disulphide bonds, and that in the native protein only one of these thiols is available for reaction with either DTT and/or DTNB.

Formation of active aspartate decarboxylase

Recombinant aspartate decarboxylase is observed to be only partially processed, both in crude lysate (Figure 2) and in our purified enzyme preparation (Figure 3, track 1). Indeed, in purified samples analysed immediately by SDS/PAGE or ESMS, the extent of processing was estimated to be approx. 10% (results not shown). This incomplete processing of aspartate decarboxylase was also reflected in the specific activity of the enzyme immediately after purification, which was determined to be between 100 and 250 $nmol \cdot min^{-1} \cdot mg^{-1}$, corresponding to 4–10% of that found in the fully processed enzyme (see below).

In their purified preparation of aspartate decarboxylase from wild-type *E. coli*, Williamson and Brown [5] detected three subunits of different sizes on SDS/PAGE. However, they did not determine their exact sizes or their relative proportions. Nonetheless, these three are likely to represent the π-, α- and β-subunits, indicating that even the non-recombinant protein is not fully processed *in vivo*. These observations are in contrast with histidine decarboxylase from *Lactobacillus* 30a, which is purified essentially completely processed to the α- and β-subunits. The π-subunit of histidine decarboxylase could be detected only in cells that were grown in media deficient in histidine and monovalent cation, and then induced with histidine [20]. Processing *in vitro* was then observed after incubation at 37 °C for several hours, and was stimulated by the addition of monovalent cations, the optimum being 0.8 M potassium ions [6,18].

When we incubated purified recombinant aspartate decarboxylase for 24 h at 37 °C, cleavage of the π-subunit, as indicated by the disappearance of the 13.8 kDa band with the concomitant appearance of the 11 kDa and 2.8 kDa bands, occurred to some extent, but was unaffected by altering the potassium concentration from 0 to 2 M (results not shown). In contrast, temperature had a significant effect, with much enhanced cleavage at 50 °C and 70 °C (Figure 4A). In a time

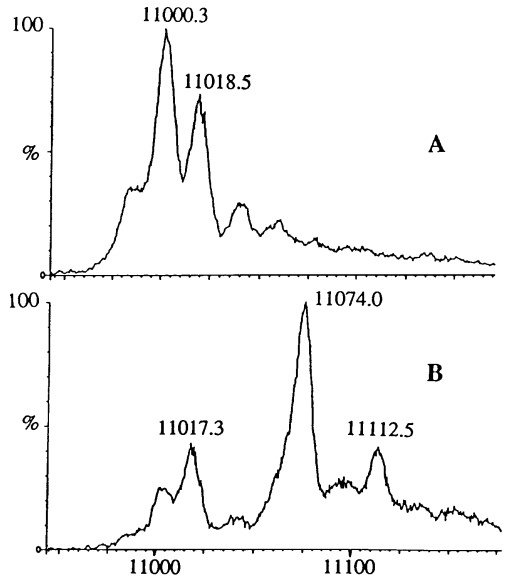

Figure 5 Electrospray mass spectrum of aspartate decarboxylase

Purified aspartate decarboxylase was completely processed by incubation at 50 °C for 48 h, and then analysed by ESMS as described in the Materials and methods section. The Figure shows the mass transformed data in the region of 11 kDa. (**A**) Aspartate decarboxylase alone. The peak at 11 000 corresponds to the α-subunit with an N-terminal pyruvoyl group. The peak at 11 018 is due to α-subunit retaining a serine at the N-terminus. (**B**) Aspartate decarboxylase to which L-aspartate had been added, followed immediately by treatment with sodium cyanoborohydride. The peaks at 11112 and 11074 are due to reductive trapping of the imine adducts with the substrate and product respectively.

course experiment (Figure 4B), the half-life was approx. 16 h at 50 °C, compared with several days at 37 °C. For further analysis, all subsequent preparations were treated at 50 °C for approx. 48 h, until judged fully processed by SDS/PAGE. The specific activity was determined to be 2400 nmol·min^{-1}·mg^{-1}, compared with 650 nmol·min^{-1}·mg^{-1} found for the enzyme purified from wild-type *E. coli* [5].

Determination of number of pyruvoyl groups

Activation of aspartate decarboxylase requires the generation of a pyruvoyl cofactor at the N-terminus of the α-subunit from the serine residue downstream of the cleavage site (Ser-25). Williamson and Brown [5] estimated that there was one pyruvoyl group per tetrameric enzyme purified from wild-type *E. coli*, implying that a large proportion of the subunits were either not processed at all, or processed without a pyruvoyl group. By analogy with other enzymes of this type, which have one pyruvoyl group per α-subunit, this is a surprising result. We therefore used two methods to determine quantitatively the amount of pyruvoyl present in our recombinant aspartate decarboxylase that had been fully processed: complete acid hydrolysis of the protein followed by measurement of the pyruvate content by HPLC, and derivatization with fluorescein thiosemicarbazide. Both methods gave similar results, namely that there is 0.75 ± 0.03 pyruvoyl group per α-subunit, i.e. an average of three per tetramer. Since this was fully processed enzyme, this result suggests that up to one-quarter of the subunits do not possess pyruvoyl at the N-terminus, but instead may have serine.

There are two lines of qualitative evidence to support this. Analysis of the fully processed enzyme by ESMS revealed a mass peak at 2834 ± 2 (results not shown), which can be assigned to

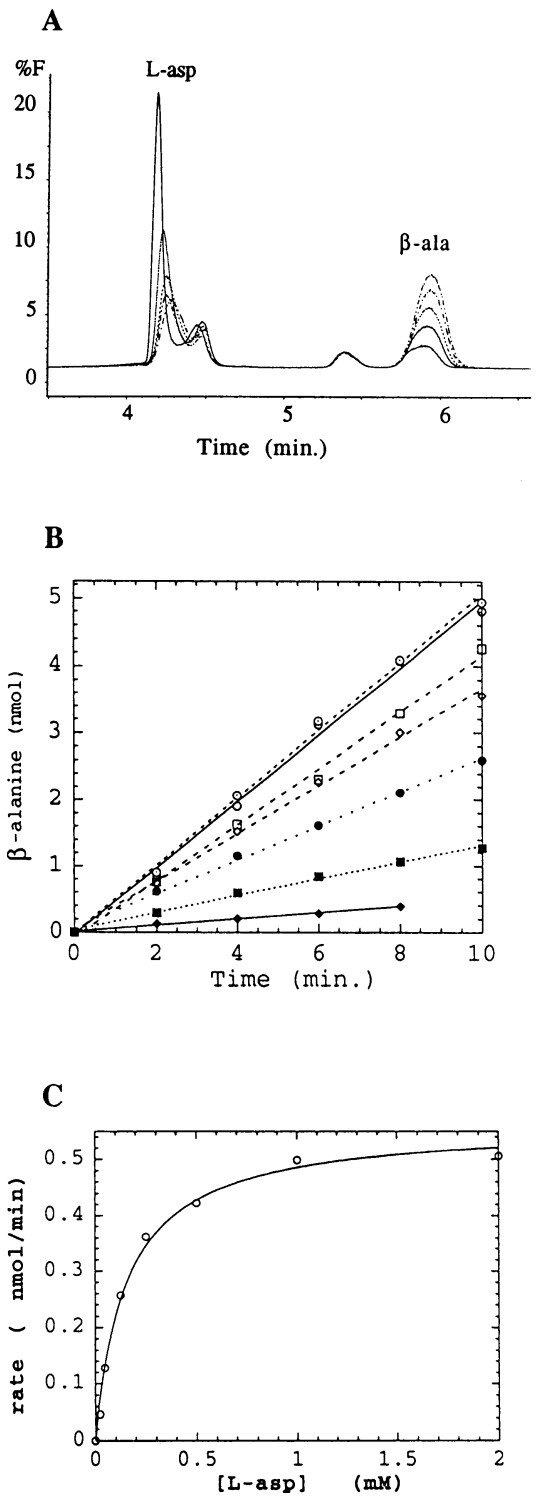

Figure 6 HPLC-based assay of aspartate decarboxylase

The assay was performed as described in the Materials and methods section in a total volume of 1 ml using 22 μg of purified aspartate decarboxylase that had been completely processed. Aliquots (100 μl) were removed at time intervals. (**A**) HPLC traces of the assay mixtures after different times of incubation (2, 4, 6, 8 and 10 min). The positions at which fluorescamine-derivatized L-aspartate and β-alanine elute are indicated. (**B**) Rate curves at increasing amounts of L-aspartate (◆, 0.03 mM; ■, 0.06 mM; ● 0.125 mM; ◇, 0.25 mM; □, 0.5 mM; ○, 1 mM and ⊙, 2 mM). (**C**) Michaelis–Menten plot of aspartate decarboxylase activity as a function of L-aspartate concentration.

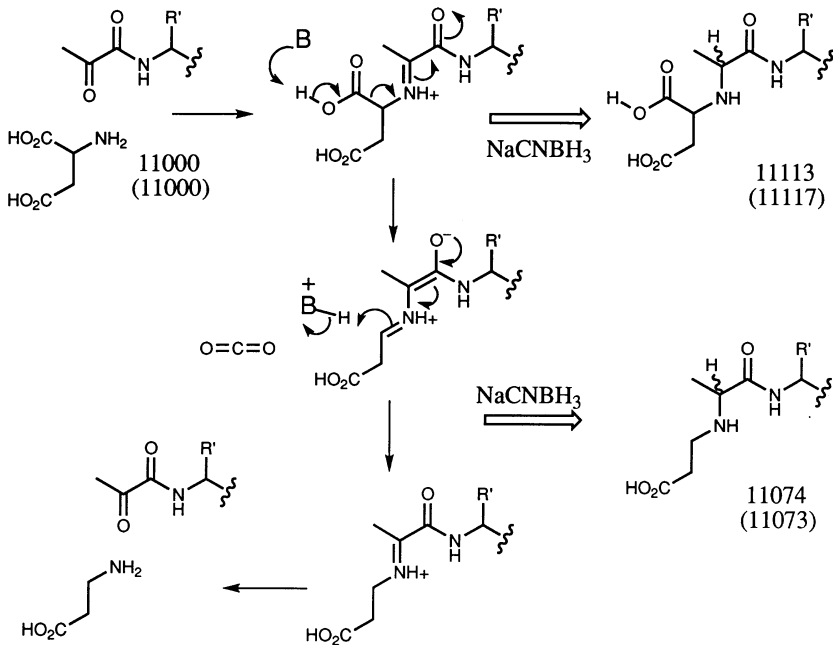

Figure 7 Proposed catalytic mechanism and trapping of intermediates by sodium cyanoborohydride of aspartate decarboxylase

The imine intermediates from the N-terminal pyruvoyl group and their trapping with sodium cyanoborohydride. The numbers shown below the structures are the observed molecular masses (Da) determined by ESMS. The values shown in parentheses are the corresponding predicted values.

the β-subunit, and two further mass peaks at 11000 ± 2 and 11018 ± 2 (Figure 5A). The former is the expected mass of the α-subunit with a covalently bound pyruvoyl (Pyr) group at the N-terminus (Figure 1, α-subunit with Pyr), whereas the other corresponds to the α-subunit with a serine residue (Figure 1, α-subunit with Ser). This method is only semiquantitative, but the proportion of the two peaks is approx. 3:1. Secondly, we carried out N-terminal amino acid sequencing of the π-, α- and β-subunits from the freshly purified protein (i.e. incompletely processed). The π- and β-subunits gave identical sequences of Met-Ile-Arg-Thr-Met, corresponding to the N-terminus of the encoded protein. The presence of a pyruvoyl group at the N-terminus of the α-subunit would prevent it from being sequenced by Edman degradation. Nevertheless, we were able to obtain some sequence of Ser-Cys-Ala-Ile-Asp, from the α-subunit, implying that a portion at least of the protein had serine at the N-terminus rather than the pyruvoyl group.

Assay of aspartate decarboxylase activity

To study the kinetics of aspartate decarboxylase we have devised a highly sensitive fluorescence-based assay. Aspartate decarboxylase has previously been assayed either by trapping radioactive carbon dioxide released from [1-^{14}C]aspartate [4,5], or by measuring the release of carbon dioxide manometrically in a stopped assay [21]. Our new assay involves taking aliquots at selected time intervals (between 1 and 10 min) and quenching the enzyme reaction by adding sodium hydroxide. The unreacted aspartate and β-alanine are then derivatized using fluorescamine [19]. This reaction is very fast (milliseconds) and unreacted fluorescamine rapidly breaks down. The derivatized mixture is then loaded onto a reversed-phase column where the fluorescamine-modified aspartate and β-alanine are easily resolved (Figure 6A). The quantification is linear at least up to 40 nmol of derivatized material, and is particularly sensitive, easily being able to give reproducible results on 0.1 nmol of material. Typical assays involve detecting approx. 0.2–5 nmol of β-alanine (Figure 6B). Using robotic sample handling on HPLC, multiple assays can be performed routinely in a matter of hours.

Using this fluorescence assay, the steady state kinetic constants determined for L-aspartate were $K_m = 151 \pm 16\ \mu M$ and $k_{cat} = 0.57\ s^{-1}$ (Figure 6C). These values are in good agreement with those obtained for the non-recombinant wild-type enzyme [5].

It has previously been reported that aspartate decarboxylase is inhibited by treatment with sodium borohydride in the presence of substrate [4,5]. These observations are consistent with a mechanism that involves the formation of an imine (Schiffs base) during catalysis (Figure 7). We have investigated this by treating aspartate decarboxylase with substrate followed quickly by sodium cyanoborohydride. The treated sample was then injected onto an electrospray mass spectrometer. Spectra of enzyme without substrate were also run as controls. The resulting spectra (Figure 5B) show that the peak at 11000 in the unmodified enzyme is significantly reduced and that two new peaks are present at 11074 ± 1 and 11113 ± 2 Da. These are values expected for protein that is covalently modified by reductive trapping of β-alanine (11073) and L-aspartate (11117) respectively (Figure 7).

DISCUSSION

We have used PCR to clone and overexpress the *panD* gene encoding L-aspartate-α-decarboxylase, so that it is now possible to purify the enzyme on a milligram scale following a procedure similar to that previously reported [5,9]. Our studies have led to increased understanding of *panD* mutants, the enzyme reaction, and in particular the generation of the catalytically active form of the enzyme from the initially formed π-enzyme.

Complementation experiments using *panD* mutant strain SJ16 were only achieved in liquid media, whereas on solid media some

growth of SJ16 was observed. The nature of the disruption in the *panD* mutant is unclear. We amplified the *panD* gene from SJ16 by PCR and found no sequence differences from the nucleotide sequence of wild-type *panD* (U. Genschel, C. Abell and A. G. Smith, unpublished work). The situation is further complicated by the possibility that an autonomous PLP-dependent aspartate-α-decarboxylase [4,5,22] may be able to rescue the *panD* mutants by producing β-alanine by an alternative route.

The pyruvoyl-dependent aspartate decarboxylase we have purified is a tetramer (Figure 3), as is 4′-phosphopantothenylcysteine decarboxylase, an enzyme involved in the later steps of CoA biosynthesis. However, unlike other members of this group, dissociation of the native aspartate decarboxylase (whether fully or partially processed) into its subunits is achieved only by harsh denaturation (boiling in 2% SDS for > 5 min), suggesting that the subunit interaction is very strong. Upon induction of the recombinant *panD* gene, the unprocessed (π) form of aspartate decarboxylase accumulates in the cell and this is the major form of the initially purified enzyme. We have shown that the π-enzyme can be processed *in vitro* to yield catalytically active aspartate decarboxylase, but the processing is very slow at 37 °C. At 50 °C, our preliminary observations suggest that processing is virtually complete after 48 h. This can be compared with histidine decarboxylase, where a half-life of 20 h at 37 °C under pseudophysiological conditions was determined [18]. The processed form of recombinant aspartate decarboxylase has essentially the same kinetic parameters as the wild-type enzyme [5].

The use of ESMS provides important insights into the processing of the enzyme. The spectrum of the overexpressed recombinant protein shows three major protein species. The peak at molecular mass 2834 Da is due to the smaller β-subunit that is formed from the first 24 amino acids of the π-enzyme. The two peaks at 11000 and at 11018 are due to differential processing to form the larger α-subunit. The peak at 11000 corresponds to the predicted molecular mass for the β-subunit with an N-terminal pyruvoyl group formed from what was formerly Ser-25. The peak at 11018 has a molecular mass expected for the β-subunit if it retains an N-terminal serine residue that has not been converted to a pyruvoyl group. Both species (the 11000 and 11018 Da proteins) could arise from the same intermediate ester by either elimination to form dehydroalanine (Figure 1, path a), which is then hydrolysed to pyruvate, or esterolysis to release the serine residue (Figure 1, path b). This intermediate is analogous to that postulated to be involved in the formation of the pyruvoyl group in histidine decarboxylase [23,24]. However, in histidine decarboxylase the processing occurs between two serine residues.

When this enzyme preparation is mixed with substrate, followed quickly by sodium cyanoborohydride, the peak at 11000 in the electrospray mass spectrum is significantly reduced and two new peaks at 11074 and 11113 appear. These peaks correspond to the species expected if the imines between the enzyme with the product (β-alanine) or substrate (aspartate) are reductively trapped. These spectra provide direct evidence for the mechanism shown in Figure 7. Similar experiments have been used to trap imine intermediates on dehydroquinase [25] and dihydrodipicolinate synthase [26], although in these studies the imine was between a lysine on the enzyme and a carbonyl group on the substrate or product. The ESMS spectrum in Figure 5(B) also shows that the peak at 11018 remains essentially unchanged, supporting the assignment that this peak is due to an α-subunit lacking the N-terminal pyruvoyl group that is therefore not able to react with the substrate.

The results described in this paper raise a number of questions. One relates to the subunit composition of the catalytically active form of aspartate decarboxylase, and the second to the kinetics of protein processing to generate the catalytically active species.

The presence of less than a stoichiometric pyruvoyl group per subunit (approx. 0.7 per α-subunit) in fully processed recombinant enzyme has been demonstrated by fluorescent labelling, hydrolysis and pyruvate determination, N-terminal amino acid sequencing, and ESMS. This could reflect the composition of the catalytically active tetramer *in vivo* as not comprising four identically processed $\alpha\beta$-subunits, but rather that only three of the α-subunits have an N-terminal pyruvoyl group. However, this would be unexpected in comparison with other pyruvoyl-dependent enzymes such as histidine decarboxylase [27] and *S*-adenosylmethionine decarboxylase [28], that have been shown to have one pyruvoyl group per subunit. An alternative explanation for the apparent deficit in pyruvoyl groups, and the presence of a serine residue detected at the N-terminus of the β-subunit, is the possibility of a competing process occurring *in vitro* that derails the correct processing of the intermediate ester implicated in the formation of the pyruvoyl group (Figure 1). If, instead of elimination across the 2,3 bond of Ser-25, there is esterolytic cleavage of the carbonyl of amino acid Gly-24, this misprocessing esterolysis would leave an N-terminal serine on the α-subunit.

Further, we have shown that the enzyme isolated soon after induction of expression of the recombinant protein is as little as 10% processed, both from its subunit composition, visualized by SDS/PAGE and ESMS, and in its enzymic activity. Similar observations have been made for the enzyme from wild-type *E. coli* cells [5]. We have been able to demonstrate slow processing of overexpressed aspartate decarboxylase *in vitro* by an autocatalytic mechanism at room temperature or 37 °C, whereas at 50 °C (Figure 4B) processing is essentially complete after 48 h. This is presumed not to be a physiologically relevant temperature, although, intriguingly, aspartate decarboxylase is catalytically very active at this temperature [5].

The question arises therefore of what the mechanism of cleavage is *in vivo*. It is possibly an exclusively autocatalytic mechanism at a specific reactive peptide bond, reminiscent of self-splicing of ribozymes. If this is the case, then by analogy with the *in vitro* process, the cleavage would not be very specific, as not all the cleavage reactions lead to pyruvoyl formation. Furthermore, the half-life for autocatalytic processing is of the order of several hours, which is difficult to reconcile with the lifetime of an *E. coli* cell before division of approx. 20–60 min. Nonetheless, a similar slow processing *in vitro* of the π-subunit of *Lactobacillus* histidine decarboxylase (half-life 21 h) has been reported [18].

An attractive alternative explanation is that there may be a specific catalyst for the π protein cleavage *in vivo* that promotes the correct cleavage mechanism leading to formation of the pyruvoyl group. In the absence of this catalyst the elimination reaction is slowed and the rate of the competing esterolytic cleavage becomes significant. This is a seductive conclusion as it could provide an explanation not only for the incorrect processing of the massively overexpressed recombinant protein and the slowness of the autocatalytic cleavage, but also for some of the ambiguities surrounding the *panD* mutant phenotype.

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