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Affinity labelling *in situ* of the bL12 protein on *E. coli* 70S ribosomes by means of a tRNA dialdehyde derivative

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In this report, we have used periodate-oxidized tRNA (tRNAox) as an affinity laleling reagent to demonstrate that: (i) the bL12 protein contacts the CCA-arm of P-site bound tRNA on the *Escherichia coli* 70S ribosomes; (ii) the stoichiometry of labelling is one molecule of tRNAox bound to one polypeptide chain of endogenous bL12; (iii) cross-linking *in situ* of bL12 with tRNAox on the ribosomes provokes the loss of activity; (iv) intact tRNA protects bL12 in the 70S ribosomes against cross-linking with tRNAox; (v) both tRNAox and pyridoxal 5′-phosphate (PLP) compete for the same or for proximal cross-linking site(s) on bL12 inside the ribosome; (vi) the stoichiometry of cross-linking of PLP to the recombinant *E. coli* bL12 protein is one molecule of PLP covalently bound per polypeptide chain; (vii) the amino acid residue of recombinant bL12 cross-linked with PLP is Lys-65; (viii) Lys-65 of *E. coli* bL12 corresponds to Lys-53 of eL42 which was previously shown to cross-link with P-site bound tRNAox on human 80S ribosomes *in situ*; (ix) finally, *E. coli* bL12 and human eL42 proteins display significant primary structure similarities, which argues for evolutionary conservation of these two proteins located at the tRNA-CCA binding site on eubacterial and eukaryal ribosomes.

Keywords: *E. coli* 70S ribosomes; *E. coli* ribosomal protein bL12; Lys-65 of bL12; periodate-oxidized tRNA; tRNA-CCA binding site.

Abbreviations: bL12 (formerly L12), eubacterial large subunit ribosomal protein L12 with a free NH_2 terminus, according to the new system for naming ribosomal proteins; L7, the NH2-terminally acetylated bL12; eL42, eukaryal or archaeal large subunit ribosomal protein L42 (formerly L42A or L42AB in yeast or L36a in human, or L44e in archaea); PLP, pyridoxal 5'-phosphate; PMP, pyridoxamine 5'-phosphate; PTC, peptidyl transferase centre; rP, ribosomal protein; tRNAox, periodate-oxidized tRNA, the 2′, 3′-dialdehyde derivative of tRNA; uL10, the large subunit ribosomal protein L10 present in the three major kingdoms of life.

In all kingdoms of life, the ribosome consists of a large and a small subunits, each of which having separate functions. Peptide bond formation occurs at the peptidyl transferase centre (PTC) of the large subunit (50S in eubacteria and archaeabacteria, and 60S in eukaryotes), whereas mRNA sequences are decoded on the small subunit (30S in bacteria and 40S in eukaryotes).

The current view of the PTC is that it is composed solely of RNA, and that no protein is involved in the mechanism of peptide bond formation (*1*, *2*). This view stemmed from the observation of the 3D structure of the 50S ribosomal subunit of *Haloarcula marismortui*, which reveals a void of protein electron density in a radius of 18 angströms of the PTC (*1*, *3*). However, crystallographic data on the full 70S ribosome from *Thermus thermophilus* show that ribosomal protein L27 extends with its N-terminus into the PTC and makes contact with the tRNA substrates (*4*). In accordance with these structural data, Polikanov *et al.* (*5*), have recently proposed that the N-terminus of *T. thermophilus* L27 might take part in peptide bond formation. In sharp contrast with these structural data, it was recently demonstrated that L27 doesn't play any significant role in peptide-bond formation (*6*). Moreover, ribosomes from archaea or eukaryotes do not have protein L27 or any homologous counterpart, indicating that L27 cannot be a part of an evolutionarily conserved peptidyl transfer mechanism.

It should be recalled that, in the past, all steps of the translation process, including the peptidyl transfer step, were thought to be supported by proteins (*7*–*12*). Accordingly, early biochemical studies had demonstrated that peptidyl transferase is an enzyme with the following characteristics: (i) the reaction of peptidyl transfer has a pH dependence with a pKa of ~7.2, which was supposed to reflect the presence on the enzyme of an ionizable functional group such as histidine (*7*–*11*); (ii) ribosomal inactivation by ethoxyformic anhydride (a reagent specific for His) was optimal at pH 7.0 (*8*), while photochemical inactivation of the ribosome with Rose Bengal dye exhibited a pH optimal at 7.5 (*9*); (iii) other small-molecule inhibitors of the activity of the ribosome known to target amino acid side chains include pyridoxal 5'-phosphate (PLP), which inactivates *Escherichia coli* ribosomes by

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covalently binding in majority to the L7/L12 protein (*12*). However, the amino acid residue(s) targeted by these reagents on the ribosome had not been identified. Since, to date, structural and biochemical studies failed to identify any ribosomal group that acts in chemical catalysis of peptide bond formation (*1*–*12*), new tools are needed. Among these, affinity labelling represents a suitable strategy for the identification of the amino acid residues involved in the molecular mechanisms of biological processes. In this context, we have previously demonstrated that the lysyl residue 53 of the human large subunit ribosomal protein eL42 could be affinity labelled with periodate-oxidized tRNA (tRNAox), the 2′,3′-dialdehyde derivative of tRNA at the P/E hybrid site, and that this covalent reaction completely abolished the poly(Phe) synthesis activity of the human 80S ribosomes (*13*). This result suggests that Lys-53 might play a functional role. Since the eubacterial ribosomes do not have the eukaryote-specific eL42 protein or any homologous counterpart, we have applied in the present report the same affinity labelling strategy to *E. coli* ribosomes, with the goal of identifying the protein(s) located at the tRNA-CCA binding site on these eubacterial ribosomes. Here, we demonstrate that the *E. coli* large subunit ribosomal protein bL12 can be affinity labeled with tRNAox and that this 2′,3′-dialdehyde derivative of tRNA fulfills all the criteria for a site-directed affinity label of the bL12 protein inside the *E. coli* 70S ribosomes. Notably, (i) the stoichiometry of covalent binding of tRNAox to bL12 is one molecule of tRNAox bound to one polypeptide chain of endogenous bL12; (ii) cross-linking *in situ* of bL12 with tRNAox on the *E. coli* ribosomes provokes the loss of activity; (iii) intact tRNA protects bL12 in the *E. coli* 70S ribosomes against cross-linking *in situ* with tRNAox; (iv) both tRNAox and PLP compete for the same or for proximal cross-linking site(s) on bL12 inside the ribosome, while the amino acid residue of recombinant bL12 cross-linked with PLP is Lys-65; (v) Lys-65 of the 62GANK65 motif of *E. coli* bL12 corresponds to Lys-53 of the 49GGQTK53 motif of the large subunit ribosomal protein eL42, which was previously shown to cross-link with P-site bound tRNAox on human 80S ribosomes *in situ*. Note that the new system for naming ribosomal proteins (*14*) is used in this report. Accordingly, the eubacterial large subunit ribosomal protein L12 with a free NH2 terminus, as well as the NH2-terminally acetylated L7 chain whose amino acid sequence is identical to that of L12 are both called bL12. Similarly, the eukaryal or archaeal large subunit ribosomal proteins of the L44E family (formerly L42A or L42AB in yeast or L36a in human, or L44e in archaea) are called eL42 (*14*).

Materials and Methods

Materials

E. coli 70S ribosomes were obtained as already described (*15*). 40S and 60S ribosomal subunits with intact rRNAs were isolated from unfrozen human placenta (*16*). 80S ribosomes were obtained by association of re-activated 40S and 60S subunits and used in control cross-linking experiments, as previously reported in (*13*, *17*). Recombinant large subunit ribosomal protein bL12 from *E. coli* was purified from the overproducing strain BL21(DE3) carrying recombinant plasmid pET21L12C-6His (a generous gift of Dr S. Sanyal). The purification procedure consisted mainly in a chromatographic step on a Ni-NTA superflow column (Qiagen). PLP was purchased from Sigma Aldrich. Trypsin was from Promega. The short mRNA analogues (oligoribonucleotides GAA UUU GAC AAA and GAA AUG GAC AAA) as well as *E. coli* $tRNA^{Phe}$ were purchased from Sigma Aldrich. $tRNA^{Asp}$ from yeast was purified by counter-current chromatography, followed by separation by polyacrylamide gel electrophoresis. Its amino acid acceptance capacity was 1,400 pmol/A_{260} unit. ^{32}P-labelling of tRNA at the 5′-end was carried out after dephosphorylation by alkaline phosphatase (Roche), in the presence of γ-[^{32} P]ATP and polynucleotide kinase. tRNAox was prepared as described previously in (*13*, *17*).

Methods

Cross-linking of E. coli *70S ribosomes with tRNAox or PLP*. On one hand, *E. coli* 70S ribosomes (0.5 μM) were incubated at 37 °C in buffer A (50 mM Tris–HCl, pH 7.5, 100 mM KCl, 10 mM $MgCl_2$ and 0.5 mM EDTA) containing 5 μM of the dodecaribonucleotide GAA UUU GAC AAA as an mRNA, in a total volume of 25 μl. The labelling reaction was initiated by the addition of 10 μM [^{32}P]$tRNA^{Asp}$ox and 5 mM sodium cyanoborohydride ($NaBH_3CN$), and the incubation continued for 1 h. After the termination of the tRNAox-labelling reaction, the incubation mixtures were quenched by the addition of 1 μl of 0.5 M sodium borohydride ($NaBH_4$) freshly prepared in 50 mM phosphate buffer (pH 7.5). 20 μl portions of the $NaBH_4$-quenched samples were applied onto a 10% polyacrylamide gel which was run by SDS-gel electrophoresis. The gels were dried under vacuum and subjected to autoradiography. Control experiments for cross-linking of ribosomal proteins with tRNAox contained [^{32} P]$tRNA^{Asp}$ox (10 μM) incubated in the same conditions, in the absence of ribosomes. tRNAox-labelling of recombinant *E. coli* bL12 (2 μM) with [^{32}P]$tRNA^{Asp}$ox (10 μM) was performed in the same conditions as for the ribosomes. In order to identify the ribosomal proteins cross-linked *in situ* with tRNAox by mass spectrometric analyses, the 10% polyacrylamide gel was run by Urea-gel electrophoresis for the preparation of those proteins. On the other hand, *E. coli* 70S ribosomes (0.5 μM) in 25 μl were pre-incubated with 1 mM PLP, 1 mM pyridoxamine 5′-phosphate (PMP) or 20 μM native $tRNA^{Asp}$ at 25 °C in buffer A, prior to the addition of [^{32}P]$tRNA^{Asp}$ox (10 μM).

Poly(Phe) synthesis activity of E. coli *70 S ribosomes*. Poly(Phe) synthesis was determined as incorporation of L-[^{3}H]Phenylalanine into hot trichloroacetic acid-insoluble material, as previously described in (*13*). The final reaction mixture (70 μl) contained 40 mM Tris-HCl (pH 7.5), 7 mM $MgCl_2$, 80 mM NH_4Cl, 1 mM dithiothreitol, 1 mM ATP, 1 mM phosphoenolpyruvate, 0.5 mM GTP, 50 μg/ml pyruvate kinase, 2.5 μM $tRNA^{Phe}$ (first charged during a 30 min incubation at 30 °C with a 3 fold excess of L-[^{3} H]Phenylalanine (37 GBq/mmol) and a saturating amount of partially purified phenylalanyl-tRNA synthetase), 6 μg poly(U), 0.5 μM EF-Tu, 0.2 μM EF-Ts and 0.3 μM EF-G. To check the effect of the cross-linking of the *E. coli* 70S or the human 80S ribosomes with $tRNA^{Phe}$ox on the poly(U)-dependent synthesis of poly(Phe), the reaction (70 μl) was started with 14 μl of mixture containing 14 pmol of the *E. coli* 70S or the human 80S ribosomes that were cross-linked with $tRNA^{Phe}$ox, with final concentrations of 0.3 μM ribosomes and 4.4 μM tRNAs. The concentrations of elongation factors with the eukaryotic 80S dependent poly(Phe) synthesis were 0.5 μM EF-1α, 0.15 μM EF-1β and 0.35 μM EF-2. During incubation at 30 °C with the *E. coli* system, or at 37 °C with the eukaryotic system, 15 μl samples were withdrawn at indicated times and spotted on glass fibre filters and hot trichloroacetic acid insoluble radioactivity was determined.

Cross-linking of recombinant E. coli *bL12 protein with PLP*. Recombinant bL12 protein (350 μM) in 100 μl was incubated with PLP (350 μM) during 30 min at 30 °C in 50 mM phosphate buffer (pH 7.5). The incubation mixtures were quenched by the addition of 1 μl of 2 M sodium borohydride ($NaBH_4$) freshly prepared in 50 mM phosphate buffer (pH 7.5). The $NaBH_4$-quenched samples (100 μl) were applied to a Superdex 75 column 10 × 300 GL (G&E Healthcare) equilibrated in 50 mM sodium phosphate buffer (pH 7.5) containing 150 mM NaCl. The flow rate was 0.4 ml/min.

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Elution was monitored by the absorbance at 280 nm (for the protein) and 325 nm (for the covalently bound PLP) (*18*, *19*).

Sample preparation for mass spectrometric analyses. After elution from the Superdex column, the phosphopyridoxylated recombinant bL12 protein absorbing at 325 nm was analysed by ESI mass spectrometry in order to measure the stoichiometry of cross-linking with PLP, or digested with trypsin prior to mass spectrometric analysis, to search for the phosphopyridoxylated peptides of bL12.

Analysis by ESI mass spectrometry of bL12 cross-linked with PLP. Solutions of bL12 protein or of bL12-PLP covalent complex were desalted using a Ziptip C4 (Millipore Saint-Quentin-en-Yvelines, France), the proteins were eluted with 10 µl of a solution of 39% water 1% formic acid and 60% acetonitrile. These eluates were loaded into NanoESI spray capillaries (Thermo Fisher Scientific, Les Ulis, France). A 1.5 kV voltage was applied on the capillary to produce a stable spray in front of an orbitrap mass spectrometer. Analyses of the multi-charged ions produced were done in the orbitrap (Thermo Fisher Scientific) settled with a resolution of 100,000 at m/z 400. Multi-charged spectra were deconvoluted using the Xtract software (Thermo Fisher Scientific) in order to determine the molecular weight of the protein and the covalent complex.

Analysis of the tryptic digest of the bL12-PLP complex by MALDI MS. The covalent complex was digested overnight by trypsin in a 0.1 M ammonium carbonate buffer. After digestion the solution was desalted using a Ziptip C18 and the peptides were eluted and loaded on a MALDI plate using a solution of 5 mg/ml of α-Cyano-4-hydroxycinnamic acid in 30% (water 0.1% TFA) 70% acetonitrile. MALDI-TOF MS and MALDI-TOF/TOF MS/MS analyses were performed using a 5800 MALDI-TOF/TOF mass spectrometer (AB Sciex, Les Ulis, France). The instrument was equipped with an Nd:YAG laser (operating at 355 nm wavelength, 500 ps pulse and 200 Hz repetition rate). Acquisitions were performed in the positive ion mode MS were processed using DataExplorer 4.4 (AB Sciex).

Results

Affinity labelling in situ of rp bL12 with tRNAox on the *E. coli* 70S ribosomes

We had previously used tRNAox as an affinity labelling reagent, to identify lysine and arginine residues at the tRNA-CCA binding site on enzymes of the translation apparatus, especially the aminoacyl-tRNA synthetases (*20–25*). This 2′,3′-dialdehyde derivative of tRNA had been obtained by the periodate treatment of native tRNA, which specifically oxidizes the 2′,3′-cis-diol function of the 3′-terminal ribose of tRNA. This approach had led to the discovery of the KMSKS motif that is considered today as a signature for the active site of class 1 aminoacyl-tRNA synthetases (*26*). More recently, we have demonstrated that the lysyl residue 53 of the human large subunit ribosomal protein eL42 could be affinity labelled with tRNAox at the P/E hybrid site, and that this covalent reaction completely abolished the poly(Phe) synthesis activity of the human 80S ribosomes, suggesting that Lys-53 might play a functional role (*13*). We have applied in the present report the same affinity labelling strategy to *E. coli* ribosomes, with the goal of identifying lysine or arginine residues at the binding site for the CCA arm of tRNA.

When we incubated *E. coli* 70S ribosomes or the Human 80S ribosomes (0.5 µM) in the presence of the dodecaribonucleotide GAAUUUGACAAA as mRNA (5 µM) and of 10 µM [^{32}P]tRNA^{Asp}ox positioned at the P-site, only one ribosomal protein was found cross-linked in *E. coli* and in human, respectively, as revealed by the analysis of the incubation mixtures of cross-linking by SDS-PAGE on 10% polyacrylamide gels (Fig. 1A and (*13*, *17*)). The radioactive band corresponding to unreacted [^{32}P]tRNAox in the reaction mixtures was assigned by the position of a control [^{32}P]tRNA^{Asp}ox loaded alone in Lane 3 (Fig. 1A). These proteins were identified as the *E. coli* large subunit ribosomal protein bL12 and the human eL42 protein, respectively, by MALDI mass spectrometric analyses performed as described in (*27*). In the case of the *E. coli* bL12 protein, five peptides corresponding

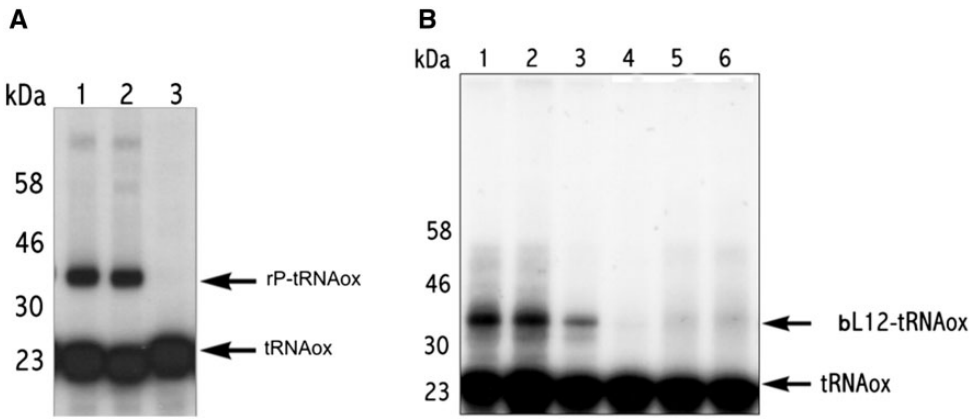


Fig. 1 Autoradiogram of the SDS gel electrophoresis analysis in 10% polyacrylamide of [^{32}P]tRNA^{Asp}ox-labelled endogenous proteins of *E. coli* 70S or human 80S ribosomes. The tRNAox-labelling incubation mixtures were as described in Materials and Methods. (**A**) Lane 1: *E. coli* 70S ribosomes incubated with [^{32}P]tRNA^{Asp}ox. The protein contained in the rP-tRNAox band in Lane 1 was identified as the large subunit ribosomal protein bL12 by mass spectrometry NanoLC-MS/MS (see text); Lane 2: human 80S ribosomes incubated with [^{32}P]tRNA^{Asp}ox. The protein contained in the rP-tRNAox band in Lane 2 was identified as the human large subunit ribosomal protein eL42 (see text); Lane 3: [^{32}P]tRNA^{Asp}ox alone. (**B**) Lane 1: *E. coli* 70S ribosomes (0.5 µM) incubated with [^{32}P]tRNA^{Asp}ox (10 µM); Lane 2: *E. coli* 70S ribosomes (0.5 µM) incubated with 1 mM PMP, prior to the addition of [^{32}P]tRNA^{Asp}ox (10 µM); Lane 3: *E. coli* 70S ribosomes (0.5 µM) incubated with a mixture of PLP (0.2 mM) and [^{32}P]tRNA^{Asp}ox (10 µM); Lane 4: *E. coli* 70S ribosomes incubated with PLP (1 mM) during 30 min, prior to the addition of [^{32}P]tRNA^{Asp}ox (10 µM); Lane 5: *E. coli* 70S ribosomes incubated with [^{32}P]tRNA^{Asp}ox-red (10 µM), the [^{32}P]tRNA^{Asp}-dialdehyde derivative reduced with $NaBH_4$; Lane 6: protection with native tRNA^{Asp} (20 µM) against labelling by [^{32}P]tRNA^{Asp}ox of ribosomal protein bL12 inside the *E. coli* 70S ribosomes. Incubation time was 1 h.

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to about 50% of the sequence coverage were found after in-gel digestion of the Urea-PAGE rP-tRNAox gel band in Lane 1 of Figure 1A, followed by analysis by MALDI mass spectrometry. The amino acid sequences deduced from the analyses are shown in Supplementary Table S1. As for eL42 (used here as a control in Lane 2 of Fig. 1A), the identification that was already reported (*13*, *17*), was reproducibly achieved in this study (results not shown). As previously described for the preparation of ribosomal proteins cross-linked *in situ* with tRNAox, in order to identify those proteins by mass spectrometric analyses, the 10% polyacrylamide gel was run by urea-gel electrophoresis. Indeed, as discussed in a previous report (*17*), an advantage in running polyacrylamide gel electrophoresis in urea is that the majority of the rPs migrate towards the (−) pole because they are positively charged, whereas rP-tRNAox covalent complexes with their additional 75 negative charges of the phosphate groups of tRNA migrate in the opposite direction. As a consequence, the rP-[^{32}P]tRNAox band can be easily detected alone on the Urea-PAGE gel.

tRNAox is a site-directed affinity label of bL12 in the *E. coli* 70S ribosomes

Our data show that tRNAox fulfills all the criteria for a site-directed affinity label of the bL12 protein inside the *E. coli* 70S ribosomes. As shown in Figure 1, the bL12-tRNAox covalent complex has an apparent molecular weight of 36,000 ± 1,000 Da that agreed well with the one expected for a covalent complex containing one polypeptide chain of endogenous *E. coli* bL12 (about 12,000 Da) and one molecule of tRNAox (25,000 Da; calculated MW of the binary rP-tRNAox complex 37,000 Da). Interestingly, the eL42-tRNAox covalent complex has also an apparent molecular weight of 36,000 ± 1,000 Da comprising one molecule of eL42 (molecular weight 12,000 Da) and one molecule of tRNAox (25,000 Da), as previously reported (Fig. 1 and (*13*, *17*)). The 1:1 stoichiometry of covalent binding of tRNAox to bL12 is compatible with the presence of one binding site for the CCA-arm of tRNA and for the translation factors, in the C-terminal domain (CTD) of each of the four subunits of the protein. In fact, the bL12 protein from the *E. coli* 70S ribosomes is composed of two dimers bound to one copy of protein uL10 (formerly L10), which anchors the pentamer to the large 50S ribosomal subunit (*28*–*30*). Each monomer of bL12 contains one binding site for tRNA and for the elongation factors (*31*). In addition, intact tRNA completely protected bL12 in the *E. coli* 70S ribosomes against cross-linking *in situ* with tRNAox (Lane 6 in Fig. 1B). Since it is well known that native tRNA binds first to the P-site, this result might reflect competition between native tRNA^{Asp} and the dialdehyde derivative thereof for the binding to the P-site. This result suggests at the same time that the tRNA-bL12 covalent complex formation occurred specifically at the P-site of 70S ribosomes. It should be recalled that the 2′,3′-dialdehyde derivative of tRNA had also been previously shown to bind to the P-site as follows: [^{32} P]tRNA^{fMet}ox from *E. coli* was positioned onto the P-site of *E. coli* 70S ribosomes in the presence of the mRNA analogue G AAAUGGACAAA containing the universal P-site triplet AUG, and the same band representing the bL12-tRNAox covalent complex was observed on the polyacrylamide gel (*32*). This result strongly suggests that the 2',3'-dialdehyde derivative of [^{32}P]tRNA^{fMet} is bound to the P-site of *E. coli* 70S ribosomes, in a ternary complex with the AUG codon of mRNA (*32*). However, at that time, the protein labelled by tRNAox on the *E. coli* 70S ribosomes could not be identified due to technical problems (*32*). Finally, cross-linking *in situ* of bL12 with tRNAox in the *E. coli* 70S ribosomes provokes the loss of the activity of poly(U)-dependent poly(Phe) synthesis, suggesting that the lysyl residue cross-linked with tRNAox might play a functional role in eubacterial ribosomes (Fig. 2).

Affinity labelling *in situ* of rP bL12 with tRNAox on the *E. coli* 70S ribosomes with or without PLP

As previously reported in the case of human eL42, MALDI mass spectrometric identification of

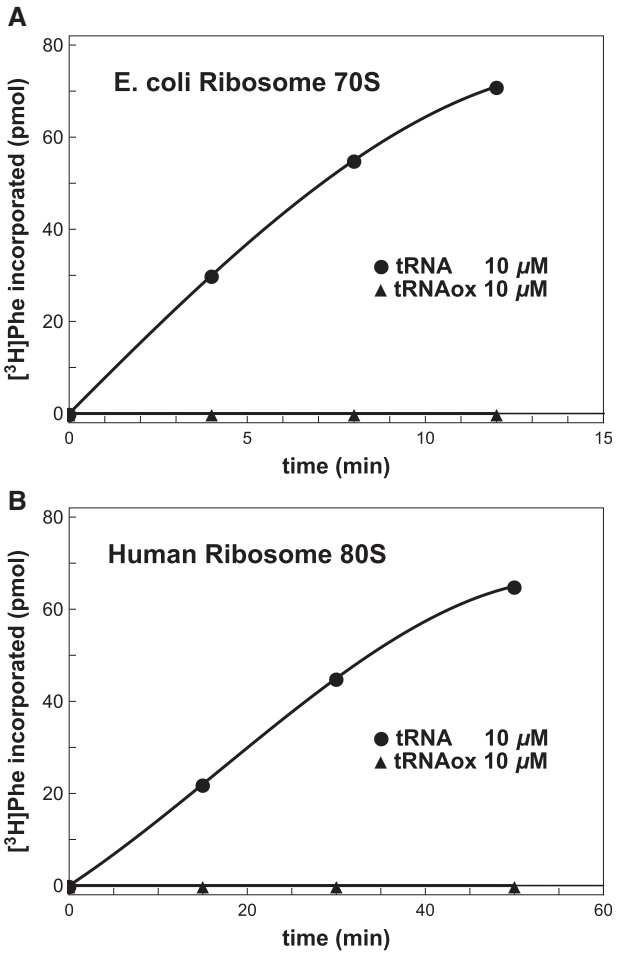


Fig. 2 Effect of tRNAox-labelling of the *E. coli* bL12 or the human eL42 proteins at the P-site on poly(Phe) synthesis by poly(U)-programmed *E. coli* or human ribosomes. Values on the ordinate axis represent the amount (pmol) of [^{3}H]Phenylalanine incorporated into the poly(Phe) polypeptide in the presence of tRNA^{Phe}ox (triangles) or of native tRNA^{Phe} (circles). For details, see Materials and Methods' Section.

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tRNAox-labelled peptides turned out to be very difficult due to the weakness of the signal of the cross-linked peptide and to mass deviations between calculated and measured masses. Therefore, the amino acid side chains targeted by tRNAox were identified indirectly by allowing this tRNA analogue to compete through the cross-linking reaction with small-molecule inhibitors of the activity of the ribosome whose cross-linking sites on bL12 can be more easily identified. Among these molecules, PLP had been previously shown to inactivate *E. coli* ribosomes by covalently binding in majority to the bL12 protein (*12*). Interestingly, it was previously shown that both tRNAox and PLP proceed by Schiff base formation with the side chain of a lysine (*12*, *18*, *20*, *27*), suggesting that they might target the same amino acid residues on bL12. Competition between tRNAox and PLP for the cross-linking with bL12 is supported by the demonstration that, in the affinity labelling reaction of aminoacyl-tRNA synthetases with tRNAox in the 1980s, there was generally only one lysine residue labelled in majority at the enzyme active site that exhibited a higher reactivity through Schiff base formation due to an abnormally low pKa value (*21*, *24*, *25*). This reactive lysine residue was always found equally cross-linked with tRNAox or with PLP (*18*, *21*, *24*, *25*). As shown in Figure 1B (Lane 5), treatment of [^{32}P]tRNA^{Asp}ox with $NaBH_4$ yielded a reduced [^{32}P]tRNA^{Asp}ox-red derivative, which failed to cross-link with bL12 on the *E. coli* 70S ribosomes. This result suggests that cross-linking of this protein with tRNAox proceeds through the formation of a Schiff base, as expected. Moreover, preincubation of *E. coli* 70S ribosomes with 1 mM PLP during 30 min prior to the addition of [^{32}P]tRNA^{Asp}ox prevented the cross-linking reaction *in situ* between bL12 and the tRNA dialdehyde derivative (Lane 4 in Fig. 1B). This result is compatible with the cross-linking of PLP proceeding equally through the formation of a Schiff base, as expected. Interestingly, as previously proposed by Ohsawa *et al*. (*12*), the cross-linking reaction *in situ* between PLP and the bL12 protein on the *E. coli* 70S ribosomes was shown to proceed through the formation of a Schiff base, by the following observation. PMP, which lacks the aldehyde group capable of forming a Schiff base with a lysyl ϵ-amino group could not prevent the cross-linking reaction *in situ* between bL12 and tRNAox (Lane 2 in Fig. 1B). Altogether, the above-cited data suggest that both PLP and tRNAox are capable of cross-linking with the ϵ-amino group of the same lysyl residue(s) of bL12. Competition between PLP and tRNAox for the cross-linking of bL12 was further demonstrated by the fact that, in the presence of 0.2 mM PLP and 5 µM [^{32}P]tRNA^{Asp}ox, the cross-linking yield is decreased (Lane 3 in Fig. 1B). We have used this PLP concentration because it corresponds to the K_D(app), that is the concentration for half-maximal amount of PLP-labelled lysyl residues at the phosphate binding site on the aminoacyl-tRNA synthetases (*18*). Application of such a K_D(app) value to the *in situ* cross-linking of bL12 with PLP on *E. coli* 70S ribosomes would explain why the yield of cross-linking with tRNAox was decreased (Lane 3 in Fig. 1B).

Analysis by mass spectrometry of bL12 cross-linked with PLP

The possibility that tRNAox and PLP might compete for the same cross-linking site(s) on the *E. coli* 70S ribosomes prompted us to search for the lysyl residue(s) cross-linked with PLP on bL12. However, a prerequisite for this experiment is to verify that bL12 effectively cross-links with PLP as previously reported by the group of Gualerzi (*12*). For this purpose, we have used the recombinant protein rather than the whole ribosome for the following reasons: (i) the ternary and quaternary structures of the bL12 protein, as deduced from intensive structural studies in solution and in the ribosome, or from the crystal structure of the whole protein were previously shown to be comparable in solution and in the ribosome, suggesting that the 3D structure of the pentameric $(bL12)_4$-uL10 complex is independent of the whole 50S ribosomal subunit (*29–31*, *33*, *34*); (ii) protein bL12 can be removed easily and selectively from the large ribosomal subunit (*35*); (iii) wild-type bL12 can be replaced by variant bL12 proteins (*35*). Taking advantage of this special feature, Sanyal's group in Uppsala (Sweden) has constructed a novel *E. coli* strain JE 28, in which a (His)6-tag has been inserted at the C-terminus of bL12 (*35*). These data suggest that the bL12 protein might behave similarly on and off the ribosome, and that the extent of PLP incorporation into this protein would be most probably the same on and off the *E. coli* 70S ribosomes. The calculated molecular mass of the polypeptide chain of the recombinant *E. coli* bL12 protein used in this study was obtained as follows: (i) the molecular mass of the *E. coli* bL12 polypeptide chain without any post-translational modification was 12,295 Da, as indicated in the Protein Data Bank; (ii) this polypeptide chain was His-tagged (at the C-terminus) with the sequence Leu-Glu-$(His)_6$ of molecular mass 1,064 Da, giving rise to a His-tagged bL12 protein of molecular mass 13,359 Da; (iii) since this recombinant protein was expressed in an *E. coli* strain, post-translational modification by removal of the N-terminal methionine (131 Da) would result in a mature bL12 polypeptide chain of molecular mass 13,228 Da. Supplementary Figure S1 shows the ESI orbitrap spectrum of the bL12 protein (Supplementary Fig. S1A) as well as the one of the bL12-PLP covalent complex (Supplementary Fig. S1B). The monoisotopic mass of the bL12 protein was found equal to the calculated monositopic mass: 13,220.95 Da (Supplementary Fig. S2), corresponding to the *E. coli* bL12 polypeptide chain with a free NH_2 terminus, His-tagged (at the C-terminus) with the sequence Leu-Glu-$(His)_6$ and lacking the N-terminal methionine. Determination of the molar ratio of bound PLP to bL12, as well as the identification of peptides modified by PLP were achieved simply by measuring the mass increase due to PLP (molecular mass of 231 Da). Supplementary Figure S2 shows the mass spectra of the control recombinant bL12 protein (Supplementary Fig. S2A) and of the bL12 protein

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cross-linked with PLP (Supplementary Fig. S2B). On one hand, the peak of monoisotopic mass 13,451.96 Da showed a mass value increased by 231 Da relative to the 13,220.94 Da peak corresponding to the molecular mass of the monomer bL12 with a free NH_2 terminus, indicating that the bL12 polypeptide chain is cross-linked with one molecule of PLP (Supplementary Fig. S2B). On the other hand, the 13,493.97 Da and the 13,262.95 Da peaks showed each a mass value increased by 41–42 Da relative to the 13,451.96 Da (covalent bL12-PLP complex) and the 13,220.94 Da (bL12) peaks, indicating that the NH_2-terminally acetylated polypeptide chain is also crosslinked with one molecule of PLP (Supplementary Fig. S2B). In conclusion, each polypeptide chain of bL12 was found to be cross-linked with one PLP molecule. However, the presence of both acetylated and non acetylated polypeptides that were not modified by PLP (Supplementary Fig. S2B) would suggest that not all the four chains of the tetramer are cross-linked with PLP. This situation is likely to be the consequence of the chemical modification of the recombinant bL12 protein (350 μM) with non-saturating amounts of PLP (350 μM). The same result was obtained when bL12 (350 μM) was allowed to react with 1 mM PLP.

Identification of the amino acid side chains targeted by PLP on the recombinant *E. coli* bL12 protein

Mass spectrometric analysis of tryptic peptides from 350 μM bL12 cross-linked with 350 μM PLP gave a $[M + H]^+$ signal at m/z 1,272.67 presenting a mass value increased by 231 Da (the molecular mass of PLP) relative to unmodified peptide with a calculated mass 1,041.64 Da corresponding to stretch 60AAGANKV AVIK70 (results not shown). Non cleavage of the peptide bond between Lys-65 and Val-66 implies that Lys-65 is actually the amino acid residue whose side chain is covalently labelled with PLP. Another peptide cross-linked with PLP was found at m/z 1598.88 (Fig. 3). It matched with fragment 60AAGANKVAVIKAVR73 (theoretical mass of 1,367.85 Da) whose mass was increased by 231 Da (Fig. 3). Owing to the 1:1 stoichiometry of modification of the protein with PLP on Lys-65 (Supplementary Fig. S2B), this fragment was assumed to result from a miscleavage at Lys-70. Finally, the 1,502.91 Da peak (Fig. 3) resulted from a metastable fragment characteristic of the loss of the phosphate group (98 Da) of PLP (*36*) from the 1,598.88 Da fragment, and may be considered as a signature of peptides cross-linked with PLP. It should be noted that, when bL12 (50 μM) was cross-linked with 1 mM PLP, only Lys-65 was found cross-linked with the reagent.

pK of the tRNAox-labelled lys-65 residue

We had previously demonstrated that the ribosomal protein eL42 could be affinity labelled *in situ* with tRNAox on the human 80S ribosomes (*13*, *17*, *27*). We had also demonstrated that the human recombinant eL42 protein could be equally affinity labelled with tRNAox (*27*). We had concluded that human eL42 is the only ribosomal protein that can interact with tRNA on and off the ribosome (*27*). Affinity labelling *in situ* with tRNAox of the eL42 protein from the human 80S ribosomes as a function of the pH of the incubation mixture indicated a pK value of 6.9 ± 0.2 for Lys-53 (*27*). Similarly, affinity labelling with tRNAox of the human recombinant eL42 protein as a function of the pH indicated a pK value of 6.9 ± 0.1

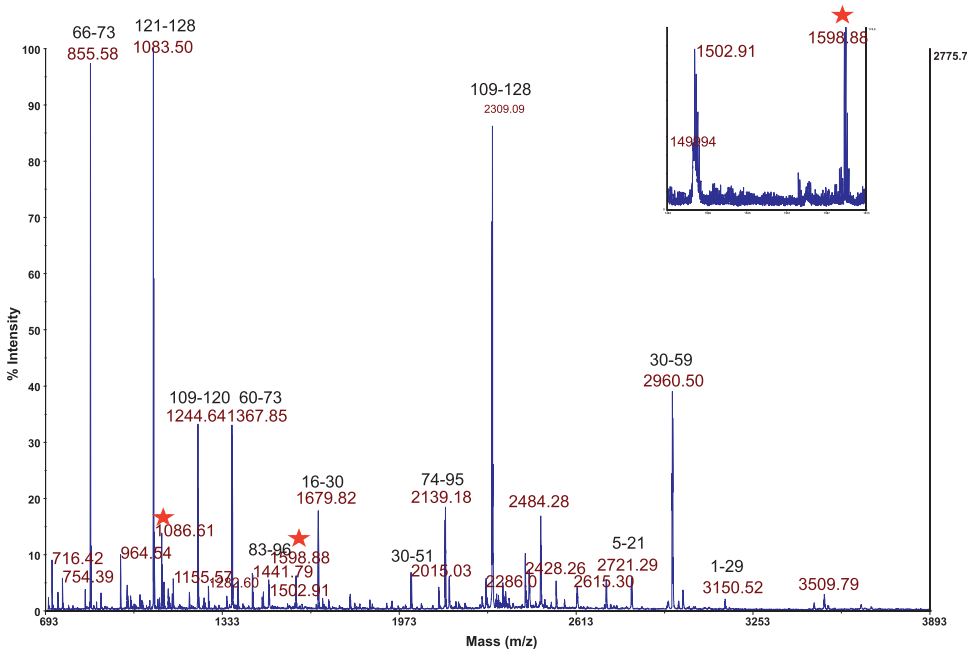


Fig. 3 MALDI mass fingerprint of tryptic peptides from bL12 cross-linked with PLP. The sequence coverage by the trypsin was 97% of the theoretical sequence. The main peaks are annotated. The stars correspond respectively to peptides 66VAVIKAVR73 (theoretical mass of 855.58 Da) and 60AAGANKVAVIKAVR73 (theoretical mass of 1367.85 Da) whose mass was increased by 231 Da. The zoom on the peak 1598.7 Da shows the metastable fragmentation of the peptide cross-linked with PLP.

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for Lys-53 (unpublished data). In the present report, we have applied these affinity labelling approaches to the bL12 protein on and off the *E. coli* 70S ribosomes, in order to check whether this eubacterial protein has the same behaviour as that of the eukaryal eL42. We have followed the tRNAox-labelling reaction between the free *E. coli* recombinant bL12 protein and [^{32}P]tRNAox as a function of the pH. Figure 4A shows the incorporation of [^{32}P]tRNAox into the recombinant bL12 as a function of the pH of the incubation mixture. The data from Figure 4A were used to plot the molar fraction of [^{32}P]tRNAox incorporated into (or cross-linked with) recombinant *E. coli* bL12 versus increasing pH values, and a pK value of 7.2 ± 0.2 was deduced for the ϵ-amino group of Lys-65 (Fig. 4B). [^{32}P]tRNAox incorporation into bL12 inside the *E. coli* 70S ribosomes as a function of the pH could be also followed. It indicated a pK value of 7.2 ± 0.1 for Lys-65 (results not shown). Similar pK values for the ϵ-amino group of Lys-65 found with the free recombinant and the ribosome-bound bL12 protein suggest that, similarly to human eL42 (*27*), bL12 is capable of interacting with tRNA on and off the ribosome. The pK value of 7.2 which is 3 units lower than is normally found for the side chain of a lysine residue (pK 10.5) describes the dissociation of the protonated form of the ϵ-amino group of Lys-65 and its greater reactivity through the tRNAox-labelling reaction, in accordance with the data obtained with eL42 from human 80S ribosomes.

Common features of the *E. coli* bL12 and the human eL42 proteins

First, these two proteins have comparable apparent molecular weights of about 12,000 Da. Comparison of the migration of the cross-linked complexes from human or *E. coli* ribosomes to that of protein markers on the same gels indicated apparent molecular weights of 36,000 ± 1,000 Da (Fig. 1). These molecular weights agreed well with that expected for a covalent complex containing one molecule of endogenous human eL42 or *E. coli* bL12 (about 12,000 Da each, according to the PDB) and one molecule of tRNAox (25,000 Da; calculated MW of the binary rP-tRNAox complex 37,000 Da). Second, as shown in Figure 2, cross-linking *in situ* of these proteins with tRNAox in the *E. coli* 70S or the human 80S ribosomes, provokes the loss of the activity of poly(U)-dependent poly(Phe) synthesis, suggesting that the lysyl residues cross-linked with tRNAox might play a functional role in eubacterial or eukaryal ribosomes, respectively. Since bL12 is the only *E. coli* large subunit ribosomal protein cross-linked with tRNAox, similarly to the human eL42 protein, one can conclude that cross-linking of these proteins with tRNAox is directly or indirectly responsible for the activity loss observed in Figure 2, as already proposed for human eL42 in a previous report (*12*). This conclusion corroborates the finding that the removal of bL12 from *E. coli* 70S ribosomes reduces the rate of protein synthesis (*35*). However, it has been shown in a previous study that the mutation K65A in bL12 reduces the binding affinity to the ternary complex EF-Tu:GTP:aminoacyl-tRNA by only a factor of 4, which is a very moderate effect (*37*). Third, they display significant primary structure similarities, with an average of 40-50% of identities and conservative replacements depending on the insertion of gaps into the amino acid sequences (Fig. 5A). In particular, the 62GANK65 motif of bL12, the lysyl residue of which is shown to cross-link with PLP in the present report, matches with the 49GGQTK53 motif of eL42 (Fig. 5B). The latter motif was found cross-linked with tRNAox through the formation of a Schiff base with Lys-53 in a previous study (*12*). The score of sequence similarity around the GANK motif was about 63% (Fig. 5B). Proteins eL42 are only found in archaea and eukaryotes. As previously reported, alignment of the amino acid sequences of eukaryotic eL42 proteins with those of the archaebacterial counterparts indicates only 44% average amino acid identities and

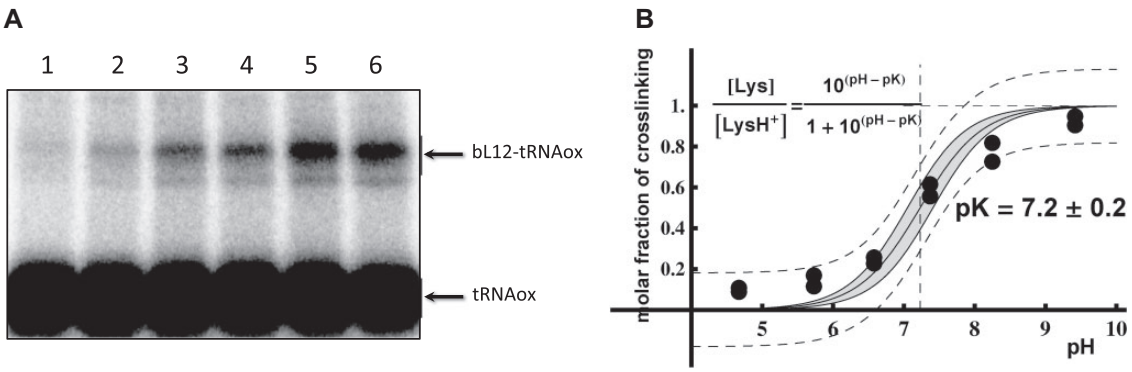


Fig. 4 Labelling of recombinant *E. coli* bL12 with [^{32} P]tRNAox as a function of the pH of the incubation mixture. (**A**), the incubation mixtures were analysed by SDS-PAGE on 10% polyacrylamide gels, and the yields of labelling were calculated as described earlier in (*27*). The tRNAox-bL12 covalent complex formed is shown. Lanes 1-6 correspond to pH 4.7, 5.8, 6.5, 7.4, 8.3 and 9.4, respectively. (**B**), plot of the molar fraction of [^{32}P]tRNAox incorporated into (or crosslinked with) recombinant *E. coli* bL12 as a function of the pH of the incubation mixture. The extents of incorporation of [^{32}P]tRNAox at pH 4.7, 5.8, 6.5, 7.4, 8.3 and 9.4 were determined from (A). Two datasets from two separate experiments as in (A) were used to obtain this graph. The data were fitted with Mathematica to the function inscribed in the figure with the following features: the dashed lines defines the 95% CIs for the prediction of a single value, whereas the continous lines and the gray-shaded region define the 95% CIs for the prediction of the average curve with pK = 7.2 ± 0.2.

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conservative replacements (*17*). Since it is generally believed that proteins sharing more than 30% primary structure similarity may be phylogenetically related, the 40–50% of identities and conservative replacements between eL42 and bL12 reported here (Fig. 5A) might reflect an evolutionary conservation of the eubacterial bL12 and the eukaryotic or the archaeal eL42 ribosomal proteins. Fourth, the tRNAox-labelled Lys-65 of *E. coli* bL12 and Lys-53 of human eL42 exhibit an abnormally low pK, as compared with the one that is normally found for the side chain of a lysine residue (pK 10.5). These results are consistent with the fact that both residues are reactive toward the reaction with tRNAox. Fifth, the GANK and GGQTK motifs occupy respectively comparable positions in the 3D structures of these proteins (Fig. 6A and B). In the 3D structure of human eL42 modelled by homology with the archaeal counterpart from *H. marismortui*, the GGQTK peptide is located in an extended loop at one extremity ((*27*) and Fig. 6B). Similarly, the GANK peptide is located at the tip of the crystallographic structure of the CTD of bL12 ((*30*, *31*, *33*) and Fig. 6A). Altogether, these structural similarities argue for the evolutionary conservation of the eL42 and bL12 proteins located at the tRNA-CCA binding site on eukaryal or eubacterial ribosomes, respectively.

Discussion

The *E. coli* large subunit ribosomal protein bL12 contacts the CCA-arm of P-site bound tRNA

Recently, we have applied an affinity labelling strategy to the human 80S ribosome, whereby we have demonstrated that the lysyl residue 53 of the large subunit ribosomal protein eL42 could covalently react with tRNAox at the P/E hybrid site, and that this reaction completely abolished the poly(Phe) synthesis activity of the human 80S ribosomes. In this report, application of the same affinity labelling strategy to *E. coli* ribosomes has led to the demonstration that the *E. coli* rP bL12 contacts the CCA-end of P-site bound tRNA, similarly to rP eL42 on the human 80S ribosomes. The bL12 protein had been localized within the *E. coli* 70S ribosome by Cryo-electron Microscopy (*29*) as two dimers bound to one copy of protein uL10 (formerly L10), which anchors the pentamer to the large

A

```
bL12     17 MDVVELISAMEEKFGVSAAAAVAVAAGPVEA--AEEKTEFDVILKAAGANKVAVIKAVRG 74
eL42      2 VNVPKTRRTFCKKCGKHQPHKVTQYKKGKDSLYAQGRRRYDRKQSGYGGQTKPIFR--KK 59
            ::*      .  * *   .  *       :   *: :  :*    : *::  .:.:  :

bL12     75 ATGLGLKEAKDLVESAPAALKEGVSKDDAEALKKALE-EAGAEVEVK 120
eL42     60 A-----KTTKKIVLRLECVEPNCRSKR-MLAIKRCKHFELGGDKKRK 100
            *     *  * :* .   .  :  **  . *:*:    *.*::   *
```

B

```
Human eL42      47GYGGQTKP I55
                  +  *++  *++
E. coli bL12    60AAGAN - KVA67

Human eL42      47GYGGQTKP IFR57
                  +  *++   ++ +
E. coli bL12    60AAGANKVAVIK70
```

C

```
                          *   **  *** **  **
E. coli                   GANKVAVIKAVRGAT
H. influenzae             GANKVAVIKAVRGAT
P. putida                 GDKKVNVIKAVRE LT
R. prowazekii             GDKRVE VIKI VKD I T
S. typhimurium            GANKVAVIKAVRGA T
T. maritima               GQNK I QVIKVVRE I T
A. aeolicus               GKNK I QVIKVVRE I T
B. stearothermophilus     GAQK I KVIKVVRE I T
B. burgdorferi            GDS K I NVIKEVRA I T
B. abortus                GANK I NVIKEVRA LT
D. vulgaris               GANK I GVIKVVRA LT
H. praevalens             GGKK I KVIKAVRE LT
H. pylori                 GAEK I KVIKVVRE I T
M. luteus                 GAEK I KVIKVVRE I T
M. leprae                 GDKK I GVIKVVRE I V
R. spheroïdes             GANK I NVIKEVRA I T
S. griseus                GEKK I QVIKVVRE LT
```

Fig. 5 Alignment of the amino acid sequences of the human eL42 and the *E. coli* bL12 large subunit ribosomal proteins. (**A**) comparison of fragment 2–100 of eL42 with fragment 17–120 of bL12. The bottom line labels residues as either strictly conserved (*), highly conserved (:) or weakly conserved (.). The alignment was generated with the program ClustalX. The regions containing the GANK motif of bL12 or the GGQ motif of eL42 are shown (in red and bold). (**B**) Alignment of the amino acid sequences of eL42 and bL12 in the region encompassing the GGQ motif of eL42. Identical or chemically similar amino acid residues (in red) are labelled as either strictly conserved (*) or highly or weakly conserved (+). (**C**) Alignment of the amino acid sequences of eubacterial rPs bL12 in the region containing the GANK motif. The strictly or strongly conserved Lys or Arg residues including the labelled Lys-65 residue are shown in bold and green. Other strictly or strongly conserved residues are shown in bold and blue. All conserved residues are labelled with (*). Val is considered as isosteric with Thr.

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50S ribosomal subunit. This pentamer is called the ribosomal bL12 'stalk'. It can be isolated either as an NH_2-terminally acetylated 12 kDa polypeptide chain, L7 or as a polypeptide with a free NH_2 terminus, L12, both forms of the protein being currently named bL12. The first ribosomal protein whose X-ray crystallographic structure was solved, is the CTD of protein bL12 (*30*, *31*). Each monomer contains two distinct domains linked by a flexible hinge (residues 37–52): an elongated N-terminal domain (residues 1–36) that is responsible for dimer interaction and binding to uL10, and a large globular CTD (residues 53–120) that interacts with elongation factors (*30*, *31*).

Identification of the residue of bL12 cross-linked *in situ* with tRNAox on *E. coli* 70S ribosomes

To overcome the difficulty in identifying tRNAox-labelled peptides by mass spectrometry, we have identified indirectly the amino acid side chain(s) targeted by tRNAox by allowing this tRNA analogue to compete through the cross-linking reaction with PLP. We have first identified the amino acid residue of bL12 cross-linked with PLP. Then, we observed that preincubation of *E. coli* 70S ribosomes with 1 mM PLP prior to the addition of [^{32}P]tRNA^{Asp}ox prevented the cross-linking reaction *in situ* between bL12 and tRNAox. As the stoichiometry of cross-linking of bL12 is one molecule of tRNAox or PLP covalently bound per polypeptide chain, the latter result suggests that both PLP and tRNAox might be cross-linked with the ϵ-amino group of the same lysyl residue of the protein. PLP has proven to be a suitable affinity label for lysyl residues near phosphate-binding sites (*12*, *20–22*). In general, formation of a Schiff base between the aldehyde group of PLP and a lysyl ϵ-amino group is preceded by initial binding of the 5′-phosphate group (*12*, *20–22*). Conversion of the Schiff base to a stable secondary amine is obtained by reduction with sodium borohydride ($NaBH_4$). Schiff base formation between the aldehyde group of PLP and a lysyl ϵ-amino group of a protein is a mimicry of the mode of binding of this coenzyme to the catalytic lysyl residue through an aldimine (Schiff base) at the active site of transaminases. During the transamination reaction, the transient form of the PLP bearing the amino group to be transferred onto a keto acid is PMP. Therefore, PLP is a useful tool to affinity labels the enzyme active sites containing catalytic lysyl residues. Previous chemical modification *in situ* of *E. coli* 50S ribosomal proteins by PLP (*12*) had led to the following observations in the 1980s: (i) mainly seven ribosomal proteins were found to be modified by PLP, the bL12 protein being cross-linked in majority (*12*); (ii) upon modification with PLP of the 50S ribosomal subunit, the interaction with the 30S ribosomal subunit, the binding of the elongation factor EF-G and the subsequent GTPase and translocation activities were strongly impaired; (iii) the poly(U)-dependent poly(Phe) synthesis activity was completely lost; (iv) the PLP-induced inhibition of EF-G binding was shown to be due to the modification of the bL12 protein (*12*). However, the lysyl residue(s) cross-linked with PLP had never been identified. In the present report, taking advantage of the fact that the bL12 protein was previously shown to behave similarly on and off the ribosome (*29–31*, *33–35*), we have used the recombinant protein rather than the whole ribosome to demonstrate the following: (i) the *E. coli* bL12 protein can be cross-linked with PLP, as previously reported by the group of Gualerzi (*12*); (ii) the stoichiometry of cross-linking of PLP to the recombinant *E. coli* bL12 protein is one molecule of PLP covalently bound per polypeptide chain; (iii) the amino acid residue of recombinant bL12 cross-linked with PLP is Lys-65; (iv) when Lys-65 of the endogenous bL12 protein of *E. coli* 70S ribosomes was cross-linked *in situ* with PLP, the 2′,3′-dialdehyde derivative of tRNA (tRNAox) bound to the P-site could not cross-link any more with this ribosomal protein. This result suggests that both PLP and tRNAox are capable of cross-linking with the ϵ-amino group of Lys-65. However, one cannot exclude that the failure of tRNAox to cross-link with the bL12 protein in the presence of PLP is due to a steric hindrance that would prevent the tRNAox molecule from reaching its cross-linking site on bL12. Given that PLP is a small-size molecule, which cannot span a wide region in the 3D structure of the protein, another possibility is that the lysyl residue targeted by tRNAox lies in proximity with Lys-65. Lys-65 of *E. coli* bL12 belongs to the large globular CTD (residues 53–120) that interacts with tRNA and the elongation factors.

Possible common functional ribosomal or extra-ribosomal roles for the L42 and L7/L12 proteins

An example of common functional role of the eL42 and bL12 proteins on the ribosome concerns their involvement in the translocation step of translation elongation. As discussed earlier, we have previously demonstrated that the CCA end of a P-tRNA can be cross-linked with the human large subunit ribosomal protein eL42. This cross-link was shown to be specific for a tRNA at the P/E hybrid site, as a tRNA in all other tRNA positions of pre-translocational ribosomes could not cross-link with any ribosomal protein (*13*). These data suggest that eL42 is involved in the translocation step, i.e. the movement of the bound tRNA from the P- to the E-site. As for L7/L12, it was previously demonstrated in several studies that this ribosomal protein is involved in the translocation of the peptidyl-tRNA from the A- to the P-site (*38–40*). The fact that the GANK and GGQTK motifs occupy comparable positions in the 3D structures of the *E. coli* bL12 and the human eL42 proteins, respectively, is compatible with their functional role in translocation on the translating ribosomes (Fig. 6). Finally, a common functional role for Lys-53 of human eL42 and Lys-65 of *E. coli* bL12 on the ribosome is most probable because the former is strictly conserved (with very few Lys/Arg conservative replacements) in the amino acid sequences of all the rPs of the eL42 family (*13*, *27*), while the latter is strictly conserved in the amino acid sequences of all eubacterial bL12 (Fig. 5C and (*41*)). Regarding the common extra-ribosomal role of these proteins, it was previously reported that eL42 is overexpressed in human hepatocellular carcinoma as well as in several human tumour cell-

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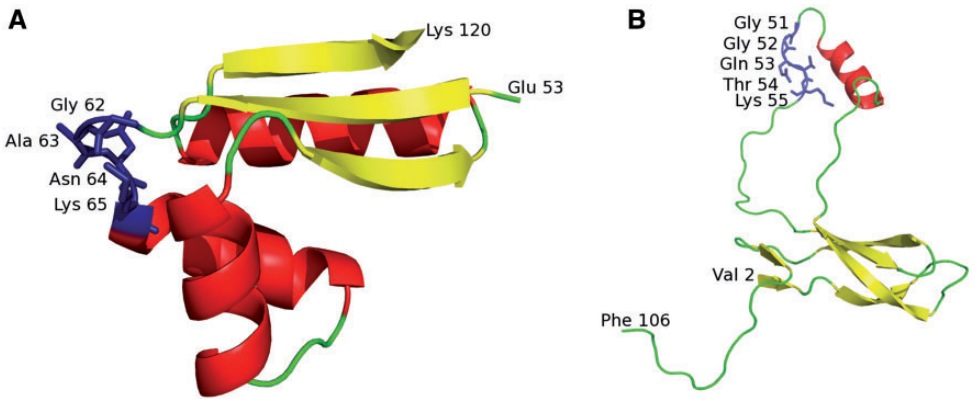


Fig. 6 Comparison of the 3D structure of *Saccharomyces cerevisiae* eL42 with that of the C-terminal factor binding domain of *E. coli* bL12. (**A**) C-terminal factor binding domain of *E. coli* bL12 (PDB entry 1CTF); (**B**) *S. cerevisiae* eL42 (PDB entry 4U4R chain q2). Secondary structures: α-helix (red), β-sheet (yellow) and coil (green). The GANK motif of bL12 and the GGQTK motif of eL42 are in stick representation (blue). The structures were visualized with pymol (https://www.pymol.org).

lines (*42*). Similarly, an increased exposure of the intestine to bacterial bL12 was previously observed in early stage colorectal cancer patients (*43*). Therefore, the common extra-ribosomal role of the eukaryal eL42 and the eubacterial bL12 proteins might be related to tumour cell proliferation (*42*, *43*). It is generally believed that overexpression of components of the translation machinery in cancer cells could lead to the discovery of new anticancer drugs. An interesting example in this respect is rapamycin, which specifically inactivates the mammalian target of rapamycin (mTOR) and had been shown to act as a powerful tumour-suppressive drug (*44*). Therefore, eL42 and bL12 might represent targets of choice for anticancer therapy.

Location of the *E. coli* bL12 and the human eL42 proteins in the 3D structures of the ribosomes

Along with the quaternary structure, the most important difference between human eL42 and *E. coli* bL12 is their location in the 3D structures of the ribosomes. Protein bL12, the only multicopy component of the eubacterial ribosomes, is located in the so-called bL12 stalk (formerly L7/L12 stalk) nearby the A-site, in sharp contrast to eL42 which would be located at the E-site of eukaryotic ribosomes, similarly to its counterpart of the archaeon *H. marismortui*. However, it was reported that the CTD of each monomer of bL12 is highly mobile and capable of visiting the functional A-, P- and E-sites of the ribosome, thanks to the marked flexibility of the whole protein (*28–30*, *45*, *46*). Therefore, it is not surprising that the CTD of the protein, which is involved in the binding of translation factors and of the CCA-arm of tRNA would be capable of contacting the CCA-end of P-site bound tRNAox.

Conclusion

In this report, we have used tRNAox, the 2′,3′-dialdehyde derivative of tRNA as a zero-length affinity labelling reagent to demonstrate that the large subunit ribosomal protein bL12 contacts the CCA-arm of P-site bound tRNA on the *E. coli* 70S ribosomes. This reactive tRNA analogue was shown to fulfill all the criteria for a site-directed affinity label of the bL12 protein inside the ribosome: (i) the stoichiometry of covalent binding of tRNAox to bL12 is one molecule of tRNAox bound to one polypeptide chain of endogenous *E. coli* bL12, in accordance with the presence of one binding site for the CCA-arm of tRNA and for the translation factors, in the CTD of each of the four subunits of the protein; (ii) cross-linking *in situ* of bL12 with tRNAox in the *E. coli* 70S ribosomes provokes the loss of the activity of poly(U)-dependent poly(Phe) synthesis, which argues for a functional role of this rp in eubacterial ribosomes; (iii) intact tRNA completely protected bL12 in the *E. coli* 70S ribosomes against cross-linking *in situ* with tRNAox, suggesting a competition between native $tRNA^{Asp}$ and the dialdehyde derivative thereof for the binding to the P-site. Both tRNAox and PLP were shown to compete for the same or for proximal cross-linking site(s) on bL12 inside the ribosome. Consistent with the latter observation, the stoichiometry of cross-linking of PLP to the recombinant *E. coli* bL12 protein is one molecule of PLP covalently bound per polypeptide chain similarly to the stoichiometry of tRNAox-labelling of the endogenous bL12. The amino acid residue of recombinant bL12 cross-linked with PLP is Lys-65. Lys-65 of the 62GANK65 motif of *E. coli* bL12 matches with Lys-53 of the 49GGQTK53 motif of the large subunit ribosomal protein eL42 which was previously shown to cross-link with P-site bound tRNAox on human 80S ribosomes *in situ*. Finally, *E. coli* bL12 and human eL42 proteins display significant primary structure similarities, with an average of 40–50% of identities and conservative replacements, which argues for evolutionary conservation of these two proteins located at the tRNA-CCA binding site on eubacterial and eukaryal ribosomes, respectively.

Supplementary Data

Supplementary Data are available at *JB* Online.

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Conflict of Interest

None declared.

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