PHOTOAFFINITY LABELLING OF 23 S RNA AT THE DONOR-SITE OF THE *ESCHERICHIA COLI* RIBOSOME

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1. Introduction

The ribosomal tRNA binding sites have been the subject of general studies employing affinity labelling [1–3]. Some derivatives of aminoacyl-tRNA were found to react with ribosomal proteins on the 50S subunit [4, 5]. Recently several authors have described photoaffinity labels which, upon photoactivation, generate carbenes or nitrenes and react with ribosomal RNA [6–8].

In this paper we describe a different kind of photoaffinity labelling which is based on the photoreaction of the carbonylgroup in aromatic ketones [9]. A benzophenone-propionic acid derivative of phenylalanyl-tRNA (BP-Phe-tRNA)* is shown to bind to the ribosomal donor-site and to react with 23S RNA upon photoactivation. The reaction is specific as demonstrated by the dependence on poly U and the inhibition by puromycin. Our results show that the formation of the covalent bond occurs within the 3′ terminal three-fifths portion of the 23S RNA molecule.

2. Materials and methods

2.1. *Materials*

70S ribosomes ('tight couples') from *E. coli* strain D10 were prepared according to Noll [10]. *p*-Benzophenone-propionic acid was synthesized [11] and converted into the *N*-hydroxysuccinimide ester (m. p. 116°C) [12]. $tRNA^{Phe}$ from yeast (specific for phenylalanine) was purchased from Boehringer, Mannheim, [^{3}H]phenylalanine (spec. act. 11Ci/mmol) and [^{14}C]phenylalanine (spec. act. 522 mCi/mmol) from Amersham. Poly U and pancreatic ribonuclease bound to CM-cellulose were obtained from Miles Seravac Ltd.

2.2. *Synthesis of the BP-Phe-tRNA derivative*

5 nmol of $tRNA^{Phe}$ charged with [^{3}H] or [^{14}C] phenylalanine [13] was dissolved in 0.4 ml 0.05 M phosphate buffer pH 6.8, mixed with 2 ml of dimethylsulfoxide containing 3 mg of benzophenone-propionic acid *N*-hydroxysuccinimide ester and incubated for 5 hr at 37°C [12]. The degree of substitution was determined by hydrolysis in the presence of Cu ions [14] and was found to vary between 70–95% in different experiments.

2.3. *Photoaffinity reaction*

A standard reaction mixture contained in 1 ml: 2.5 mg ribosomes, 0.5 mg poly U and 7 μCi of BP-[^{3}H]Phe-tRNA or 0.5 μCi of BP-[^{14}C]Phe-tRNA in 50 mM NH_4Cl, 50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) pH 7.4, 10.5 mM magnesium acetate, 0.5 mM EDTA and 6 mM

Abbreviations: BP-Phe-tRNA, *p*-benzophenone-propionyl-phenylalanyl-transfer ribonucleic acid.

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mercaptoethanol. Incubation was carried out for 15 min at 37°C. In the control experiment puromycin was added to 1 mM and the sample incubated for additional 15 min at 37°C. The mixture was then cooled to 8°C and deoxygenated for 10 min by bubbling through pure nitrogen. The sample was maintained under nitrogen atmosphere during the subsequent photoreaction. The irradiation was carried out at 8°C for 45 min using a Philips SP 500 Watt high pressure mercury lamp equipped with a WG 320 cut-off filter which completely absorbs light of wavelengths below 300 nm and a quartz focusing lens of 5 cm diameter. RNA was obtained by extraction with phenol at 55°C and precipitation with 66% ethanol. The RNA was then dissolved and centrifuged through a 15–30% sucrose gradient. The A_{260} profile and the radioactivity were determined as described [5]. For the isolation of 50S subunits the irradiated incubation mixtures were dialyzed and centrifuged on sucrose gradients [5]. 50S subunits had specific activities of 4.4×10^5 cpm [^{3}H]phenylalanine and 7.6×10^4 cpm [^{14}C] phenylalanine per mg, which corresponds to 0.12 mol [^{3}H]phenylalanine and 0.16 mol of [^{14}C] phenylalanine incorporated per mole of 50S subunit respectively. 23S RNA was extracted from isolated 50S subunits. The specific activities were 6.1×10^5 cpm of [^{3}H]phenylalanine and 1.2×10^5 cpm of [^{14}C]phenylalanine per mg of 23S RNA. Since the 50S subunit contains 63% RNA [15] essentially all radioactivity must have been incorporated into RNA.

2.4. *Limited hydrolysis of the 50S subunit*

The 50S subunit was split into two specific ribonucleoprotein fragments (18S and 13S) according to Allet and Spahr [16]. The conditions of hydrolysis were changed slightly: 50S subunits at a concentration of 2 mg/ml were digested using pancreatic ribonuclease bound to CM-cellulose with an enzyme/substrate ratio of 0.04 (w/w) for 90 min at 0°C. The two fragments released were fractionated on a sucrose gradient in an SW 27 rotor and fractions were collected: one half was analysed for radioactivity by dissolving it in Bray's scintillation fluid and counting in an Intertechnique spectrometer, the other half was checked for absorbance at 260 nm.

2.5. *Limited hydrolysis of 23S RNA*

This hydrolysis was performed under the conditions described by Branlant et al. [17]: After incubation for 1 hr at 60°C in 0.05 M Tris–HCl pH 7.5, 0.02 M $MgCl_2$, 0.35 M KCl, 23S RNA was hydrolysed with T1 ribonuclease using an enzyme/substrate ratio of 1/3000 (w/w) for 15 min at 0°C. The digestion products were fractionated by centrifugation through a 15–30% linear sucrose gradient [17]. The fractions were analysed for radioactivity and absorbance at 260 nm as above.

3. Results and discussion

Radioactive BP-Phe-tRNA was incubated with ribosomes in the presence of poly U and the sample

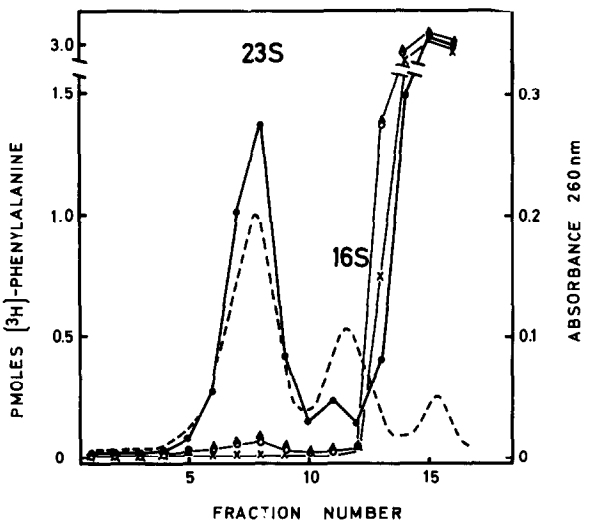

Fig.1. Sucrose gradient centrifugation of labelled 23S ENA. Ribosomes were incubated with poly U and BP-[^{3}H] Phe-tRNA for 15 min at 37°C and irradiated as described in Methods. RNA was isolated by phenol extraction and centrifuged through a 15–30% sucrose gradient in 100 mM NaCl, 10 mM Tris–HCl pH 7.4, 1 mM EDTA, 0.5% SDS in an SW 40 rotor at 27 000 rev/min for 17 hr at 19°C. The absorbance was monitored at 260 nm and fractions were analyzed for radioactivity; (●——●) complete incubation mixture; (○——○) poly U was omitted from the incubation mixture; (▲——▲) the complete incubation mixture was treated with 1 mM puromycin for additional 15 min at 37°C before being irradiated; (×——×) the complete incubation mixture, but omitting irradiation; (– – –) absorbance at 260 nm.

irradiated as described in Methods. RNA was subsequently isolated and fractionated on sucrose-SDS gradients. As shown in fig.1, a considerable amount of radioactive phenylalanine becomes incorporated into 23S RNA upon irradiation of the complete system. The small amount of radioactivity recovered in the 16S RNA peak presumably reflects a small amount of degradation of 23S RNA since it was not found in RNA extracted from purified 30S subunits. Omission of poly U results in a more than thirtyfold reduction in incorporation into 23S RNA indicating the high specificity of the labelling reaction. As expected, no radioactivity is incorporated when the sample is not irradiated. The addition of puromycin after binding of BP-Phe-tRNA but before irradiation inhibits the incorporation by more than 95%. This indicates that the photoaffinity reaction occurs with BP-Phe-tRNA bound to the ribosomal donor-site. Direct proof of this is provided by the demonstration that BP-Phe-tRNA covalently attached to 23S RNA after the photoaffinity reaction can still form a peptide bond with [^{3}H]Phe-tRNA (unpublished results).

50S subunits labelled with the radioactive phenylalanine derivative were submitted to the mild hydrolysis conditions as described in Methods. The profile of RNA in the sucrose gradient was the same as that obtained by Allet and Spahr [16]: two partially resolved peaks, corresponding to 18S and 13S core fragments and, in front of the 18S peak, a trace of undigested material. Radioactivity was only recovered in the 18S peak (fig.2).

Alet and Spahr [16] suggested that the RNA fragment contained in the 18S peak encompasses the 3′ terminal three-fifths portion of 23S RNA. This was confirmed by the work of Spierer et al. [18] and by our sequence studies on 23S RNA ([17] and G. Branlant et al., unpublished results). Since the experiment, repeated with different batches of 50S subunits labelled with [^{3}H] or [^{14}C]phenylalanine, gave consistent results, we can assume that the covalent linkage between the phenylalanine derivative and 23S RNA occurs specifically within the three-fifths portion of 23S RNA at the 3′ part of the molecule. It should be pointed out that 5S RNA in the presence of proteins L2, L6, L18 and L25 binds to the same 23S RNA region as shown by Gray and Monier [19].

23S RNA labelled with the radioactive phenylalanine derivative was hydrolysed using the conditions described in Methods. The RNA profile [17] obtained

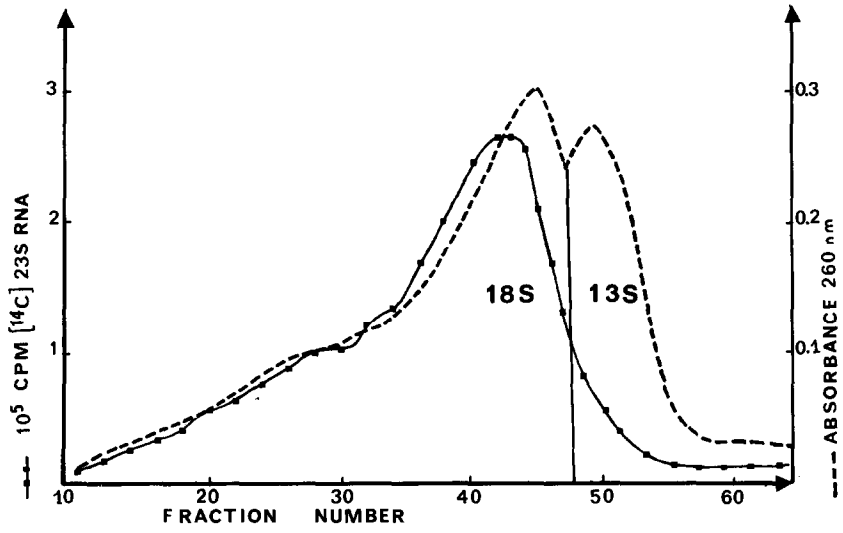

Fig.2. Limited hydrolysis of 50S subunits labelled with the [^{14}C] phenylalanine derivative as described in Methods: 50S subunits digested with pancreatic RNase bound to CM-cellulose, were treated with 2 M LiCl and pelleted. The pellet was then centrifuged through a 5–20% sucrose gradient in 10 mM Tris–HCl pH 7.5, 1 mM EDTA, in an SW27 rotor, at 26 000 rev/min for 27 hr at 4°C. Fractions were analysed for radioactivity and absorbance at 260 nm.

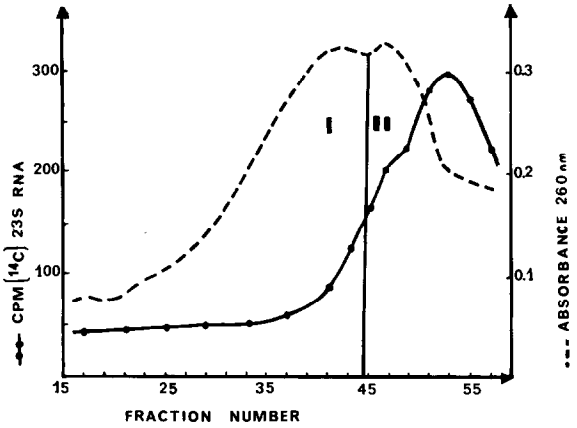

Fig.3. Limited hydrolysis of 23S RNA labelled with the [^{14}C] phenylalanine derivative as described in Methods: The 23S RNA digest was centrifuged through a 15–30% linear sucrose gradient in 0.05 M Tris–HCl pH 7.5, 0.02 M $MgCl_2$, 0.35 M KCl in an SW25 rotor, for 16 hours, at 23 000 rev/min and at 4°C. Fractions were analysed for radioactivity and absorbance at 260 nm.

after centifugation of the digest on a sucrose gradient consists of two poorly separated, broad peaks I and II. Some radioactivity was recovered within peak II, but the majority was contained in the fractions from the top of the gradient (fig.3). This infers that labelling occurred in a region quite sensitive to T1 ribonuclease hydrolysis. Control experiments (unpublished) have shown that the covalent linkage between the phenylalanine derivative and the 23S RNA was not broken by the pretreatment for 1 hr at 60°C indicating that the bond itself is stable under these conditions.

It has been shown [17] that peak I contains essentially large fragments from the 5′ end of 23S RNA, whilst peak II contains a high amount of two large fragments located within the 550 nucleotides at the 3′ end of 23S RNA: fragment 3 encompassing about 350 nucleotides, located at the 3′ end and fragment 7b encompassing 120–130 nucleotides. Since the radioactivity is not found within these large fragments, the covalent linkage must have occurred in that part of the 18S region excluding fragments 3 and 7b (fig.4).

Model experiments have shown that upon photoactivation benzophenone can react with peptides as well as nucleosides ([9] and our unpublished results). The preferential labelling of RNA

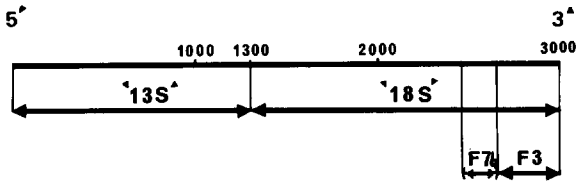

Fig.4. A scheme of the 23S RNA showing the distribution of various RNA fragments: '13S' and '18S' represent the two large RNA fragments contained in the 13S and 18S peaks obtained upon limited hydrolysis of 50S subunit according to Allet and Spahr [16]. The fragments F3 and F7b were obtained upon limited hydrolysis of 23S RNA.

obviously indicates that at the ribosomal donor-site, the 23S RNA is more easily available for reaction than the proteins known to be present at this site [20,21]. This is in agreement with the recent work by other authors ([6,7] and Zamir, A., personal communication). Our results emphasize the importance of region of the 23S RNA molecule, situated in the 18S fragment but excluding the 3′-terminal end, as a constituent of the ribosomal donor-site.

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