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Full Length Research Paper

Recent advances in the characterization of peptidyl transferase center: zero-distance labeling of proteins at or near the catalytic site of human 80S or *Escherichia coli* 70S ribosomes by means of periodate-oxidized tRNA

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Periodate-oxidized tRNA (tRNAox), the 2',3'-dialdehyde derivative of tRNA, was used as a zero-length active site-directed affinity labeling reagent, to covalently label proteins at the peptidyl transferase center (PTC), the catalytic site of the large ribosomal subunit. When human 80S or *Escherichia coli* 70S ribosomes were reacted separately with tRNAox positioned at the P-site, in the presence of an appropriate 12 mer mRNA, a set of two tRNAox- labeled ribosomal proteins was observed. These proteins referred to in this work as rPox1 and rPox2 exhibited comparable physico-chemical properties including apparent molecular weights. In the case of human 80S ribosome, the protein present in the major labeled tRNA- rPox1 covalent complex was identified as the 60S ribosomal protein L36a-like (RPL36AL) by mass spectrometry. The molecular weight of the minor labeled tRNA-rPox2 covalent complex was estimated from the data of the 1-D SDS-PAGE, and a deduced molecular weight of 34,000 + 2,000 Da for the ribosomal protein referred to as rPox2 designated protein RPL5 as the candidate minor labeled protein of human 80S ribosome. Search for candidate ribosomal proteins for the tRNAox-labeled proteins rPox1 and rPox2 of 70S ribosome from *E. coli* designated RPL2 (M.W. 29,860 Da), the largest eubacterial rP as the tRNAox-labeled protein corresponding to the minor labeled human RPL5, and RPL15 (M.W. 14,980 Da) or RPL16 (M.W. 15,281 Da) as corresponding to the major labeled human RPL36AL.

Key words: Human 80S or *E. coli* 70S ribosomes, peptidyl transferase center, human RPL36AL, RPL5/P-site or A-site tRNA, periodate-oxidized tRNA.

INTRODUCTION

One of the most fundamental molecular processes in biology is catalysis of peptide bond formation during protein biosynthesis. In all cells, the ribosome is the ribonucleoprotein complex responsible for the translation process in which messenger RNA (mRNA) serves as the template, and aminoacylated transfer RNAs (tRNAs) serve as the substrates. All ribosomes are composed of two subunits of unequal size. Bacterial ribosomes have a relative sedimentation rate of 70S and can be separated into a large 50S subunit and a small 30S subunit. In the eubacteria *Escherichia coli* one third of the mass of a ribosome consists of proteins and the other two thirds of ribosomal RNA (rRNA): the 50S subunit contains both a 5S (120 nucleotides) and a 23S rRNA (approximately 2900

nucleotides), while the 30S subunit contains a single 16S rRNA (about 1500 nucleotides) . The protein fraction consists of 21 different proteins in the small subunit and 33 proteins in the large subunit. Eukaryotic ribosomes are larger : *Saccharomyces cerevisae* (yeast) ribosomes, for example, sediment at 80S and are separable into 60S and 40S subunits. They have longer rRNAs, an additional rRNA and 20 - 30 extra ribosomal proteins, which together account for the 30% increase in size relative to *E. coli* ribosomes.

In all kingdoms of life, the catalytic ''heart'' of the ribosome is the peptidyl transferase center (PTC) of the large ribosomal subunit where peptide bond formation takes place during protein synthesis. It has two major components, an A-site which interacts with the CCA end of aminoacylated tRNAs and a P-site, where the CCA end of peptidyl-tRNAs is bound when peptide bonds form (Baram and Yonath, 2005; Steitz, 2005; Selmer et al., 2006; Beringer and Rodnina, 2007). The reaction it catalyses is the nucleophilic attack of the α-amino group of an A-site-bound aminoacyl-tRNA on the carbonyl car-bon of the ester bond that links a nascent peptide to a tRNA in the P-site. Historically, much controversy has surrounded the question regarding the peptidyl trans-ferase center. Specifically, the questions posed related to whether this catalytic site was made of RNA or protein (or both). In the past beliefs, the most important mechanistic aspects of translation, including peptidyl transferase acti-vity, were taught to be exclusively the domain of proteins (Maden and Monro, 1968; Baxter and Zahid, 1978; Wan et al., 1975). Accordingly, early biochemical evidence had supported the idea that peptidyl transferase is an enzyme with an ionizable functional group such as histidine. Indeed, the peptidyl transferase reaction has a pH dependence with an acidity constant (pKa) of approximately 7.2 (Maden and Monro, 1968) and ribosomal inactivation by ethoxyformic anhydride which was thought to react with an active-site histidyl residue, had a pH maximum at 7.0 (Baxter and Zahid, 1978), while photochemical inactivation of the ribosome with Rose Bengal dye exhibited a pH optimal at 7.5 (Wan et al., 1975) . However, none of the isolated ribosomal proteins, nor any of their mixtures, have ever been shown to possess any detectable peptidyl transferase activity. Crystallographic studies have revealed that the chemical reactions catalyzed by the ribosome, peptide bond formation and peptide release, both occur at the peptidyl transferase center of the large ribosomal subunit and appear to be promoted by RNA (Ban et al., 2000; Nissen et al., 2000; Yusupov et al., 2001; Yusupova et al., 2001; Bashan et al., 2003; Steitz and Moore, 2003) . In particular, the refined three dimensional structure of a 50S ribosomal subunit at a resolution of 2.4 angströms of the archaeon *Haloarcula marismortui* reveals a void of protein electron density in a radius of 18 angströms of the PTC (Ban et al., 2000; Nissen et al., 2000). This led to the current view of ribosomal peptidyl transfer that ribosome is a ribozyme (Nissen et al., 2000; Steitz and Moore, 2003; Youngman et al., 2004) and that ribosomal proteins are not involved in catalysis of peptide bond formation. Similarly, no protein appears to surround the 3'-terminus of tRNA at either the A or the P site on human 80S ribosome. Indeed, recent photoaffinity cross-linking studies have shown that a photoactivatable tRNA analogue bearing a 4-thiouridine residue at its 3'-terminus is found cross- linked only to nucleotides in domain V of the 28S rRNA of the large 60S subunit (Bulygin et al., 2008). However, most attempts to demonstrate catalytic activity for isolated rRNA were unsuccessful (Khaitovich et al., 1999). Therefore, the authors have proposed that the nature of ribosomal peptidyl transferase might be of two possible types: peptidyl transferase might be a ribozyme, whose functional conformation is stabilized by ribosomal proteins, or a ribonucleoprotein enzyme, where binding of substrates is insured by RNA and the chemical step is catalyzed by a ribosomal protein (Khaitovich et al., 1999). In contrast to the above cited organisms, several previous studies on the eubacteria have demonstrated that ribosomal proteins are located at or near the active site. First, previous studies on *Thermus aquaticus* have shown that 23S rRNA and ribosomal proteins L2 and L3 are the possible essential macromolecular components of the peptidyl transferase center (Khaitovich et al., 1999). Second, the protein L27 had been found cross -linked along with L33 to the 3' ends of both the A- and P- site tRNAs in the PTC (Wower et al., 1989; Wower et al., 1998; Kirillov et al., 2002). Third, an L27 deletion mutant of *E. coli* had been shown to be viable, but exhibits a growth speed five to six times weaker than the wild- type and shows deficiencies in peptidyl transfer activity as well as an impaired binding of tRNA to the A-site (Maguire et al., 2005). Fourth, recent 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 (Selmer et al., 2006) . Finally, Trobro and Aqvist (2008) have investigated the role of L27 in peptidyl transfer in *T. thermophilus* by carrying out computer simulations. However, no structural data have yet elucidated the possible role of these proteins in peptidyl transfer.

Thus, despite the structural insights from the crystallo-

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Abbreviations: PTC, peptidyl transferase center; **tRNAox,** periodate-oxidized tRNA, the 2',3'-dialdehyde derivative of tRNA; **rP,** ribosomal protein; **rPox1** and **rPox2,** the two ribosomal proteins found labeled by tRNAox in this work as tRNA-rP covalent complexes; **RPL36AL,** human ribosomal protein L36a-like; **RPL5,** human ribosomal protein L5.

lographic studies, the catalytic mechanisms of the PTC are still not fully understood in molecular terms and the question of whether the catalytic ''heart'' of the ribosome is made of RNA or protein, or both, is still open. In order to take benefit from the progress that has been made in ribosome crystallography (Selmer et al., 2006; Yusupova et al., 2001; Bashan et al., 2003; Nissen et al., 2000; Yusupov et al., 2001; Spahn et al., 2001) and contribute to defining the PTC in molecular terms, novel biochemical tools are required. More than 20 years ago, we had successfully used periodate-oxidized tRNA as a zero-length active site-directed affinity laleling reagent, to identify lysine and arginine residues at the binding site for the CCA arm of tRNA on aminoacyl-tRNA synthetases (Hountondji et al., 1979; Hountondji et al., 1985; Hountondji et al., 1986a; Hountondji et al., 1987; Sanni et al., 1991; Baouz et al., 2009). After having affinity labeled a few aminoacyl-tRNA synthetases, we had discovered the KMSKS motif which is the catalytic signature of class I aminoacyl-tRNA synthetases (Hountondji et al., 1986b). Lys-335, the second lysyl residue of the KMSKS catalytic motif of *E. coli* methionyl-tRNA synthetase had been shown to represent a catalytic residue involved in methionyl-adenylate formation and $tRNA^{Met}$ amino-acylation (Mechulam et al., 1991). Since during peptide bond formation, the 3' terminal ribose bearing A76 of the conserved CCA terminus of tRNA, as well as its attached aminoacyl and peptidyl groups are supposed to come in close proximity to the PTC, periodate-oxidized tRNA with its zero-length 2',3'-dialdehyde group is expected to react with basic aminoacid side chains involved in proper positioning of the tRNA acceptor stem and CCA arm within the PTC. In this paper, we report the labeling of ribosomal proteins from human 80S or *E. coli* 70S ribosomes, by means of periodate-oxidized tRNAs directed to the PTC of these ribosomes and we identify the human large subunit ribosomal proteins L36a-like and L5 as components of the catalytic site of human 80S ribosome.

EXPERIMENTAL PROCEDURES

Ribosomes, mRNA, tRNAs and periodate-oxidized tRNAs

40S and 60S ribosomal subunits with intact rRNAs, isolated from unfrozen human placenta, were a kind gift from Prof. G. Karpova (Institute of Chemical Biology and Fundamental Medicine, SB RAS, ICBFM Novosibirsk, Russia). Prior to use, the subunits were re-activated by incubation in binding buffer A (50 mM Tris-HCl, pH 7.5, 100 mM KCl, 10 mM $MgCl_2$ and 0.5 mM EDTA) at 37°C for 10 min. 80S ribosomes were obtained by association of the re-activated 40S and 60S subunits taken in the equimolar ratio. 70S ribosomes from *E. coli* MRE 600 were a generous gift from Dr J.B. Créchet (Ecole Polytechnique, Département de Chimie et de Synthèse Organique, Palaiseau, France). Short mRNA analogues (oligoribonucleotides GAA UUU GAC AAA and GAA AUG GAC AAA) were purchased from Sigma Aldrich (Evry, France). $tRNA^{Asp}$ from yeast and $tRNA^{Phe}$ from beef were purified by conter-current chromatography, followed by separation by polyacrylamide gel electrophoresis. Their amino acid acceptance capacities were 1400 pmol/A_{260} unit and 1300 pmol/A_{260} unit for $tRNA^{Asp}$ and $tRNA^{Phe}$, respectively. *E. coli* $tRNA^{fMet}$ (1500 pmol/A_{260} unit) was a kind gift from Dr Y. Mechulam (Ecole Polytechnique, Laboratoire de Biochimie, Palaiseau, France). ^{32}P-labeling of tRNA at the 5'-end was carried out after dephosphorylation by alkaline phosphatase (Roche), in the presence of γ-[^{32}P]ATP and T4 polynucleotide kinase. Periodate-oxidized tRNA (tRNAox) was prepared a discribed previously (Fayat et al., 1979).

Design of the ribosomal complexes for tRNAox-labeling of ribosomal proteins

For the labeling experiments, the following ribosomal complexes were designed:
Complex 1: human 80S ribosomes + GAA UUU GAC AAA + [^{32}P]$tRNA^{Asp}$ox at the P site.
Complex 2: human 80S ribosomes + GAA UUU GAC AAA + intact $tRNA^{Phe}$ at the P site
+ [^{32}P]$tRNA^{Asp}$ox at the A site.
Complex 3: human 80S or *E. coli* 70S ribosomes + GAA AUG GAC AAA + [^{32}P]$tRNA^{fMet}$ox at the P site.

Ribosomal complexes with tRNA analogues (tRNAox) were obtained in buffer A (50 mM Tris-HCl, pH 7.5, 100 mM KCl, 10 mM $MgCl_2$ and 0.5 mM EDTA) at 37°C. For human 80S ribosomal complex 1 (with [^{32}P]$tRNA^{Asp}$ox at the P-site), the reaction mixtures for the labeling experiments contained: 0.5 μM of 80S ribosomes, 5 μM of the dodecaribonucleotide GAA UUU GAC AAA as an mRNA and 5 mM sodium cyanoborohydride in a total volume of 25 μL buffer A. These mixtures were preincubated for 5 min at 37°C. The labeling reaction was initiated by the addition of 5 μM [^{32}P]$tRNA^{Asp}$ox and the incubation continued for 1 hour. In the control experiment, [^{32}P]tRNAox at the P-site was replaced by intact [^{32}P]$tRNA^{Asp}$ (5 μM) which was added to a reaction mixture corresponding to complex 1 (see above). For the protection of 80S ribosomes against labeling with [^{32}P]$tRNA^{Asp}$ox, 15 μ M intact $tRNA^{Asp}$ was added to the reaction mixture of complex 1, followed by preincubation for 5 min at 37°C, before the initiation of labeling reaction with 5 μM [^{32}P]$tRNA^{Asp}$ox. To obtain complex 2 (with [^{32}P]$tRNA^{Asp}$ox at the A-site), at first a ternary complex of human 80S ribosomes with intact $tRNA^{Phe}$ at the P-site and the dodecaribonucleotide GAA UUU GAC AAA was obtained as described above; then, this complex was added with [^{32}P]$tRNA^{Asp}$ox (5 M) directed to the A-site and the incubation was continued for 1 hour. Human 80S or *E. coli* 70S ribosomal complex 3 (with [^{32}P]$tRNA^{fMet}$ox at the P-site) was obtained in the same incubation mixture as above, except that the dodecaribonucleotide GAA AUG GAC AAA was used as an mRNA. In the control experiments, [^{32}P]$tRNA^{fMet}$ox at the P-site was replaced by intact [^{32}P]$tRNA^{fMet}$ (5 μM) which was added to reaction mixtures.

Analysis of the tRNAox-labeled proteins

After the termination of the labeling reaction, the mixtures were applied onto a 12% polyacrylamide gel which was run by urea-gel and/or SDS-gel electrophoreses (Laemmli, 1970). To carry out the RNase T1 treatment, 1 μl of RNase T1 (10 mg/ml) was added to the reaction mixture, followed by incubation at 37°C for 30 min, prior to analysis by PAGE. To carry out treatment with proteinase K, 1 μl of proteinase K (10 mg/ml) was added to the reaction mixture, followed by incubation at 37°C for 30 min. The gels were dried under vacuum and subjected to autoradiography.

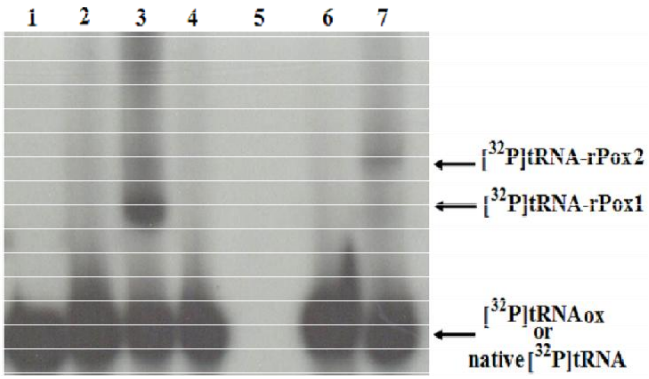

Figure 2. Autoradiogram of the SDS gel electrophoresis analysis in 12% polyacrylamide of [^{32}P]tRNA^{Asp}ox-labeled proteins of human 80S ribosomes. The tRNAox-labeling incubation mixtures were as described in Experimental Procedures.
Lanes 1 and 2 : controls are respectively, [^{32}P]tRNA^{Asp}ox alone, and native [^{32}P]tRNA^{Asp} incubated with ribosomes as described in Experimental Procedures; lane 3 : [^{32}P]tRNA^{Asp}ox-labeling of human 80S ribosomal proteins; lane 4 : Ribosomes incubated with [^{32}P]tRNA^{Asp}ox-red, the [^{32}P]tRNA^{Asp}-dialdehyde derivative reduced with $NaBH_4$; lanes 5 and 6: RNase T1 and proteinase K treatments of the incubation mixtures containing [^{32}P]tRNA^{Asp}ox-labeled ribosomal proteins; lane 7: protection with native tRNA^{Asp} of ribosomal proteins against labeling by [^{32}P]tRNA^{Asp}ox. The arrows indicate the [^{32}P]tRNA^{Asp}-rPox1 and [^{32}P]tRNA^{Asp}-rPox2 covalent complexes, as well as the controls [^{32}P]tRNA^{Asp}ox and native [^{32}P]tRNA^{Asp}.

Identification of proteins by mass spectrometry NanoLC-MS/MS

Unstained bands corresponding to the tRNAox-rP covalent complex referred to as [^{32}P]tRNA-rPox1 in Figure 2 were cut into small pieces of approximately 1mm from Urea-PAGE gels. The position of these non radioactive bands was defined comparing gels loaded with radioactive versus non radioactive samples. Gel pieces were thoroughly washed, reduced with DTT and alkylated with iodoacetamide using the Progest robot (Genomic Solution) and following standard protocols (Cavusoglu et al., 2003). The proteins were then digested with trypsin (Promega), the resulting peptides were extracted with a solution of acetonitrile, water and formic acid (60/38/2) and dried under vacuum.

Peptide mixtures were injected on a nanoLC and submitted to electrospray tandem mass spectrometry. The analyses of peptides were performed on a U3000 Dionex nanoflow system connected to a LTQ Orbitrap mass spectrometer equipped with a nano-electrospray source (Thermo-Fischer, Bremen, Germany). Chromatographic separation took place in a C18 pepmap 100 column (75 µm ID, 15 cm length, 5 µm 100 A Dionex). Peptides mixtures were injected on pre-concentration column with a flow rate of 20 µl/min of water TFA 0.1%. After three minutes wash with the same solvent, the peptides were eluted and separated on the analytical column with a flow of 200 nl/min and a 30 min gradient from 2 to 60% MeCN in 0.1% formic acid. The mass spectrometer was operated in the data dependent mode to automatically switch between orbitrap MS and MS^2 in the linear trap. Survey full scan MS spectra from 500 to 2000 Da were acquired in the orbitrap with resolution R = 60000 at m/z 400, after accumulation of 500,000 charges on the linear ion trap. The most intense ions (up to six, depending on signal intensity) were sequentially isolated for fragmentation, in the linear ion trap using CID at a target value of 100,000 charges. The resulting fragments were recorded in the linear trap. Proteins identifications were performed using high accuracy MS and MSMS Data using in parallel Sequest (Thermo-Fischer) or Mascot (matrix science). The mass accuracy for MS and MS/MS Data was set to 5 ppm and 0.8 uma respectively. Data mining was done in human Uniprot database.

RESULTS

TRNAox-labeling of ribosomal proteins

The periodate treatment specifically oxidizes the 2',3'-cis-diol function of the 3'-terminal ribose of tRNA, to create a 2',3'-dialdehyde derivative of tRNA (tRNAox) . Labeling of a protein with tRNAox proceeds through the formation of a reversible Schiff's base between its 2',3'- aldehyde groups and amino groups of lysine and arginine residues on the protein. Sodium cyanoborohydride ($NaBH_3CN$), a mild reducing agent converts the imino moiety of the Schiff's base specifically and continuously into a stable secondary amine, but leaves intact the reacting tRNA dialdehyde. Periodate-oxidized [^{32}P]tRNA was incubated with human 80S ribosomes or with *E. coli* 70S ribosomes, as described under Experimental Procedures.

Design of complexes for ribosomal protein (rP) labeling experiments was based on the well known fact that tRNA binds at first to the P-site. Thus, in the complex obtained in the presence of dodecaribonucleotide GAAUUUGACAAA as mRNA, [^{32}P]tRNA^{Asp} dialdehyde cognate to GAC codon was bound at the P-site of human 80S ribosomes (Figure 1, complex 1), while on human 80S or *E. coli* 70S ribosomes, [^{32}P]tRNA^{fMet}ox was bound at the AUG codon (P-site) of dodecaribonucleotide GAA AUG GAC AAA (Figure 1, complex 3) . To direct [^{32}P]tRNA^{Asp} dialdehyde to the A-site (Figure 1, complex 2), at first a ternary complex of 80S ribosomes with the dodecaribonucleotide GAAUUUGACAAA and tRNA^{Phe} that targeted UUU codon to the P-site was obtained, followed by occupation of the A site GAC triplet by cognate [^{32}P]tRNA^{Asp} dialdehyde.

Labeling of ribosomal proteins by P-site bound [^{32}P]tRNA^{Asp}ox

Human 80S ribosome was reacted with periodateoxidized 5'-[^{32}P]labeled tRNA^{Asp} ([^{32}P]tRNA^{Asp}ox) positioned at the P-site, in the presence of dodecari bonucleotide GAAUUUGACAAA mRNA (Figure 1, complex 1) and two radiolabeled bands appeared on the autoradiogram of the SDS-polyacrylamide gel: a fast migrating major label-ed band and a slow migrating minor labeled one referred

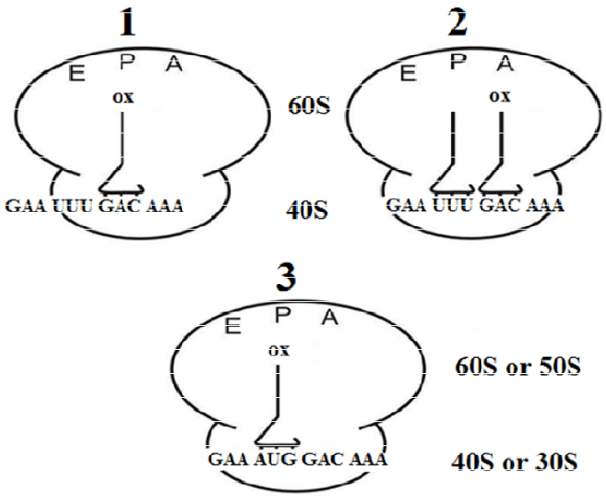

Figure 1. Types of complexes of human 80S or *E. coli* 70S ribosomes with tRNA.
complex 1: Human 80S ribosomes + GAA UUU GAC AAA + [^{32}P]tRNA^{Asp}ox at the P site.
complex 2: Human 80S ribosomes + GAA UUU GAC AAA + intact tRNA^{Phe} at the P site + [^{32}P]tRNA^{Asp}ox at the A site.
complex 3: Human 80S or *E. coli* 70S ribosomes + GAA AUG GAC AAA + [^{32}P]tRNA^{fMet}ox at the P site.

to as [^{32}P]tRNA-rPox1 and [^{32}P]tRNA-rPox2, res- pectively (Figure 2 and lane 3). When [^{32}P]tRNA^{Asp}ox was replaced by intact [^{32}P]tRNA^{Asp} in the incubation mixtures contain-ing 80S ribosomes, no [^{32}P]tRNAox-rP covalent complex was visible on the gel (Figure 2, lane 2). The radioactive band corresponding to unreacted [^{32}P]tRNA dialdehyde derivative in the reaction mixtures for tRNAox-labeling of ribosomal proteins was assigned by the posi-tion of a control [^{32}P]tRNA^{Asp}ox loaded alone in lane 1 (Figure 2). Treatment of the [^{32}P]tRNA-dialdehyde deriva-tive with $NaBH_4$ yielded a reduced [^{32}P]tRNAox- red deri-vative which failed to label any ribosomal protein (Figure 2, lane 4), indicating that tRNAox-labeling proceeded through the formation of a reversible Schiff's base between the 3'-dialdehyde group of tRNAox and amino group(s) belonging to lysine or arginine residues of the ribosomal proteins, as expected. Intact tRNA^{Asp} com- peted with [^{32}P]tRNA^{Asp}ox for the binding to the P-site, by preventing the formation of [^{32}P]tRNA^{Asp}-rPox1 covalent complex (Figure 2, lane 7), while rPox2 was not protected against labeling with [^{32}P]tRNAox, as judged by compa-rison of lanes 3 and 7 on Figure 2.

Demonstration of the presence of ribosomal proteins (rP) in the [^{32}P]tRNAox-rP covalent complex (Figure 2, lane 3) was carried out as follows: prior to the analysis by SDS-PAGE, the reaction mixture was treated with either proteinase K or RNase T1. Comparison of lanes 3 and 6 from Figure 2 indicates the disappearence due to protein-ase K of the radioactive bands containing the [^{32}P]tRNAox-rP complexes and the concomitant increase of the radioactive band containing [^{32}P]tRNAox. As shown in Figure 2, lane 5, treatment with RNase T1 resulted in the complete loss of the label in all bands on the gel.

Labeling of a ribosomal protein by A-site bound [^{32}P]tRNA^{Asp}ox

Figure 3 describes the formation of only the [^{32}P]tRNA^{Asp}-rPox2 covalent complex (lane 3) in the presence of intact tRNA^{Phe} directed to the P- site, prior to addition of [^{32}P]tRNA^{Asp}ox directed to the A-site of human 80S ribo-some (Complex 2, Figure 1) . Because the [^{32}P]tRNA^{Asp}-rPox1 complex disappeared from lane 3, as compared with lane 2, we conclude that the fast migrating major labeled protein referred to as rPox1 was protected by P-site bound intact tRNA^{Phe} against labeling by A-site bound [^{32}P]tRNA^{Asp}ox.

tRNAox-labeling of ribosomal proteins on human 80S or *Escherichia coli* 70S ribosomes

To address the question of whether periodate-oxidized tRNA is capable of reacting at a zero-distance with pro-teins of eubacterial 70S ribosomes, similarly to eukaryotic 80S ribosomes, as reported in the present study, *E. coli* 70S or human 80S ribosomes were reacted each with periodate-oxidized [^{32}P]labeled tRNA^{fMet} ([^{32}P]tRNA^{fMet}ox) positioned at the P-site, in the presence of the dodecari-bonucleotide GAA AUG GAC AAA as an mRNA (complex 3, Figure 1). In the control, [^{32}P]tRNA^{fMet}ox was replaced by intact [^{32}P]tRNA^{fMet} in the reaction mixture for protein labeling in the presence of *E. coli* 70S ribosomes. As shown in Figure 4, two radio- active bands similar to the afore-mentioned human 80S ribosomal [^{32}P]tRNA^{Asp}ox-labeled tRNA-rPox1 and tRNA-rPox2 covalent complexes appeared on the autoradiogram of the SDS-polyacryl-amide gel in lanes 1 and 2 cor- responding to human and *E. coli* ribosomes respectively. Interestingly, the mobility of the two bands in SDS-PAGE was similar in human and *E. coli*. Accordingly, these two bands were assumed to contain [^{32}P]tRNAox-labeled ribosomal proteins with com-parable apparent molecular weights in human and *E. coli* respectively.

Identification of the tRNAox-labeled proteins of human 80S ribosome

The major labeled band (Figure 2, lane 3) referred to as [^{32}P]tRNA-rPox1 was identified by mass spectrometry as the human 60S ribosomal protein L36a-like (RPL36AL, Acc. N. Q969Q0). Four peptides corresponding to 33% of the sequence coverage of the human RPL36AL were

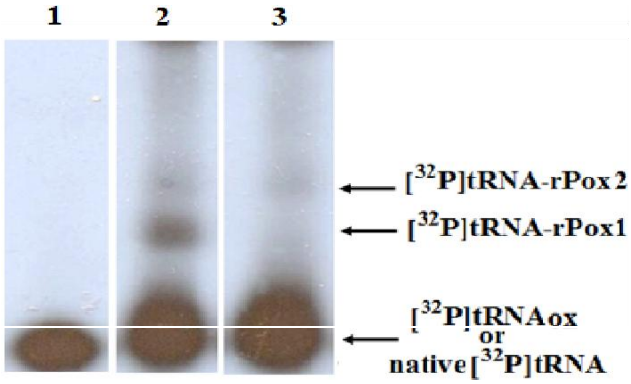

Figure 3. Absence of [^{32}P]tRNA-rPox1 covalent complex on the autoradiogram of the SDS gel after targetting of oxidized [^{32}P]tRNA^{Asp} to the A-site.
Lane 1: Control native [^{32}P]tRNA^{Asp} ; lane 2 : formation of the two [^{32}P]tRNA^{Asp}-rPox1 and [^{32}P]tRNA^{Asp}-rPox2 covalent complexes in the presence of [^{32}P]tRNA^{Asp}ox directed to the P-site of human 80S ribosome ; lane 3 : formation of only the [^{32}P]tRNA^{Asp}-rPox2 covalent complex in the presence of intact tRNA^{Phe} directed to the P-site, prior to addition of [^{32}P]tRNA^{Asp}ox directed to the A-site of human 80S ribosome. Because the [^{32}P]tRNA^{Asp}-rPox1 complex disappeared, as compared to lane 2, we conclude that the fast migrating major labeled protein rPox1 was protected by P-site bound intact tRNA^{Phe} against labeling by A-site bound [^{32}P]tRNA^{Asp}ox.

found after in-gel digestion of the corresponding Urea-PAGE gel band. Amino acid sequences were obtained for peptides ^{28}GKDSLYAQGR^{38}, ^{69}LECVEPNCR^{78}, ^{89}HFELGGDKKR^{106} and ^{101}GQVIQF^{106} . The differences between experimental and theoretical masses was less than 3 ppm. The upper band corresponding to the minor labeled [^{32}P]tRNA-rPox2 covalent complex in Figure 2 (lanes 3 and 7) was not in sufficient amount to permit protein identification either from Urea-PAGE or from SDS-PAGE. However, the molecular weight of the [^{32}P]tRNA-rPox2 covalent complex could be estimated from comparison of the molecular weights of [^{32}P]tRNA (M.W. 25,000 Da) and the [^{32}P]tRNA-rPox1 covalent complex (M.W. 37,000 Da consisting of [^{32}P]tRNA, M.W. 25,000 Da plus RPL36AL, M.W. 12,000 Da), on one hand and from comparison of their relative mobilities in SDS-PAGE, on the other hand. A molecular weight of 59,000 $\pm$ 2,000 Da for the [^{32}P]tRNA-rPox2 covalent complex could be deduced which corresponded to a molecular weight of 34,000 $\pm$ 2,000 Da for the ribosomal protein referred to as rPox2 in the present work. This molecular weight estimate of rPox2 was the average of values determined from 10 electrophoresis runs. This value is somewhat comparable to the calculated molecular weight of human L5 (34,463 Da), L6 (32,728 Da) or L7 (29,226 Da), sug-suggesting that the candidate labeled protein of the [^{32}P]tRNA-rPox2 covalent complex is RPL5, RPL6 or RPL7.

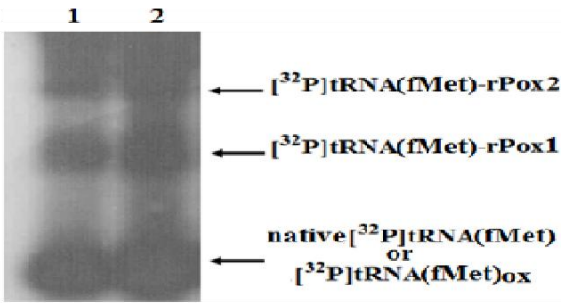

Figure 4. Presence of two [^{32}P]tRNA^{fMet}-rPox1 and [^{32}P]tRNA^{fMet}-rPox2 covalent complexes in *E. coli* 70S or human 80S ribosomes. Human 80S (lane 1) or *E. coli* 70S (lane 2) ribosomes were incubated with [^{32}P]tRNA^{fMet}ox in the incubation mixtures described in Experimental Procedures. The arrows indicate the positions of [^{32}P]tRNA^{fMet}-rPox1 and [^{32}P]tRNA^{fMet}-rPox2 covalent complexes, as well as that of the controls [^{32}P]tRNA^{fMet}ox or native [^{32}P]tRNA^{fMet}.

Search for candidate ribosomal proteins for the peptidyl transferase center of 70S ribosome from *E. coli*

In *E. coli*, the highest known calculated molecular weight for an rP is that of L2 (M.W. 29,860 Da) which is comparable with the aforementioned molecular weights of human RPL5, RPL6 or RPL7. As for candidate rPs from *E. coli* that exhibit comparable apparent molecular weights as human RPL36AL, as many as 15 large subunit rPs were concerned.

DISCUSSION

The use of periodate-oxidized tRNA (tRNAox) as a zero-length active site-directed affinity laleling reagent for human 80S or *E. coli* 70S ribosomes was designated by the fact that interactions between the CCA ends of the peptidyl- and aminoacyl-tRNA substrates and ribosomal elements play a central role in positioning the substrates for catalysis within the peptidyl transferase center (PTC). Our data show that, when human 80S or *E. coli* 70S ribosomes were reacted each with [^{32}P]tRNAox positioned at the P-site, in the presence of an appropriate 12 mer mRNA, a set of two radiolabeled bands appeared on the autoradiogram of the SDS-polyacrylamide gel: a fast migrating major labeled band and a slow migrating minor labeled one referred to as tRNA- rPox1 and tRNA- rPox2 covalent complexes, respectively. Interestingly, the mobilities of the two bands were similar in human 80S and *E. coli* 70S ribosomes. Similarly, polyacrylamide/urea gel electrophoresis run in parallel, showed two similar tRNAox- labeled ribosomal proteins in human 80S and *E. coli* 70S ribosomes, respectively (data not shown). These results suggest that two ribosomal proteins with compara-

ble physico chemical properties referred to as rPox1 and rPox2 are actually being tRNAox-labeled on human 80S and *E. coli* 70S ribosomes, respectively.

In the case of human 80S ribosome, the ribosomal protein present in the major labeled tRNA-rPox1 covalent complex was identified as the 60S ribosomal protein L36a-like (RPL36AL) by mass spectrometry. The minor labeled [^{32}P]tRNA-rPox2 covalent complex was not in sufficient amount to permit protein identification.
However, its molecular weight could be estimated from comparison of the molecular weights of [^{32}P]tRNA and of the [^{32}P]tRNA- rPox1 complex, on one hand, and from comparison of their relative mobilities in SDS-PAGE, on the other hand. A molecular weight of 59,000 $\pm$ 2,000 Da for the [^{32}P]tRNA-rPox2 covalent complex could be deduced which corresponded to a molecular weight of 34,000 $\pm$ 2,000 Da for the ribosomal protein referred to as rPox2. Comparison of this value with the calculated molecular weights of human large ribosomal subunit rPs designated proteins RPL5, RPL6 or RPL7 as the candidate labeled proteins of the [^{32}P]tRNA-rPox2 covalent complex. In previous studies, Fabijanski and Pellegrini (1981) had identified rPs of the rat liver large ribosomal subunit involved in the PTC by using a mercurated 3'-terminal pentanucleotide fragment C-A- C-C-A(Acetyl[^{3}H]Leu) in which mercury atoms ([^{203}Hg]) had been added at the C-5 position of all three cytosine residues. The major proteins labeled by this reagent are RPL36A (identical to human RPL36AL) and RPL5, while RPL3, RPL4, RPL7, RPL10, RPL25 and RPL31 are labeled to a lesser extent (Fabijanski and Pellegrini, 1981). Among these mammalian PTC rPs, except RPL36AL which was identified in the present work, only proteins L5 (M.W. 34,463 Da) and L7 (M.W. 29,226 Da) are likely candidates for tRNAox-labeling on human 80S ribosome, on the basis of their calculated molecular weights. The fact that the mercurated fragment is charged with Acetyl[^{3}H]Leu makes it likely that each of these rPs might be located in the P-site of the rat liver large ribosomal subunit (Fabijanski and Pellegrini, 1981). However, due to the small size of this fragment as compared with that of a whole tRNA molecule, its tRNA mimicry is poor and one cannot exclude that some of the rat liver rPs are non- specifically labeled, especially the minor labeled ones. Therefore, in the present work, tRNAox-labeling of RPL5 (along with RPL36AL) obtained with a whole tRNA molecule reactive at its 3'-end and spanning both subunits of human 80S ribosome is most probable and might be the reflect of a more specific interaction of the tRNA CCA-end with the PTC. The presence of RPL36AL at or near the binding site for the CCA end of the peptidyl-tRNA substrate positioned at the P-site of human 80S ribosome was demonstrated by the following data: (i) Intact tRNA^{Asp} competed with tRNA^{Asp}ox by preventing the formation of tRNA-RPL36AL covalent complex, not of tRNA-RPL5. Since it is well known that native tRNA^{Asp} binds first to the P-site, the latter result might reflect competition between native tRNA^{Asp} and the dial- dehyde derivative thereof for the binding to the P-site. This result suggests at the same time that tRNA-RPL36AL covalent complex formation occured specifically at the P-site of 80S ribosomes; (ii) When the P-site was occupied by intact tRNA^{Phe}, prior to addition of oxidized tRNA^{Asp} directed to the A-site, no tRNA-RPL36AL covalent complex was observed, indicat-ing that RPL36AL is not in proximity of binding to the CCA arm of a tRNA positioned at the A-site. The latter data argue for a specific binding of native tRNA^{Asp} or its dialdehyde derivative to the P-site with the CCA arm in close proximity to RPL36AL. In turn, labeling of only RPL5 with oxidized tRNA^{Asp} directed to the A-site suggests that this rP is in proximity of binding to the CCA arm of a tRNA positioned at the A-site.

Rat or human RPL36A (or RPL36AL) is strongly conserved among eukaryotes (Davies et al., 1986; Gallagher et al., 1988; Wool et al., 1995). For example, yeast RPL42A (or RPL42B) previously named L44 is related to RPL36A, with 77 identities in 110 aligned amino acid residues (percentage identity of 70%) (Davies et al., 1986). These strongly conserved eukaryotic ribosomal proteins belong to the L44e family of rPs, a repre-sentative of which is protein RPL44e of *H. marismortui* (*Hma*). The large ribosomal subunit of this archaeon had been analyzed by X- ray cristallographic studies (Ban et al., 2000). Alignment of the amino acid sequences of rat or human proteins L36a with those of the archaebacterial RPL44E indicates 45% average amino acid identity and conservative replacement (results not shown). Since it is generally observed that proteins sharing more than 30% primary structure similarity are somewhat similarly folded, it can be reasonably expected that rat protein L36a or human protein L36a-like and the *Hma* L44e protein present similar overall three-dimen-sional structures on ribosomes. Finally, all known eukar-yotic RPL36A and archaebacterial RPL44E have a zinc binding motif. For example, the zinc binding motif of RPL44E from *H. marismortui* was shown to interact with RNA (Klein et al., 2004).

It is interesting to note that, in the 3-D structure of the *Hma* large ribosomal subunit in complex with a CCA trinucleotide, the two unstacked cytosine residues were shown to interact with conserved E- site nucleotides of 23S rRNA and with a polypeptide loop of the L44e protein (Steitz, 2008). The discrepancy between location of *Hma* L44e protein at the large ribosomal subunit E-site, apart from the P-site according to the crystallographic data, and location of the human or rat L36a homologous proteins at the P-site, as suggested by previous studies (Fabijanski and Pellegrini, 1981) and by our labeling data in the present report might be explained as follows: (i) First of all, Wower et al. (1995) have observed that the peptidyl transferase center and the E-site are closer to one

one another than previously supposed, and that consequently, they share certain ribosomal components in common. This observation suggests that RPL44E from *H. marismortui* might be located at the P-site of the archaeal ribosome as well; (ii) the CCA trinucleotide that mimics the CCA end of tRNA bound to *Hma* large ribosomal subunit (Steitz, 2008) on one hand and the CCA of a whole bound tRNAox molecule that spans both ribosomal subunits in this work, on another hand, might present different orientations within the ribosomal catalytic pocket, so that the isolated CCA trinucleotide might be artifactually bound to the E-site instead of the P-site; (iii) the well-known flexibility of the CCA- arm of tRNA with its full length of 15 angströms, might explain why the 2',3'-dialdehyde derivative of tRNA positioned on the P-site is capable of migrating from P- to E-site where, consistent with *Hma* large subunit, it can interact with nearby proteins.

Contrasting with RPL5 which is conserved in eukaryal, eubacterial and archaebacterial kingdoms, the rPs of the L44e family are only found in archaea and eukaryotes. However, the conservation of the zinc binding capacity of all the rPs of the L44e family, as well as the conservation of their amino acid sequences point to an important common functional role in ribosome structure and function in eukaryotes and archaebacteria.

As previously reported, some ribosomal proteins are bifunctional or even multifunctional (Wool et al., 1995). For example, it was reported that RPL36A is over-expressed in human hepatocellular carcinoma as well as in several human tumor cell-lines, and that its functional role may be related to tumor cell proliferation (Kim et al., 2004). One explanation of the over-expression of RPL36A in cancer is that this rP essential for the elongation step of protein biosynthesis could be over-produced to enhance tumor cell proliferation. These observations would suggest that RPL36A might represent a target for anticancer therapy. Moreover, in apoptotic cells where ribosomes were structurally altered as a consequence of the inactivation of protein synthesis, a decrease in the amounts of some ribosomal proteins including RPL36A and RPL5 was observed, as well as the expression of these rPs at the cell surface (Nishida et al., 2002). Finally, in a variety of cells, RPL5 was shown to be complexed with the mdm-2 proteins, the function of which consists of binding to the p53 protein in order to block its ability to act as a transcription factor (Maréchal et al., 1994).

The presence of ribosomal proteins at or near the PTC of human 80S ribosome contrasts with recent photoaffinity cross-linking studies where a photoactivatable tRNA analogue bearing a 4-thiouridine residue at its 3'-terminus was found cross- linked only to nucleotides in domain V of the 28S rRNA of the large subunit (Bulygin et al., 2008). Similarly, our results contrast with crystallographic data on the 50S ribosomal subunit of the archaeon *H. marismortui* (*Hma*) which revealed a void of protein electron density in a radius of approximately 18 angströms of the peptidyl transferase center, leading to the current view of ribosomal peptidyl transfer that ribosome is a ribozyme, and that ribosomal proteins are not involved in catalysis of peptide bond formation (Steitz and Moore, 2003). As for the eubacterial ribosomes, several studies suggest that proteins play a supporting role in catalysis and are closer to the active site than previously reported. For example, Schulze and Nierhaus (1982) have reconstituted an *E. coli* ribosomal particle from highly purified proteins and RNA and have reported that the minimal set of ribosomal proteins for the peptidyl transferase activity is composed of L2, L3, L4, L15 and L16. Moreover, experimental data have shown that 23S rRNA and ribosomal proteins L2 and L3 are the possible essential macromolecular components of the peptidyl transferase center from *T. aquaticus* (Khaitovich et al., 1999). Finally, several previous biochemical studies suggest that the eubacterial protein L27 is located at or near the catalytic site of 70S ribosome (Wower et al., 1989; Wower et al., 1998; Kirillov et al., 2002; Maguire et al., 2005; Selmer et al., 2006; Trobro and Aqvist, 2008). Altogether, these data suggest that the set of eubacterial ribosomal proteins located at or near the PTC is composed of L2, L3, L4, L15, L16, L27 and L33.

However, no structural data have yet elucidated the pos-sible role of these proteins in peptidyl transfer. In the present study, the search for candidate ribosomal proteins for the peptidyl transferase center of 70S ribosome from *E. coli* was performed by comparing the calculated molecular weights of human RPL36A (M.W. 12,441 Da) and RPL5 (M.W. 34,463 Da) with those of this set of PTC related rPs. The highest calculated molecular weight for an *E. coli* rP is that of L2 (M.W. 29,860 Da) which is comparable with the molecular weight of human RPL5. Therefore, it is most probable that the tRNAox-labeled rP from *E. coli* referred to as rPox2 in the present work is RPL2, the largest eubacterial rP. As for *E. coli* candidate rPs exhibiting comparable apparent molecular weight as human RPL36AL, as many as 15 large subunit rPs exhibit a calculated molecular weight in the range 10,000-15,000 Da. Among these, only two rPs belong to the set of PTC related eubacterial rPs : these are *E. coli* RPL15 (M.W. 14,980 Da) and RPL16 (M.W. 15,281 Da) . PTC related *E. coli* RPL27 (M.W. 9,124 Da) and RPL33 (M.W. 6,372 Da) exhibit calculated molecular weights lower by 26 and 49%, as compared with that of RPL36A. Therefore, they are not likely candidate rPs for tRNAox-labeling. Work is in progress, to identify the tRNAox-labeled rPs from *E. coli* by means of mass spectrometric analyses.

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