Part of the 23S RNA located in the 11S RNA fragment is a constituent of the ribosomal peptidyltransferase centre

Andrea Barta and Ernst Kuechler*

Institut für Biochemie, University of Vienna, Währingerstr. 17, A-1090 Vienna, Austria

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Upon irradiation, 3-[4-benzoylphenyl]propionyl-PhetRNA bound to the P-site of poly(U)-primed ribosomes is exclusively cross-linked to 23S RNA. It is shown that the photoreaction only occurs with pyrimidine nucleotides. The site of the cross-link is located within an 11S RNA fragment, which comprises the 1100 nucleotides at the 3'-end of 23S RNA. The cross-linked Phe tRNA derivative is still functionally active in peptide bond formation. The site labelled on the 11S fragment is therefore an integral part of the peptidyltransferase centre.

E. coli *ribosome* *Photoaffinity labelling* *Benzophenone derivative* *23S RNA* *Cross-link* *Peptidyltransferase*

1. INTRODUCTION

Identification of the components of functional sites of the ribosome is a prerequisite for the elucidation of the molecular mechanism of protein biosynthesis. For the characterization of ribosomal constituents located at the binding sites for mRNA, tRNA and antibiotics, the techniques of affinity and photoaffinity labelling have proved themselves to be particularly useful (reviews [1,2]). Affinity and photoaffinity reagents designed for the peptidyltransferase centre have labelled proteins as well as RNA to various extents [3–12]. Several investigators [8–12] have described derivatives of PhetRNA containing a photoreactive group attached to the amino group of the phenylalanyl moiety. Upon irradiation the photoaffinity labels were shown to react predominantly with 23S RNA. Photoreagents in general are less specific in terms of their chemical reactivities than conventional affinity labels. Therefore, the results of photoaffinity labelling experiments can be evaluated with greater confidence. These experiments indicate that some region in 23S RNA is an important part of the ribosomal peptidyltransferase centre.

Barta et al. [12] have demonstrated previously that 3-[4-benzoylphenyl]propionyl-PhetRNA (BP-PhetRNA) bound to the ribosome in the presence of poly(U) reacts exclusively with the 18S fragment of the 23S RNA. The reaction occurred at the ribosomal P-site, because labelling was prevented by preincubation with puromycin. In this paper the site of reaction of BP-PhetRNA on 23S RNA is further characterized and additional evidence for the specificity of the reaction is presented.

Abbreviation: BP-PhetRNA, 3-[4-benzoylphenyl]propionyl-PhetRNA

* To whom correspondence should be addressed

2. MATERIALS AND METHODS

2.1. *Materials*

BP-[^{3}H]PhetRNA was synthesized as in [13]. 70S ribosomes ('tight couples') from *E. coli* strain MRE 600 were prepared as in [14].

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[^{3}H[Phenylalanine (spec. act. 70 Ci/mmol) and [^{14}C]phenylalanine (spec. act. 520 mCi/mmol) were purchased from Amersham, poly(U) and RNase A bound to CM-cellulose from Miles Servac. $tRNA^{Phe}_{yeast}$ was obtained from Boehringer, Mannheim.

2.2. *Synthesis of BP-[^{14}C]Phe and the photoreaction with mononucleotides*

BP-[^{14}C]Phe was synthesized as in [15]. 0.45 mmol [^{14}C]phenylalanine (spec. act. 33 μCi/mmol) and 0.3 mmol $NaHCO_3$ were dissolved in 3 ml water. After the addition of 0.3 mmol 3-[4-benzoylphenyl]propionyl-*N*-hydroxysuccinimide ester [13] dissolved in 4 ml 1,2-dimethoxyethane, the mixture was incubated overnight at room temperature. The solution was acidified to pH 2 with diluted HCl and allowed to crystallize for 1 h at 0°C. The white precipitate was collected and recrystallized in methanol. Yield: 104 mg (87%), m.p. 151–153°C. For each mononucleotide (UMP, GMP, CMP, AMP) the photoreaction with BP-[^{14}C]Phe was carried out separately. 2–3 mg BP-[^{14}C]Phe was dissolved in 0.5 ml of 0.5 M mononucleotide solution and irradiated for 1 h with UV light of 320 nm under standard conditions [13]. An aliquot of each of the irradiated mixtures was spotted on Whatman 3MM paper and developed by descending chromatography using 1 M ammonium acetate (pH 7.5): 95% ethanol (30:70, v/v). After drying the chromatogram was cut in strips of 2 cm and the radioactivity of the strips was determined in a toluene-based scintillator.

2.3. *Photoaffinity labelling of ribosomes and isolation of 23S RNA fragments*

Irradiation of ribosomal complexes was performed as in [13]. When peptide bond formation was tested, unlabelled BP-PhetRNA was bound to poly(U)-primed ribosomes. Subsequently an equimolar amount of [^{3}H]PhetRNA was added and the incubation continued for 15 min at 37°C. 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 SW40 rotor at 27 000 rpm for 17 h at 19°C. The absorbance was monitored at 260 nm and fractions were analysed for radioactivity. Limited hydrolysis of 23S RNA with carrier-bound RNase A was done essentially as in [16]. To obtain high yields of the 18S and 11S RNA fragments, the conditions were changed slightly. Photoaffinity labelled 50S subunits at a concentration of 3–4 mg/ml in TMK buffer (0.1 M KCl, 0.01 M Tris-HCl (pH 7.4), 0.001 M magnesium acetate) were digested with RNase A bound to CM-cellulose at an enzyme/substrate ratio of 0.01 (w/w) for 45 min at 0°C. The resulting RNA fragments were isolated by extraction with phenol and separated on a 5–20% (w/v) sucrose gradient containing 10 mM Tris-HCl (pH 7.4), 1 mM EDTA in an SW40 rotor, at 30 000 rpm for 18 h at 4°C. The fractions corresponding to the 18S and 13S RNA peaks were collected separately and RNA was precipitated by adjusting the solution to 0.2 M NaCl and adding 2 vols of ethanol. The RNA was further analysed by electrophoresis on 3.5% polyacrylamide disc gels in 0.04 M Tris-acetate (pH 7.5), 0.02 M sodium acetate, 0.002 M EDTA. The gels were scanned for absorbance at 260 nm, sliced and their radioactivity determined [7].

3. RESULTS AND DISCUSSION

Upon irradiation at 320 nm a free electron pair of the carbonyl group of aromatic ketones is activated resulting in a triplet state [17,18]. The energy of this triplet state is about 70–75 kcal. As shown in [18] aromatic ketones can react with simple peptide analogues by insertion into the α C–H bond of the amino acid. The energy of the activated state is, however, too low to cause reaction with water molecules. To study the reactivity of BP-Phe with RNA, model experiments were first carried out with the four mononucleotides. Solutions of AMP, CMP, GMP and UMP were prepared separately, synthetic BP-[^{14}C]Phe was added to each and the mixtures were irradiated under standard conditions at 320 nm [13]. Aliquots were spotted on Whatman 3 MM paper. Following chromatography, the sheets were cut and the radioactivity determined. As can be seen in fig. 1, non-irradiated BP-[^{14}C]Phe migrated close to the solvent front ($R_f = 0.95$). Upon irradiation of BP-[^{14}C]Phe photoproducts were generated [17] which migrated somewhat more slowly ($R_f = 0.8$–0.9). When the photoreactions of BP-[^{14}C]Phe with the individual nucleotides were studied, only UMP

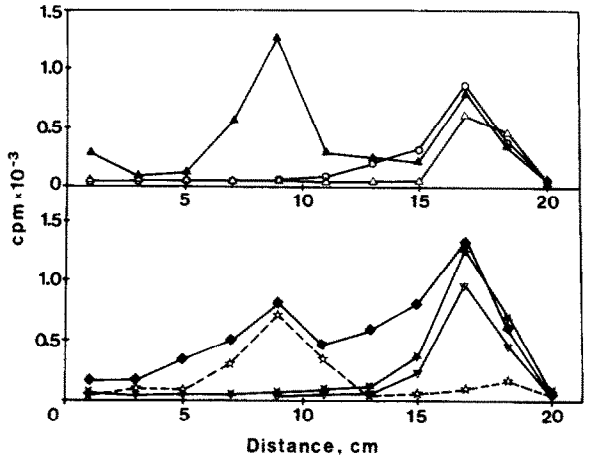

Fig.1. Analysis of the photoproducts of the reaction of BP-[^{14}C]Phe with mononucleotides. BP-[^{14}C]Phe was dissolved in a concentrated mononucleotide solution and irradiated for 1 h with UV light of 320 nm. Descending chromatography of the photoproducts was performed on Whatman 3MM paper using 1 M ammonium acetate (pH 7.5): 95% ethanol (30:70). The paper was cut and the strips were analysed for radioactivity. (△) BP-[^{14}C]Phe, not irradiated; (○) BP-[^{14}C]Phe, + UV; (▲) UMP + BP-[^{14}C]Phe, + UV; (◆) CMP + BP-[^{14}C]Phe, + UV; (★) AMP + BP-[^{14}C]Phe, + UV; (▽) GMP + BP-[^{14}C]Phe, + UV; (☆) 23S RNA photoaffinity labelled by irradiating ribosomal complexes of BP-[^{3}H]PhetRNA. The radioactive 23S RNA was hydrolysed by incubation with KOH. The neutralised mixture was spotted on Whatman 3MM paper and analysed as described above.

and CMP were found to react. The major photoproduct showed R_f values of around 0.5. The photoproduct with UMP appeared as a rather homogeneous peak, whereas photoreaction with CMP seemed to generate more by-products. In a model experiment 23S RNA was photoaffinity labelled with BP-[^{3}H]-PhetRNA on poly(U)-primed ribosomes. The RNA was then subjected to total hydrolysis with KOH and chromatography was carried out as before. The peak of ^{3}H radioactivity was found to comigrate with the peak of the modified pyrimidine nucleotides. This was taken to indicate that on the ribosome BP-[^{3}H]Phet RNA might photoreact with pyrimidine(s) on 23S RNA.

Evidence for the specificity of the labelling is provided by demonstrating peptide bond formation after the photoaffinity reaction has occurred. For this purpose, unlabelled BP-PhetRNA was bound to poly(U)-primed ribosomes. Following irradiation [^{3}H]PhetRNA was added and the mixture was incubated. As seen in fig.2, radioactivity was found to be incorporated into 23S RNA. [^{3}H]PhetRNA can only be attached through peptide bond formation generating BP-Phe-[^{3}H]Phet RNA covalently bound to 23S RNA. In a control experiment the order of reactions was reversed. Ribosomal complexes of BP-PhetRNA were incubated with [^{3}H]PhetRNA and irradiated subsequently. As shown in fig.2, a similar amount of radioactivity was found to be incorporated into 23S RNA under both conditions. The following conclusions can be drawn from these experiments: Photoaffinity reaction of BP-PhetRNA occurs at the P-site. This is in agreement with previous results demonstrating that preincubation with puromycin prevents the photoaffinity labelling [12]. Peptide bond formation still occurs after the cross-linking indicating that BP-PhetRNA is

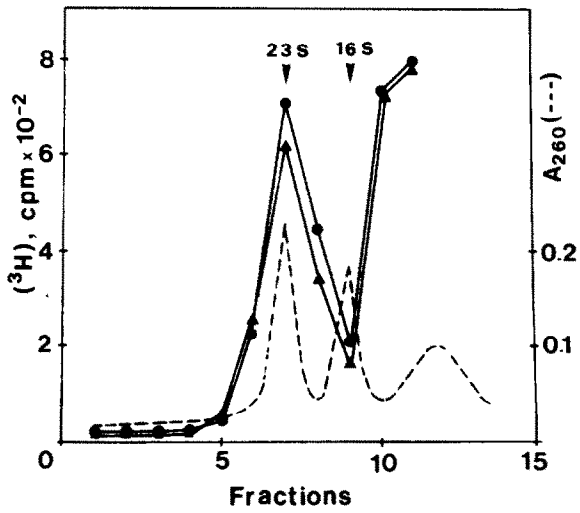

Fig.2. Sucrose gradient centrifugation of photoaffinity labelled 23S RNA and test for peptide bond formation. The absorbance was monitored at 260 nm and fractions were analysed for radioactivity. (●) Non-radioactive BP-PhetRNA was bound to poly(U)-primed ribosomes and [^{3}H]Phet-RNA was added. The incubation was continued for 15 min at 37°C to allow peptide bond formation and then irradiated with UV light of 320 nm under standard conditions. (▲) Again a complete ribosomal complex was formed with poly(U) and BP-PhetRNA and irradiated as described above. After the photoreaction [^{3}H]PhetRNA was added and the mixture was incubated for additional 15 min at 37°C.

bound in the proper configuration at the P-site even after covalent attachment to 23S RNA. The site labelled on 23S RNA must therefore be in close proximity to the peptidyltransferase centre.

This site has previously been localized in the 18S RNA fragment which comprises the 3′-terminal two-thirds of the 23S RNA [12]. An 11S subfragment of 18S can be obtained by prolonged incubation of 50S subunits with matrix-bound RNase A [16]. The 11S corresponds to a length of about 1100 nucleotides from the 3′-terminus of the 23S RNA. Fig.3A presents a sucrose gradient of fragmented ribosomal RNA obtained after treatment of BP-[^{3}H]PhetRNA-labelled 50S subunits with matrix-bound RNase A. A major peak of radioactivity was found in 18S RNA. The gradient was fractionated according to the optical density and the regions corresponding to the major digestion products 18S and 13S RNA were collected. The two samples were then further analysed on 3.5% polyacrylamide gels. Fig.3B shows the profile from the 18S RNA region. As expected the peak of the radioactivity corresponded exactly to the peak of the OD of 18S RNA; a minor peak of 11S was also observed. When the RNA of the 13S region was analysed (fig.3C), essentially all of the radioactivity was recovered in a peak of 11S RNA clearly separated from the OD peak of 13S RNA. This supports our previous conclusion that there is no incorporation into the 13S RNA fragment from the 5′-terminus of 23S RNA. A comparison of the specific activities calculated on a molar basis of the labelled 11S and 18S RNA peaks indicates that cross-linking takes place exclusively within the region corresponding to the 11S RNA fragment.

Our previous finding that the site labelled on the 18S RNA fragment is near the peptidyltransferase centre was later supported by similar results in [11]. None of the other photoaffinity labels of 23S RNA has been analysed to the level of the 11S RNA subfragment. There is, however, circumstantial evidence to support the concept that a region located within the 11S RNA fragment participates in the peptidyltransferase centre. Protein L27, a protein labelled by a variety of affinity labels which are specific for the peptidyltransferase centre [3,7,9], has a binding site located within the region of the 11S RNA fragment as identified by direct binding studies [16] as well as protein-RNA cross-linking [19]. Furthermore, several chloram-

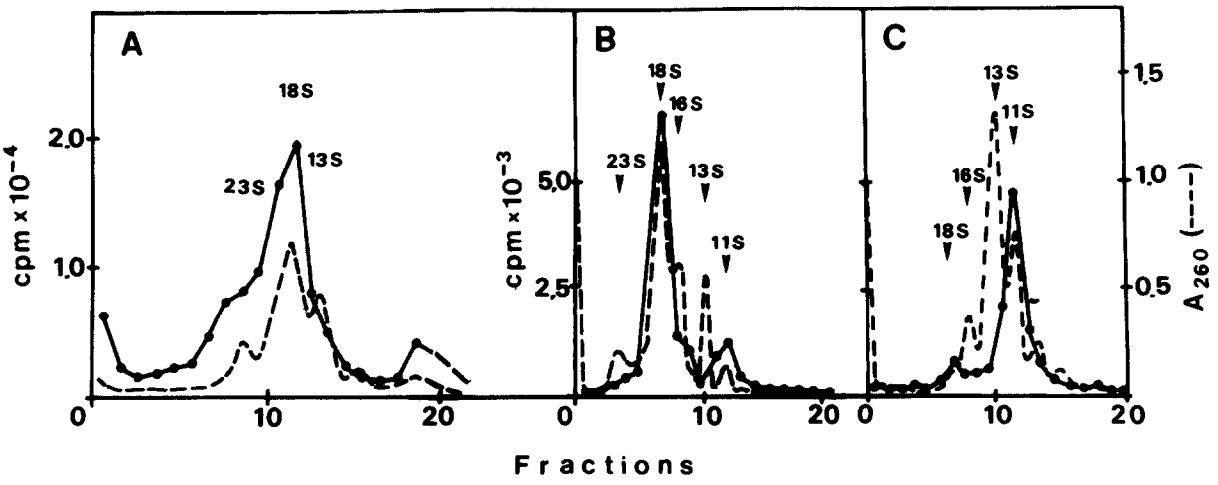

Fig.3. Limited hydrolysis of photoaffinity labelled 50S subunits. Digestion with RNase A bound to CM-cellulose was carried out as in section 2. (A) RNA fragments were isolated by phenol extraction and centrifuged through a 5–20% sucrose gradient. The absorbance was monitored at 260 nm (– – –) and the fractions were analysed for radioactivity. Fractions corresponding to the 18S and 13S RNA peaks were pooled separately and the RNA was precipitated. (B) The 18S RNA was further analysed on a 3.5% polyacrylamide gel in 0.04 M Tris-acetate (pH 7.5), 0.02 M sodium acetate, 0.002 M EDTA. The gel was scanned for absorbance at 260 nm (– – –) and the radioactivities of the 2 mm slices were determined as in [7]. (C) The analysis of the RNA from the 13S peak on a 3.5% polyacrylamide gel was done as described above.

phenicol-resistent mutants have been mapped in a region of mitochondrial ribosomal RNA which corresponds to the loop V domain of *E. coli* 23S RNA located within the 11S RNA fragment [20,21].

The experiments presented demonstrate that BP-PhetRNA cross-linked to 23S RNA at the P-site is bound in a functionally active state. The site labelled by the photoaffinity reagent must therefore be an integral part of the peptidyltransferase centre. Further work is required to identify more precisely the location of the site of the cross-link on the 11S RNA fragment.

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