A bacterial ortholog of class II lysyl-tRNA synthetase activates lysine

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Abstract

Aminoacyl-tRNA synthetases produce aminoacyl-tRNAs, essential substrates for accurate protein synthesis. Beyond their central role in translation some of these enzymes or their orthologs are recruited for alternative functions, not always related to their primary cellular role. We investigate here the enzymatic properties of GenX (also called PoxA and YjeA), an ortholog of bacterial class II lysyl-tRNA synthetase. GenX is present in most Gram-negative bacteria and is homologous to the catalytic core of lysyl-tRNA synthetase, but it lacks the amino terminal anticodon binding domain of the latter enzyme. We show that, in agreement with its well-conserved lysine binding site, GenX can activate in vitro l-lysine and lysine analogs, but does not acylate tRNALys or other cellular RNAs.

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1. Introduction

Aminoacyl-tRNA synthetases (aaRSs) comprise a family of enzymes whose primary function is the generation of aminoacyl-tRNAs (aa-tRNAs) for protein synthesis. The aminoacylation reaction is a two step process whereby the cognate amino acid is first activated to aminoacyl-AMP, and then esterified to the 3'-terminal adenosine of the corresponding tRNA molecule. The central role of the aaRSs in ensuring accurate protein synthesis and hence cell survival, as well as their contribution to the emergence of modern days genetic code has been widely documented [1,2]. One of the outcomes of the broad genome sequencing effort was the realization of the existence of many genes with sequence homology to parts of known aaRSs [3]. While it was initially believed that these homologs may be pseudo-genes and remnants of the complex aaRS evolutionary history [2,4], a closer look at the enzymatic properties of the gene products proved this idea wrong. While none of the known aaRS orthologs was able to transfer an amino acid to the 3'-end of a tRNA molecule, they nevertheless showed different enzymatic activities. For instance, a set of small proteins present in bacteria and eukarya with sequence homology to the editing domain of bacterial type prolyl-tRNA synthetase were shown to hydrolyze the misacylated Ala-tRNAPro and Cys-tRNAPro species [5]. Self-standing homologs of alanyl-tRNA synthetase and threonyl-tRNA synthetase editing domains, present in bacteria and archaea, also deacylate incorrectly charged aa-tRNA species [5,6]. Another example was provided by the presence in more than 40 bacterial genomes of a the YadB protein, displaying homology to the catalytic core of glutamyl-tRNA synthetase (GluRS), but lacking the whole anticodon binding domain. Like GluRS, YadB activates glutamate in presence of ATP; in contrast to GluRS, it transfers the activated glutamate onto the modified nucleoside queosine, located at the first position of the tRNAAsp anticodon [7,8]. Aminoacyl-tRNA synthetase analogs have also been recruited for non-tRNA related functions such as amino acid biosynthesis. The HisZ protein with significant similarity to HisRS but lacking an anticodon binding domain and residues essential for catalysis was shown to be present in Lactococcus lactis and a number of other bacterial species [12]. While this protein is unable to activate histidine, it participates together with the HisG protein in the first step of histidine biosynthesis in these organisms [9]. Likewise, an aspargyl-tRNA synthetase homolog with a truncated anticodon binding domain is found in Pyrococcus abyssi and in a number of archaea. In these organisms, in absence of the expected characterized asparagine biosynthetic genes, this protein is responsible for the production of asparagine [10]. The analysis of bacterial genomes reveals the presence of GenX, a lysyl-tRNA synthetase (LysRS) ortholog. While the existence of this protein has been reported before [11], no function or activity is known. Thus, we undertook the investigation of its biochemical properties.

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2. Materials and methods

2.1. General

Uniformly labeled $[^{14}C]$lysine (78 Ci/mmol), sodium $[^{32}P]$pyrophosphate (15 Ci/mmol) were from Amersham Biosciences. l-lysine and lysine analogs were purchased from Sigma. The genX::kan Escherichia coli knockout strain as well as the parental wild type strain K-12 BW25113 was kindly sent by H. Mori Laboratory (Keio University, Yamagata, Japan). E. coli LysRS was purified as previously described [12].

2.2. Cloning of E. coli genX (yjeA)

E. coli genomic DNA was extracted from E. coli W3110 strain. E. coli genX encoding gene was amplified from corresponding genomic DNA under standard PCR conditions using the following primers Forward: (NdeI) 5’-CATATGAGCGGAACGGCATTGGC and Reverse: (BamHI) 5’-GGATCCTTATGCCCGGTCAACGCTAAAG; the amplified fragment was cloned into Topo TA (Invitrogen) vector and transformed into Top 10 E. coli cells. The internal Ndel restriction site in genX was removed by site-directed mutagenesis using Qiagen quick-change technology. genX was sequenced prior to sub-cloning into Pet15b (Invitrogen) expression vector.

2.3. Overexpression and purification of GenX

E. coli BL21(DE3) cells transformed were grown in Luria broth supplemented with ampicillin (100 μg/ml) at 37 °C to an optical density at 600 nm of 1.0. The expression of the recombinant protein was then induced by IPTG (isopropyl-β-D-thiogalactopyranoside), final concentration of 1 mM) for 1 h at 37 °C. Longer over-expression times had a lethal effect on the host cells. The cells were harvested by centrifugation and washed with phosphate buffered saline; this and subsequent steps were performed at 4 °C. The cell paste was resuspended into lysis buffer (50 mM Tris–HCl, pH 8.0, 500 mM NaCl, 5 mM 2-mercaptoethanol, protease inhibitor (Roche) and sonicated for 10 cycles of 30 s. The cell extract was obtained by ultracentrifugation at 100,000 g for 45 min and subsequently applied to a Ni-nitrilotriacetic acid agarose column (Qiagen), according to the standard procedure. The protein was eluted with an elution buffer (containing 50 mM Tris–HCl [pH 8.0], 500 mM NaCl, 5 mM 2-mercaptoethanol, 2 mM ATP, 25 μM radioactive amino acid $[^{14}C]$lysine, and 40–60 μg of unfraccionated tRNA or 0.7–1.3 μg of pure tRNAlys (Sigma). Enzyme concentration was 500 nM (genX) or 100 nM (LysRS). Aliquots (20 μl) were taken at different times, spotted on Whatman 3MM filter paper discs, and washed twice with 5% TCA.

2.4. ATP-[32P]PPi exchange reaction

The reaction mixture of 0.2 ml contained 100 mM Tris–HCl, pH 8.0, 10 mM MgCl2, 50 mM KCl, 2 mM 2-mercaptoethanol, 2 mM KF, ATP concentration either fixed at 2 mM or varying from 0.1 to 10 mM for $K_m$ determination, l-lysine concentration fixed at 10 mM or varying from 1 to 50 mM for $K_m$ determinations, 2 mM $[^{32}P]$PPi (1.6 cpm/pmol) and 500 nM GenX protein. After various incubation times at 37 °C, the $[^{32}P]$ATP present in 40 μl aliquots of the reaction mixture was specifically adsorbed on acid-washed Norit [0.2 ml of a 1%(w/v) in 0.4 M sodium pyrophosphate solution with 15% (v/v) perchloric acid], rinsed with 15 ml of water on Whatman GF/C fiber glass filter disks, dried, and liquid scintillation-counted. Effect of Mg$^{2+}$ on l-lysine activation by GenX was investigated by varying the concentration in MgCl2 from 0 to 10 mM in the reaction buffer. Likewise, l-lysine activation pH optimum was determined by changing the nature of the reaction buffer and its pH (Citrate–Na [pH 4.0 and 5.0], BisTris–HCl [pH 6.0 and 6.5], Hapes–Na [pH 7.2 and 7.5], Tris–HCl [pH 8.0 and 8.8], Glycine [pH 9.0 and 10.0] and CAPS [pH 11.0]) while maintaining the buffer concentration to 100 mM in all cases. The l-lysine activation temperature optimum was determined in the standard conditions by varying reaction temperature from 25 to 65 °C.

2.5. Aminoacylation assay

In vitro acylations with E. coli GenX and LysRS enzymes were carried out at 37 °C in a 120 μl reaction mixture containing 100 mM Tris–HCl, pH 8.0, 10 mM MgCl2, 50 mM KCl, 5 mM 2-mercaptoethanol, 2 mM ATP, 25 μM radioactive amino acid $[^{14}C]$lysine, and 40–60 μg of unfraccionated tRNA or 0.7–1.3 μg of pure tRNAlys (Sigma). Enzyme concentration was 500 nM (genX) or 100 nM (LysRS). Aliquots (20 μl) were taken at different times, spotted on Whatman 3MM filter paper discs, and washed twice with 5% TCA.

3. Results

3.1. GenX is a bacterial class II LysRS ortholog

Analysis of bacterial genomes revealed the presence of two distinctive types of class II LysRS orthologs. The first one, termed LysX, is restricted to organisms from the Actinobacteria group [13]. To date it is found in nine organisms including all four sequenced mycobacterial species. LysX is a fusion protein between a protein homologous to a class II LysRS (38% homology and 41% similarity to E. coli LysRS) and a polypeptide that shows homology to MprF (23% identity and 42% similarity to S. aureus MprF), a membrane protein known to transfer lysine to phospholipids in Gram-positive bacteria [14]. The domain with sequence homology to MprF is appended to the amino terminus of the LysRS-like protein. The second LysRS ortholog, known as GenX, PoxA or YjeA, is widespread among Gram-negative bacteria since homologs can be found in about 40 organisms, mostly in gamma proteobacteria (except for the Pseudomonadaceae group), in organisms from the alpha and delta proteobacteria, as well as in two human pathogen species from the spirochaetales group (Treponema pallidum and Leptospira interrogans). GenX is a well-conserved protein of 326 amino acids (in E. coli), which shows extensive sequence homology (31% identity and 51% homology) to the LysRS class II catalytic core but appears to be missing the well-characterized OB-fold anticodon binding domain of the class II LysRS, which is localized at the amino terminus of the polypeptide. Sequence alignment comparison of GenX with E. coli LysRS shows also almost complete conservation of the active site amino acids involved in l-lysine and ATP binding as identified by the crystal structure determination of the E. coli LysRS and confirmed by mutagenesis analysis (Fig. 1 and [15,16]). Amino acids shown to be crucial for lysine binding by LysRS (E240, R262, E278, Y280, E428 and N424) are strictly conserved amongst GenX homologs (Fig. 1). Likewise, amino acids involved in ATP binding and recognition (E264, E414, E421 and E480) are conserved in the GenX active site (Fig. 1). Only G216, involved in
binding of the alpha amino group of lysine in LysRS is absent from the GenX active site (Fig. 1). Furthermore, a five amino acid replacement in GenX (compared to LysRS) is clearly noticeable immediately after motif I. Based on these observations we cloned E. coli genX, purified the corresponding gene product, and analyzed its enzymatic properties.

3.2. GenX activates l-lysine

ATP-[32P]PPi exchange experiments with the pure recombinant GenX shows that the protein is able to activate l-lysine in the presence of ATP (Fig. 2A). Determination of the kinetic parameters in the ATP-PPi exchange reaction shows that GenX has a lower affinity for l-lysine (6.2 mM) than its full-length LysRS counterpart (45 μM, reported in [12] and [16]). The affinity for ATP was however similar for both enzymes: 200 μM for GenX and 50 μM and 227 μM for E. coli and Bacillus steatorrhophilus LysRS respectively ([12] and [17]). ATP is most likely the natural substrate for this enzyme since no activity was detected in the presence of the other three nucleotides. GenX is a slower enzyme than its LysRS counterpart since we found the turnover constant to be 0.9 s⁻¹ while published data for the LysRS range from 25 to 60 s⁻¹ ([12] and [16]). We also found that like LysRS, GenX requires the presence of magnesium ions to be able to activate lysine (Fig. 2B). In order to optimize the lysine activation we varied the pH and temperature conditions. We found that the GenX activity was the most robust at pH 8.0 and at temperatures ranging from 25 to 37 °C.

3.3. GenX activates l-lysine analogs

The use of cognate amino acid analogs either as substrate or inhibitor of the activation reaction has allowed mapping of the topology of the active site of a number of aminoacyl-tRNA synthetases. Extensive analysis of the way l-lysine analogs are recognized by E. coli LysRS has been conducted recently ([16] and [18]). The same approach had been used earlier to characterize B. steatorrhophilus LysRS active site [19]. Using the same set of analogs we investigated GenX amino acid recognition pattern (Fig. 3). We found marked difference between the two enzymes. E. coli LysRS readily activates S-(2-aminoethyl)-L-cysteine (AEC), ornithine, l-lysine methyl ester and to a lesser extend lysine analogs such as D-lysine, 5(R)-hydroxy-l-lysine and aminobutyric acid (Fig. 3). GenX has a much narrower substrate spectrum since only 5(R)-hydroxy-

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**Fig. 1.** Sequence alignment of GenX from various bacterial species (Styphi, Ecoli, Ypest, Hinf, Smeli, Lint refers to the following organisms: Salmonella typhimurium, E. coli, Yersinia pestis, Haemophilus influenzae, Sinorhizobium meliloti, Leptospira interrogans). EcoliS refers to the E. coli LysRS (encoded by lysS). Class II AARS defining motifs I, II and III are indicated. Residues involved in ATP and lysine binding are indicated by an open circle and a star respectively.

**Fig. 2.** GenX activates l-lysine in vitro (A) in a magnesium concentration manner (B). (A) amino acid activation reaction catalyzed by GenX was performed in the presence of 10 mM lysine and either 2 mM ATP (●) or a mix of CTP/GTP/UTP (2 mM each, □) and in the presence of a 19 amino acids mix (1 mM each, deprived of lysine) and either 2 mM ATP (●) or a mix of CTP/GTP/UTP (2 mM each, □). Lysine activation of E. coli LysRS in the presence of ATP was used as control (○). (B) Lysine (10 mM) activation by GenX in the presence of 2 mM ATP and 10 mM (●), 5 mM (▲), 1 mM (●), 0.5 mM (●) and 0 mM (○) magnesium chloride.
l-lysine and AEC could be activated (Fig. 3). Activation of 5(R)-hydroxy-l-lysine showed kinetic parameters sensibly similar to ones found for l-lysine. In terms of inhibition of lysine activation, GenX was the most efficiently inhibited by l-lysine hydroxamate and lysinamide with $K_i$ values of 150 and 280 $\mu$M respectively. These two l-lysine analogs are also among the best E. coli LysRS inhibitors [18].

5(R)-hydroxy-l-lysine is mostly found in eukarya and is not a common metabolite in bacteria. We thus looked for other potential l-lysine derivative present in bacteria. Diamino pimelic acid is the direct precursor of lysine and an important component of the bacterial cell wall peptidoglycan. Diaminopimelic acid could be activated by GenX although to a lesser extent than l-lysine and 5(R)-hydroxy-l-lysine (Fig. 3). However, as for 5(R)-hydroxy-l-lysine, diaminopimelic acid was a better substrate for GenX than for E. coli LysRS (Fig. 3).

3.4. GenX does not transfer lysine onto RNA

While pure lysine tRNA and unfractionated tRNA extracted from either wild type E. coli or the genX::Kan knockout strain could be aminocylated by E. coli LysRS, they were not substrate for GenX. Total RNA extract also containing heavier molecular weight RNA could not be lysylated by GenX either (data not shown).

4. Discussion

Evidence for the existence of GenX, also known as PoxA, was first reported in E. coli [11]. In this early work, an E. coli crude extract containing overexpressed recombinant GenX did not show more lysine activation than an E. coli crude extract control containing only an empty vector, implying that the truncated LysRS was unable to activate l-lysine [11]. We show here this is not the case since a purified LysRS-free recombinant GenX protein preparation is in fact able to activate l-lysine (Fig. 2). The lower affinity for l-lysine and lower catalytic turnover of GenX compared to LysRS may explain the result observed earlier. Given the sequence similarity between GenX and its LysRS counterpart, this dramatic difference in substrate affinity was initially surprising. Indeed, primary amino acid sequence comparison indicates that all residues of LysRS known to interact with lysine or ATP are conserved in GenX, except for glycine 216 (E. coli LysRS numbering, Fig. 1). LysRS crystal structures captured at different stages during lysine activation indicate that the oxygen of the amide peptide backbone of glycine 216 contacts the alpha amino group of the lysine substrate (Fig. 4A).

These contacts are responsible for triggering a series of complex active site conformational rearrangements [15]. Mutation of glycine 216 to alanine in LysRS lowers dramatically the affinity of the mutant enzyme for l-lysine to 4.5 mM, an affinity close to that determined for GenX [16]. The absence of glycine 216 together with a five amino acid replacement in the same region of the GenX active site is therefore a likely reason for the lower affinity for l-lysine (Fig. 4B). The low turnover number (0.9 s$^{-1}$) of GenX when compared to the value for LysRS may be a consequence of the lack of the anticodon binding domain. The deletion of the B. stearothermophilus LysRS anticodon binding domain reduced the enzyme’s $k_{cat}$ value 500-fold [19]; the $k_{cat}$ value decreased from 50 s$^{-1}$ for the full length enzyme to 0.1 s$^{-1}$ for its truncated counterpart [19]. The GenX turnover number is in line with that found for the anticodon-truncated B. stearothermophilus LysRS, and thus confirms the role of the LysRS amino terminal anticodon binding domain in stabilizing the lysine activation transition state. Lastly, the probing of the active site of GenX and LysRS using l-lysine structural analogs confirmed the notion of clear differences in the active site topology of the two enzymes. The fact that diaminopimelic acid and 5-hydroxy lysine are good substrates for GenX, but are not activated by the LysRS, suggests a more relaxed active site for the truncated homolog.

While we now have established that GenX retains the ability to activate l-lysine, its physiological role in a subset of bacteria remains unclear. GenX has been linked to expression regulation of pyruvate oxidase PoxB. Transposon insertion in genX was shown to significantly decrease PoxB expression in Salmonella typhimurium and in E. coli [20]. Inactivation of genX was also shown to provoke a severe attenuation of S. typhimurium virulence, making such a strain a possible candidate for vaccine development [21]. An E. coli GenX-inactivated mutant shows a pleiotropic phenotype, it is more sensitive than the wild type counterpart to treatment with antibiotics, a variety of amino acid analogs and dyes [20]. This paper also shows that the inactivated E. coli mutant is more sensitive to high concentrations of l-lysine than its wild type counterpart (Supplementary Fig. 1). A recent report links GenX to the formation of a post-translational modification on elongation factor P (EF-P) in bacteria [22]. In eukarya and archaea the EF-P homolog a conserved lysine is modified to hypussine. This amino acid, essential for EF-P function in organisms from the two aforementioned kingdoms, is absent in the bacterial EF-P homolog [22]. However, mass spectrometric analysis of the E. coli EF-P showed that the protein is modified at the homologous lysine with an unidentified structure of 145 Da in mass [23]. The modification of the EF-P lysine residue,
Vated amino acids have been shown to be transferred onto protein amino acid and an acceptor moiety is common in bacteria. Activated linkage (or the structurally equivalent ester) between an activated lysine analog via an amide linkage. Establishment of an amide by the coupling of lysine epsilon amine to the carbonyl group of a hydroxybutyl chain. The bacterial modification would be formed amine where the epsilon amino group of lysine carries an amino and its archaeal and eukaryotic counterparts. Hypussine is a secondary modification carried by EF-P in bacteria would differ from that of line with experimental measurement. The chemical nature of the stance, would increase the mass of EF-P by 146 Da, a number in attractive possibility [22]. The addition of hydroxy lysine, for possibly mediated by GenX, with an homolog of lysine is thus an appealing possibility [22]. The additional of hydroxy lysine, for instance, would increase the mass of EF-P by 146 Da, a number in line with experimental measurement. The chemical nature of the modification carried by EF-P in bacteria would differ from that of its archaeal and eukaryotic counterparts. Hypussine is a secondary amine where the epsilon amino group of lysine carries an amino hydroxybutyl chain. The bacterial modification would be formed by the coupling of lysine epsilon amine to the carbonyl group of a lysine analog via an amide linkage. Establishment of an amide linkage (or the structurally equivalent ester) between an activated amino acid and an acceptor moiety is common in bacteria. Activated amino acids have been shown to be transferred onto protein [24], nucleic acid [7], phospholipids [25] or other metabolites [26]. Hypussine exposes a positively charged amine to the surface of the eukaryotic EF-P molecule, mediating interaction with the ribosome. Modification of the bacterial EF-P with a lysine analog will also result in exposure of primary amine to the surface of the elongation factor, suggesting that both modifications could serve the same functional purpose [23].

While a GenX involvement in EF-P modification is an appealing suggestion, experimental proof will be needed for its biogenesis and its function in the cell.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2010.05.036.

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