SPECIFICITY OF THE PHOTO-CROSSLINKING REACTION BETWEEN POLY(U) AND PROTEIN S1 ON THE *ESCHERICHIA COLI* RIBOSOME

Peter MARGARITELLA and Ernst KUECHLER

Institute of Biochemistry, University of Vienna, Währingerstrasse 17, A-1090 Vienna, Austria

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1. Introduction

Photochemical crosslinking is an attractive method to study the environment of a ligand on a macromolecule. Following binding of an inert derivative of the ligand, the reactive form is generated on the binding site by irradiation. To study the binding site of mRNA on the ribosome of *E. coli*, poly-4-thiouridylic acid was employed [1–0]. It can be photoactivated at 330 nm. The photoreaction takes places predominantly with protein S1 and to a smaller extent with proteins S18 and S21. These data agree with the studies of chemically reactive oligonucleotide derivatives as affinity labels [4–7].

Poly(U) has also been used for photo-crosslinking. It can be photoactivated at 254 nm. When a ribosomal complex is irradiated, covalent bonds between poly(U) and S1 are formed [8].

In this paper we provide further evidence for the specificity of the photo-reaction with poly(U). Binding of polynucleotides to ribosomes can be increased by the presence of the deacylated form of the cognate tRNA [9]. Using similar conditions we demonstrate here that photochemical crosslinking of S1 on ribosomes with [^{3}H] poly(U) is strongly stimulated by purified $tRNA^{Phe}$. Four noncognate tRNA species used as controls did not show any stimulatory effect.

2. Experimental

2.1. *Materials*

E. coli tRNA (total mixture) and purified *E. coli* $tRNA^{Phe}$, $tRNA^{Tyr}$, $tRNA_1^{Val}$ and $tRNA_2^{Glu}$ were obtained from Boehringer Mannheim (FRG). Yeast $tRNA_{1+2}^{Ser}$ was kindly provided by H. G. Zachau, Munich. Ribosomes from *E. coli* D10 were prepared as in [10]. Before use ribosomes were preincubated for 5 min at 37°C. S150-supernatant proteins were obtained by centrifugation of an *E. coli* homogenate at 150 000 × *g* for 2 h followed by dialysis [3]. [^{3}H]-Poly(U) (spec. act. 1.5×10^5 cpm nmol^{-1}) was prepared as in [11]. Molar concentrations of [^{3}H]-poly(U) in the text represent concentrations of mononucleotide residues. [^{14}C] Phenylalanine (spec. act. 495 Ci mol^{-1}) was from Radiochemical Centre (Amersham, England).

2.2. *Buffers*

Buffer A contained 80 mM KCl, 30 mM Tris–HCl, pH 7.8, 5 mM magnesiumacetate and 1 mM dithiothreitol; buffer B same as A but 20 mM magnesium acetate. Buffer C was 0.1 M sodiumphosphate pH 7.1 containing 0.1% sodium dodecyl sulfate.

2.3. *Ultraviolet inactivation of ribosomes*

Ribosomes, 150 pmol, were incubated in 2 ml buffer A for 10 min at 37°C. Irradiation was carried out in a quartz test tube of 1 cm diameter using a low-pressure mercury lamp (Original Hanau NN15/44 VK, FGR) at 5 cm. The density of light at 254 nm was 4 mW/cm^2. During irradiation the samples were cooled by running tap water (8°C). Aliquots, 0.17 ml, were removed at 0, 5, 10 and 20 min and stored in ice. Mixture, 2 ml, containing 1.5 mg tRNA, 6 mg S150-supernatant proteins, 0.08 mg pyruvate kinase, 5 mM ATP, 0.4 mM GTP, 9 mM phosphoenolpyruvate, 0.075 mM each amino acid except phenyl-

alanine and 2.5 × 10^6 cpm [^{14}C]phenylalanine in buffer B was preincubated for 10 min at 37°C. Aliquots, 0.2 ml, were removed and mixed with the 0.17 ml aliquots of irradiated ribosomes, 0.04 mg poly(U) was added and the mixture incubated for 15 min at 37°C. NaOH was then added to 0.1 M and incubation continued for 30 min at 37°C. Trichloroacetic acid was added to 10%, the precipitate collected on Whatman GF/C glass-fibre filters and the radioactivity determined in toluene based scintillator.

2.4. *Photochemical crosslinking*

Mixtures, 1 ml, containing 60 pmol 70 S ribosomes or 30 S subunits, 1250 pmol [^{3}H]poly(U) and 1600 pmol respective tRNA species in buffer A were incubated for 10 min at 37°C. Samples were irradiated for 15 min as described above and treated with ribonuclease A at 0.002 mg/ml for 10 min at 0°C. Proteins were precipitated with trichloroacetic acid and collected by centrifugation.

2.5. *Gel electrophoresis*

The protein pellets were dissolved in 0.08 ml solution containing 0.06 M sodium phosphate buffer pH 7.1, 1% sodium dodecyl sulfate and 10 mM dithiothreitol. The solutions were heated for 90 s to 100°C. Samples were layered on to 7.5% polyacrylamide gels in buffer C [12]. Electrophoresis was performed for 14 h at 6 mA gel. Gels were stained with Coomassie brilliant blue and cut into 2 mm slices. Radioactivity was determined as in [8].

3. Results

Irradiation at 250 nm is known to affect ribosomal activity. The kinetics of inactivation is shown in table 1. Ribosomes were irradiated and aliquots of the mixture were removed at varying time intervals. The ribosomal activity in poly(Phe) synthesis was measured (see section 2). As can be seen in table 1, the time at which half of the ribosomes are inactivated is from 10–20 min irradiation. An irradiation time of 15 min was therefore chosen for all subsequent experiments. No alteration in sedimentation or dissociation properties of the ribosomes was observed under this condition.

Ribosomes, 70 S, were incubated with [^{3}H]poly(U)

Table 1
Effect of irradiation on ribosomal activity

Irradiation (min)	[^{14}C]Phe incorporated (cpm)	%
0	1873	100.0
5	1845	98.5
10	1310	69.9
20	482	25.7

Ribosomes were irradiated for the times indicated. Poly(U), [^{14}C]Phe and all components for in vitro protein synthesis were then added. Incorporation of [^{14}C]Phe obtained in the absence of poly(U) is subtracted

and uncharged $tRNA^{Phe}$ *(E. coli)* at 5 mM Mg^{2+} concentration. Samples were subsequently irradiated as above. Ribonuclease was then added and digestion allowed to go for 10 min at 0°C. Protein was precipitated and subsequently dissolved in buffer containing sodium dodecyl sulfate. Electrophoresis was carried out on 7.5% polyacrylamide gels. The profile of radioactivity is shown in fig.1a. A prominent peak of radioactivity is found in the S1 band. In addition there was some radioactivity in the region of the smaller ribosomal proteins which could not be allocated to any particular species. In a parallel experiment labelled ribosomes were dissociated into 50 S and 30 S subunits. As expected radioactivity attached to S1 was found only in 30 S subunits. None of the 50 S proteins were labelled (data not shown). No radioactivity was found in a control experiment in which the sample was not irradiated (fig.1a). Figure 1, panels b–e show controls in which *E. coli* $tRNA^{Phe}$ was replaced by *E. coli* $tRNA^{Tyr}$, $tRNA_1^{Val}$, $tRNA_2^{Glu}$ and yeast $tRNA_{1+2}^{Ser}$, respectively. It can be seen that in all cases the labelling of S1 was drastically reduced. Total omission of the tRNA from the incubation mixture also resulted in a reduction of radioactivity in S1 (fig.1f).

A similar photo-crosslinking experiment was performed on complexes as 30 S subunits with [^{3}H]-poly(U). As shown in fig.2 labelling of S1 on 30 S complexes was again stimulated by $tRNA^{Phe}$. In order to correct for S1 released during the dissociation step in the preparation of 30 S subunits, gels stained with Coomassie blue were scanned and the S1 peak was measured by densitometry. A comparison of the scans of 70 S and 30 S gels indicated that 30 S

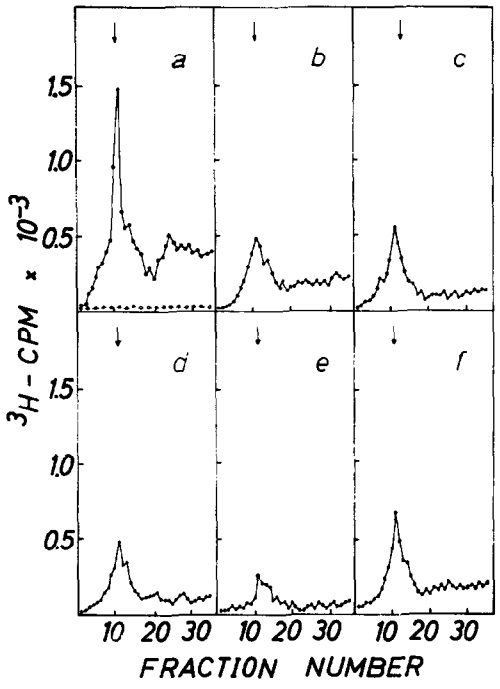

Fig.1. Stimulation of photoaffinity labelling of S1 by $tRNA^{Phe}$ on 70 S ribosomes. [^{3}H]Poly(U) was incubated with ribosomes in the presence of different tRNAs. Samples were irradiated for 15 min (full circles); control not irradiated (open circles). (a) *E. coli* $tRNA^{Phe}$; (b) *E. coli* $tRNA^{Tyr}$; (c) *E. coli* $tRNA_1^{Val}$; (d) *E. coli* $tRNA_2^{Glu}$; (e) Yeast $tRNA^{Ser}_{1+2}$; (f) no tRNA added. Proteins were extracted and separated on 7.5% polyacrylamide gels. The same amount of proteins was layered on each gel. The arrow indicates the position of the stained S1 band on the gel.

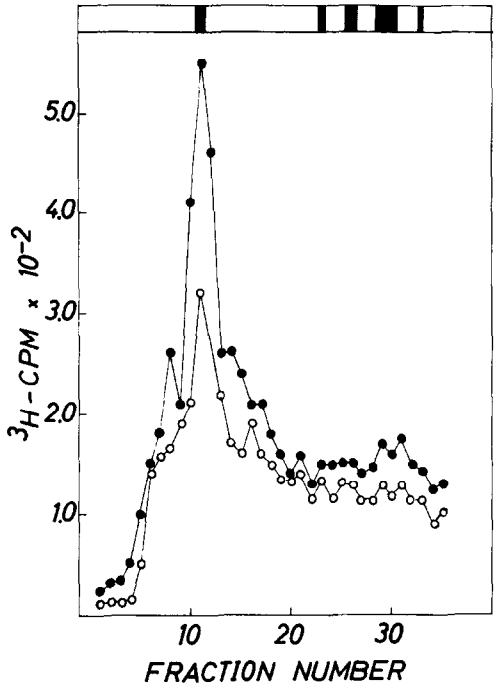

Fig.2. Stimulation of photoaffinity labelling of S1 by $tRNA^{Phe}$ on 30 S subunits. Conditions used were the same as in fig.1 except that complexes were formed with isolated 30 S subunits. Incubation and irradiation in the presence of *E. coli* $tRNA^{Phe}$ (full circles); no tRNA added (open circles). The amounts of proteins layered on the gels were identical. The staining pattern of the gel is shown schematically on top of the figure; the peak of radioactivity corresponds to the S1 band on the gel.

subunits had lost about 30% of the S1 during preparation (data not shown). This difference in S1 content of 70 S and 30 S should be taken into account when comparing the results in fig.1 and fig.2.

4. Discussion

Irradiation at 254 nm inactivates ribosomal activity as in [13,14]. High dosage of ultraviolet-light leads to extensive crosslinking between rRNA and proteins [15,16]. This photoreaction has been utilized by other authors to study the binding site of the ribosomal proteins S7 [17,18] and S4 [19] on 16 S RNA. In the investigations described in this paper a comparatively low irradiation dosage was applied.

Deacylated tRNA has a strong tendency to bind to the ribosomal peptidyl site at low Mg^{2+} concentration both in procaryotic and eucaryotic systems [20,21]. Binding of homopolynucleotides was mediated by the deacylated form of the cognate tRNA [9]. Using sucrose density gradient centrifugation these authors compared 10 different tRNA species for their capacity to stimulate mRNA binding to ribosomes. In the *E. coli* system, poly(U) binding was only stimulated by $tRNA^{Phe}$ whereas $tRNA^{Lys}$ specifically increased poly(A) binding.

In the study of the above photoaffinity reaction we have compared $tRNA^{Phe}$ with four tRNA species also previously employed in the experiments [9]. The anticodon of *E. coli* $tRNA^{Phe}$ is GAA. Although some of the tRNAs used as controls share at least one

A in the anticodon with $tRNA^{Phe}$ [22], none of them shows any stimulatory effect on the photoreaction with S1. In fact labelling seems to be somewhat suppressed when the noncognate tRNA is present as compared to the control in which tRNA is totally missing (fig.1). The specificity of the labelling reaction can be explained best by a stabilization of the ribosomal complex due to complementary base pair interaction between the UUU codon and the anticodon of $tRNA^{Phe}$.

Supernatant proteins of *E. coli* contain a factor called HF with a molecular weight similar to S1 which binds to polynucleotides, preferentially to poly(A) [23,24]. This factor dissociates into subunits of small molecular weight upon prolonged boiling in SDS in the presence of DTT. We have therefore heated the labelled protein samples for periods up to 30 min, but no change in the profile of radioactivity on the gel was observed. Furthermore, no significant difference was found when the photoreaction was carried out in the presence of supernatant proteins.

The experiments presented in this paper confirm and extend the conclusion drawn from [1–3,8]. They demonstrate that photochemical-crosslinking of protein S1 and poly(U) in the presence of $tRNA^{Phe}$ indeed occurs at the ribosomal mRNA binding site. S1 therefore constitutes part of this site. The result is in line with current ideas about the role of S1 in protein biosynthesis [25–30].

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