Biochemical and Biophysical Research Communications 436 (2013) 362–365

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Distinct physiological roles for the two L-asparaginase isozymes of *Escherichia coli*

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ARTICLE INFO

Article history:
Received 8 May 2013
Available online 31 May 2013

Keywords:
Escherichia coli
Asparaginase
Anaerobic growth
Isozymes

ABSTRACT

Escherichia coli expresses two L-asparaginase (EC 3.5.1.1) isozymes: L-asparaginse I, which is a low affinity, cytoplasmic enzyme that is expressed constitutively, and L-asparaginase II, a high affinity periplasmic enzyme that is under complex co-transcriptional regulation by both Fnr and Crp. The distinct localisation and regulation of these enzymes suggest different roles. To define these roles, a set of isogenic mutants was constructed that lacked either or both enzymes. Evidence is provided that L-asparaginase II, in contrast to L-asparaginase I, can be used in the provision of an anaerobic electron acceptor when using a non-fermentable carbon source in the presence of excess nitrogen.

1. Introduction

L-Asparaginases are widely distributed in the three domains of life and play a central role in amino acid metabolism and utilisation. L-Asparaginase enzymes catalyse the hydrolysis of L-asparagine (EC 3.5.1.1) generating L-aspartate and ammonia (NH_3). Based on studies with *Escherichia coli* and *Salmonella enterica*, bacterial L-asparaginases are of two types: a high affinity enzyme located in the periplasm (L-asparaginase II encoded by *ansB*) and a cytoplasmic low affinity enzyme (L-asparaginase I encoded by *ansA*). *E. coli* and a few other bacteria have been shown to possess both type I and type II isozymes [1–4]. The amino acid sequences are substantially diverged but retain sequence similarity around the active site residues [4]. The high affinity enzymes have attracted a great deal of interest due to their utility as anti-tumour agents in the treatment of childhood acute lymphocytic leukemia [5]. This is due to the efficient depletion of exogenous L-asparagine, which leukemic cells depend on for growth. More recently it has been reported in *S. enterica* Typhimurium that L-asparaginase II (AnsB) has a role in infection and immunity by inhibiting T-cell responses [6] and it also contributes to virulence in the human pathogens *Campylobacter jejuni*, *Helicobacter pylori* and *S. enterica* Typhimurium [6–8] via a role in colonisation.

As well as two L-asparaginase isozymes, *E. coli* also possess two distinct systems for L-asparagine uptake distinguishable on the basis of specificity and regulation [9,10]: a low-affinity system (K_m = 80 μM), and a high affinity system (K_m = 3.5 μM) which is repressed by the presence of L-asparagine in the growth medium in concentrations greater than 1 mM. These systems have not been studied at the molecular level; However, an asparagine permease, AnsP, has also been reported in *S. enterica* and *E. coli* [11,12].

There is evidence that L-asparaginase I (AnsA) plays a role in *E. coli* in the utilisation of L-asparagine as a nitrogen source [13] and it presumably functions to degrade L-asparagine when it has accumulated to an appropriate intracellular concentration. However, the role of the high affinity L-asparaginase II has never been determined. While L-asparaginase I is expressed constitutively, L-asparaginase II expression requires co-dependent activation by both anaerobiosis, via the Fnr transcriptional activator, and by the cyclic-AMP receptor protein (Crp) [3,14,15]. Several possible roles are consistent with the activity, localisation, and regulation of the L-asparaginase II. Firstly, L-asparaginase II may serve to utilise L-asparagine as a carbon source under unfavorable conditions, as in the case of, for example, exported phosphatases, proteases and lipases. Such a role is consistent with regulation by Crp. Secondly, this enzyme may be required for growth on low concentrations of L-asparagine as a nitrogen source. Thirdly, it has been suggested that during anaerobic growth on a non-fermentable carbon source, hydrolysis of L-asparagine, could lead to the provision of fumarate as a terminal electron acceptor for anaerobic respiration since the resulting L-aspartate could be taken up by an L-aspartate transporter and catabolised to fumarate by L-aspartase (see Fig. 2) [3]; this function is consistent with the anaerobic regulation of L-asparaginase II in both *E. coli*, via Fnr, and *S. enterica* by an unknown mechanism, [15] and with the anaerobic regulation of aspartase and dicarboxylate transporters [16–18].

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0006-291X/$ - see front matter © 2013 Published by Elsevier Inc.
http://dx.doi.org/10.1016/j.bbrc.2013.05.066

Table 1
Strains and plasmids used in this study. CGSC: Coli Genetic Stock Centre.

Name	Relevant genotype	Source/Reference
E. coli strains		
HB94	Wild type	Del Casale et al. (1983)
HB94K	*ansB*::*kan*	This work
RC614	*ansA*	Del Casale et al. (1983)
RC614K	*ansA, ansB*::*kan*	This work
CGSC6355(P1)	P1 lysogen	CGSC
Plasmids		
pMJ13	*ansB*	Jennings et al. (1990)
pMJ13kan	*ansB*::*kan*	This work
pPJ1	*ansA*	Jerlstrom et al. (1989)

2. Materials and methods

2.1. Media and growth conditions

Strains and plasmids used in this study are detailed in Table 1. HB94 (wild type) and its three mutant derivatives (RC614 (*ansA*$^-$); HB94K (*ansB*$^-$); RC614K (*ansA*$^-$, *ansB*$^-$) were grown as follows. For Fig. 1A, HB94 was grown in 1× M9 medium and supplemented with 10 mM Ca, 100 mM Mg, 20% glycerol, 100 mM tryptophan and 18 mM NH_4Cl. Cultures were grown anaerobically at 37 °C. Cells were harvested by centrifugation (45,000 × *g*, 15 min, RT) after overnight growth, washed twice with 1× M9 medium and resuspended in 2 ml of the same medium. This was used as a starter

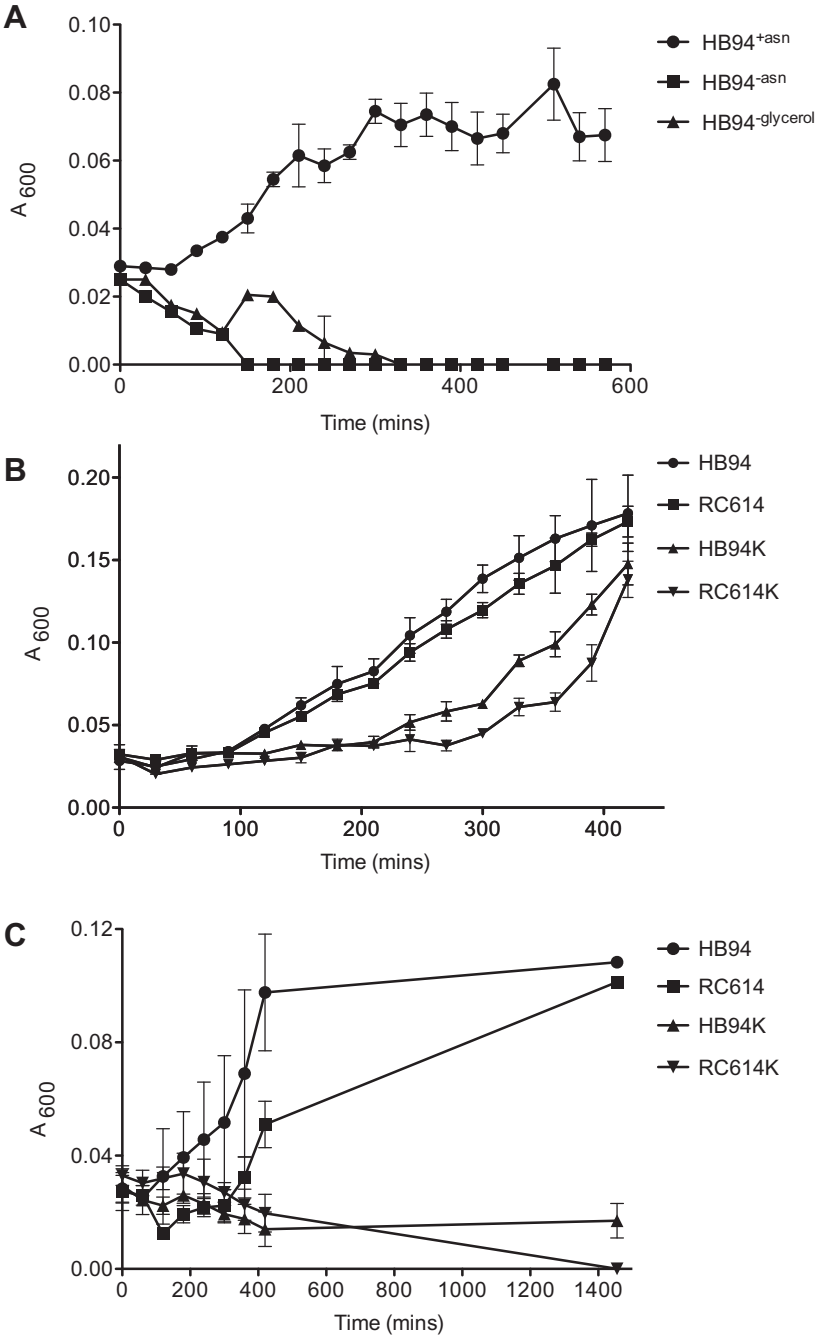

Fig. 1. The role of L-asparaginase II in anaerobic respiration. (A) Growth of HB94 in the presence or absence of asparagine and glycerol. (B) Growth of HB94 and the isogenic mutants RC614, HB94K and RC614K in the presence of asparagine and absence of NH_4Cl. (C) Growth of HB94 and the isogenic mutants RC614, HB94K and RC614K in the presence of asparagine and NH_4Cl. Growth conditions are detailed in Section 2.

culture (initial OD_{600} of 0.03) to inoculate tubes containing 15 ml of 1× M9, and supplemented with 10 mM Ca, 100 mM Mg and 100 mM tryptophan in the presence or absence of 0.05 mM L-asparagine and 20% glycerol. For Fig. 1B and C, the growth rate of HB94 and the isogenic mutants RC614, HB94K and RC614K was observed with 0.05 mM L-asparagine in the absence (Fig. 1B) or presence (Fig. 1C) of NH_4Cl. Strains were grown as above with or without 18 mM NH_4Cl. All cultures were grown anaerobically at 37 °C. Where required, kanamyacin was used at a concentration of 10 μg/ml. Cells were harvested by centrifugation (45,000 × *g*, 15 min, RT) after overnight growth, washed twice with 1× M9 medium and resuspended in 2 ml of the same medium. These were used as starter cultures (initial OD_{600} of 0.03) to inoculate tubes containing 15 ml of 1× M9, 0.05 mM L-asparagine and supplemented with 10 mM Ca, 100 mM Mg, 20% glycerol and 100 mM tryptophan. Readings were taken in triplicate for each strain and averaged.

2.2. Construction of the ansB mutant

An *ansB* mutant was constructed by insertion of a kanamycin resistance cassette, excised from pKIX (Pharmacia) by digestion with *Sma*I, into a unique *Hpa*I site present in the coding region of *ansB* on plasmid pMJ13. The resulting plasmid containing the *ansB*::*kan* allele was linearised and used to transform *E. coli* strain V355 competent cells (*recD*; [19]). Kanamycin resistant colonies were isolated and the presence of the kanamycin cassette confirmed by southern blot. The *ansB* phenotype was confirmed by Western blot using anti-AnsB rabbit polyclonal sera [20] and absence of high affinity L-asparaginase activity in anaerobically grown cultures. One of the characterised mutants, MPJ10 was used to make a P1 lysogen, and the *ansB*::*kan* mutation transduced to recipient strains HB94 and RC614 [13], resulting in strains HB94K and RC614K.

3. Results and discussion

3.1. L-Asparaginase II allows anaerobic respiration in the presence of excess nitrogen

In order to test these putative functions of the high affinity, exported L-asparaginase II, and in particular the possibility of a role in anaerobic respiration, we grew strain HB94 anaerobically with glycerol as the non-fermentable carbon source and with L-asparagine. Slow growth was observed which was dependent on both glycerol and L-asparagine (Fig. 1A). To investigate the role of L-asparaginases in the provision of L-asparagine as electron acceptor, we used isogenic mutants of *E. coli* strain HB94 which lack either L-asparaginase I (RC614; see [13]), L-asparaginase II (HB94K) or both isozymes (RC614K; see Fig. 1B and C). In medium lacking NH_4Cl, all strains were able to grow slowly, indicating that either L-asparaginase I or II can be used as a source of fumarate (Fig. 1B). However, in medium containing NH_4Cl, growth was dependent on the presence of L-asparaginase II (Fig. 1C), indicating that L-asparaginase I was unable to deaminate internal L-asparagine under these conditions. These results indicate that both L-asparaginase isozymes are capable of allowing use of L-asparagine as a source of fumarate as an anaerobic electron acceptor, but that in the presence of an excess of nitrogen, in the form of NH_4Cl, L-asparaginase II is obligatory. This may be due to a critical L-asparagine transporter being inoperative in the presence of excess nitrogen, due either to inhibition of its synthesis or transport function: it is known that the GlnLG system (encoded by *glnLG*; also known as NtrBC) is not active under conditions of high ammonia [21], and hence expression of an L-asparagine transporter would not occur if it was part of the GlnLG/NtrBC regulon. Alternatively, the global regulator Fis has been shown to repress expression of the asparagine transporter AnsP in *E. coli* under nutrient-rich conditions,

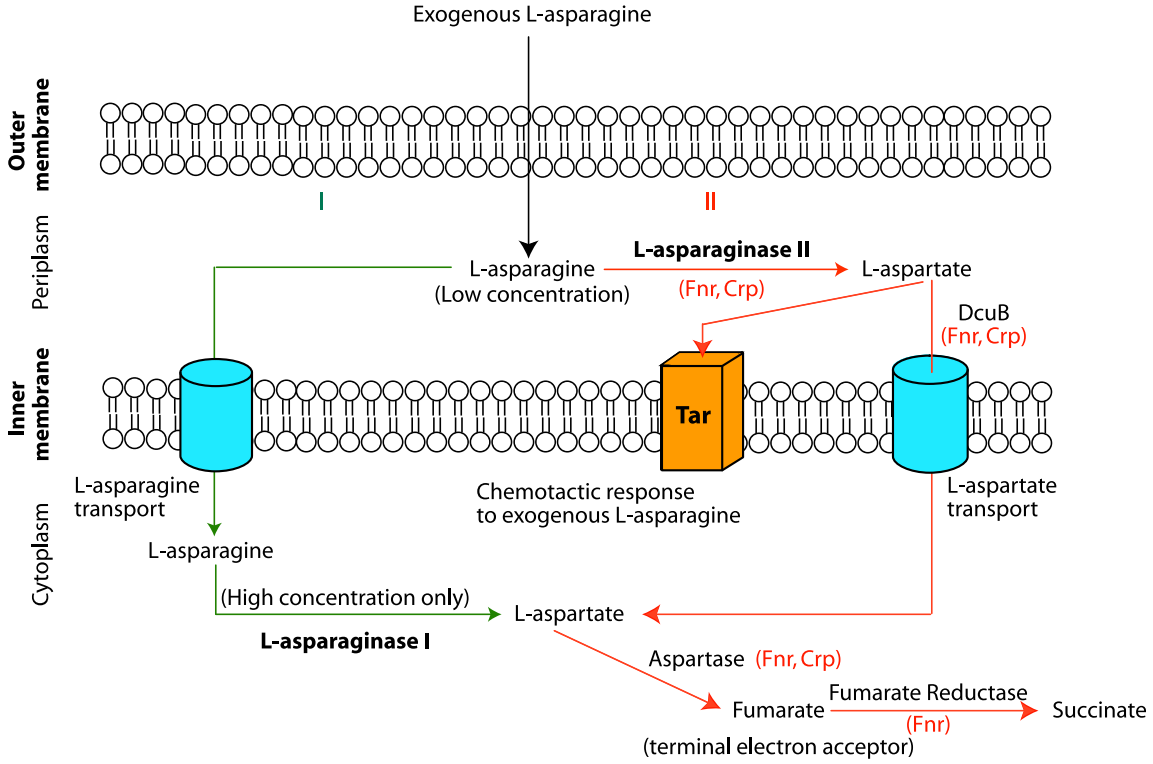

Fig. 2. Proposed role for L-asparagniase II in asparagine utilisation. Pathway I indicates utilisation of L-asparagine in the absence of L-asparaginase II. Under conditions of both anaerobiosis and high cAMP L-asparaginase II is expressed (Pathway II) and converts L-asparagine to L-aspartate in the periplasm, followed by transport into the cytoplasm and utilisation as a terminal electron acceptor. A consequence of conversion of L-asparagine to L-aspartate in the periplasm is signal conversion with respect to Tar chemotaxis.

which may include directly or indirectly, excess nitrogen [12]. The AnsP protein has been shown to be critical for asparagine transport in *S. enterica* [11], but whether AnsP is the L-asparagine transporter down-regulated in *E. coli* in response to ammonia remains to be elucidated. Nonetheless, if L-asparagine is unable to be transported, the action of L-asparaginase II in the periplasm becomes essential in the generation of aspartate, and hence fumarate as an electron acceptor, from exogenous L-asparagine.

The utilisation of L-asparagine, via pathway II, in anaerobic respiration (Fig. 2), is consistently positively regulated by anaerobiosis via Fnr: transcription of the C4-dicarboxylate transporter and L-aspartase genes (*dcuB* and *aspA*) are both regulated by Fnr and Crp [16–18]. Fumarate reductase, responsible for the terminal step of anaerobic electron transfer to fumarate, is likewise Fnr-regulated [22]. In addition, pathway II in its entirety, from L-asparagine, is repressed by nitrate via the NarXL two-component system [23,24]. Since nitrate is a preferred electron acceptor, this is consistent with the role of asparaginase II, and pathway II, in anaerobic respiration.

In addition to anaerobic regulation, it is notable that in both *S. enterica* Typhimurium and *E. coli*, production of L-asparaginase II is also absolutely dependent on Crp binding at the respective *ansB* promoters; in *E. coli* the promoter is co-dependent on Fnr and Crp whilst in *S. enterica* the upstream Fnr site has been modified to form a second Crp binding site but the promoter retains anaerobic regulation by an unknown mechanism [3,25]. The *ansB* gene is also transcriptionally up-regulated by the presence of amino acids [26]. Thus, in both enteric organisms, asparaginase II is highly expressed under Stringent nutritional conditions and anaerobiosis allowing the conversion of L-asparagine to L-aspartate and anaerobic respiration. Chemotaxis in response to L-asparagine has been demonstrated in a strain expressing the aspartate receptor (Tar) and asparaginase II, the latter under aerobic conditions from an artificial promoter [27]; since the native *ansB* promoter is very highly expressed under Crp-permissive plus anaerobic conditions [3,28], it can be assumed that chemotaxis towards L-asparagine-derived L-aspartate will be similarly enabled under these native conditions, followed by uptake and conversion to fumarate.

In summary, there are two pathways by which L-asparagine may be utilised (Fig. 2). One involves the use of L-asparagine as a source of nitrogen and/or carbon and depends on active transport of L-asparagine followed by conversion of the accumulated L-asparagine to L-aspartate by the low affinity cytoplasmic L-asparaginase I. The second (Fig. 2, pathway II), in the presence of ammonia as nitrogen source, is the utilisation of low concentrations of exogenous L-asparagine as a terminal electron acceptor following conversion by L-asparaginase II to L-aspartate and thence fumarate by L-aspartase. Initial conversion to L-aspartate by the high affinity L-asparaginase II in the periplasm will allow (a) chemotaxis to L-asparagine via the product, L-aspartate, and Tar signalling; and (b) transport of L-aspartate into the cell followed by conversion to fumarate. This becomes essential when ammonia is present which we suggest leads to abolition of asparagine transport into the cytoplasm through potential down-regulation of a likely sole asparagine transporter. Significantly, this pathway is subject to anaerobic regulation, involving Fnr, and by nitrate, via NarL, at the periplasmic, membrane transport and cytoplasmic levels, consistent with its role in anaerobic respiration.

The role of L-asparaginase II in pathogenesis in *S. enterica* serovar Typhimurium may well follow selection for this enzyme in pathway II in non-host environments, or as a commensal, and hence be regarded as 'coincidental selection' [29].

Acknowledgment

The work was supported by NHMRC Program Grant 565526 to M.P.J.

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