The EMBO Journal vol.7 no.12 pp.3949–3955, 1988

Photo-affinity labelling at the peptidyl transferase centre reveals two different positions for the A- and P-sites in domain V of 23S rRNA

Guenter Steiner, Ernst Kuechler and Andrea Barta

Institut fuer Biochemie der Universitaet Wien, Waehringer Strasse 17, A-1090 Wien, Austria

Communicated by E.Wintersberger

Photo-reactive 3-(4′-benzoylphenyl)propionyl-Phe-tRNA bound to the A- or the P-site was crosslinked to 23S RNA upon irradiation at 320 nm. The sites of reaction were identified as U-2584 and U-2585 at the A-site and A-2451 and C-2452 at the P-site. Minor crosslinks from both sites were observed at nucleotides A-2503 to U-2506. All sites identified lie in close proximity according to the secondary structure model and constitute part of the highly conserved loop region of domain V. Antibiotics known to inhibit peptidyl transferase activity had a pronounced effect on photo-crosslinking. In addition, tetracycline was also shown to photo-crosslink to this region. These experiments permit a dissection of the peptidyl transferase region on the 23S RNA into two distinct areas for the A- and P-site.

Key words: A- and P-site/antibiotics/23S rRNA/peptidyl transferase/photo-crosslink

Introduction

The secondary structures of rRNA have been strongly conserved throughout evolution (Noller, 1984; Brimacombe and Stiege, 1985). This conservation implies an important role for the RNA in the function of the ribosome. This is of particular interest in the light of the recent findings that RNAs can act as enzymes (Guerrier-Takada *et al.*, 1983; Zaug and Cech, 1986; Sharp and Eisenberg, 1987; Uhlenbeck, 1987). Furthermore, there is increasing evidence that the rRNAs play a fundamental role in the assembly as well as in the function of the ribosome. In the past few years several putative functional sites on 16S and 23S RNA have been identified both by genetic studies and by chemical or photo-chemical crosslinking (Prince *et al.*, 1982; Barta *et al.*, 1984; Cundliffe, 1986; Noller *et al.*, 1986; Ofengand *et al.*, 1986). The latter techniques have been used to characterize sites within the rRNA that are in close contact with the various components such as ribosomal proteins (Brimacombe and Stiege, 1985; Brimacombe *et al.*, 1986; Ebel *et al.*, 1986), tRNAs (Barta *et al.*, 1984; Ofengand *et al.*, 1986) and mRNA (Shine and Dalgarno, 1974; Prince *et al.*, 1982; Noller *et al.*, 1986). RNA–RNA interactions are of considerable interest, given their potential involvement in essential ribosomal functions. The role of 16S RNA in binding prokaryotic mRNAs (Shine and Dalgarno, 1974; Steitz, 1979) and its participation in the decoding site (Prince *et al.*, 1982; Noller *et al.*, 1986; Ofengand *et al.*, 1986), is well established. Moreover, there are indications that RNA–RNA interactions and conformational 'switches' might be a driving force for the translocation process (reviewed in Noller,1984; Brimacombe *et al.*, 1986). rRNAs have also been implicated in the binding of several antibiotics (reviewed by Cundliffe, 1986).

Peptide bond formation occurs on the 50S subunit. However, despite much effort the site of catalytic activity responsible for trans-peptidation has not been assigned to any particular ribosomal protein. In previous experiments, we developed a photo-affinity label for identifying components at or close to the peptidyl transferase region (Barta *et al.*, 1975; Barta and Kuechler, 1983; Barta *et al.*, 1984). Use was made of a benzophenone derivative [3-(4′-benzoylphenyl)propionic acid] covalently attached to the amino group of the Phe moiety of Phe-tRNA. Upon irradiation at 320 nm the carbonyl group of the benzophenone residue has the potential to react with both proteins and nucleotides (Barta and Kuechler, 1983). When bound to the ribosome in an mRNA dependent manner the 3-(4′-benzoylphenyl)propionyl-Phe-tRNA (BP-Phe-tRNA) was found to be crosslinked exclusively to 23S RNA (Barta *et al.*, 1975; Barta and Kuechler, 1983), with the region between nucleotides 2442 and 2625 on 23S RNA being identified as the site of crosslinking by DNA–RNA hybridization. Primer extension experiments localized the site of photo-crosslinking to U-2584 and U-2585 (Barta *et al.*, 1984). This finding correlates with the fact that 23S RNA is among the ribosomal components essential for peptidyl transferase activity (Schulze and Nierhaus, 1982). Under the conditions used, the binding of the BP-Phe-tRNA could not however be unambiguously assigned to the A- or the P-site; a tentative assignment to the P-site based on puromycin sensitivity of the photo-affinity labelling was nevertheless made. In this report we extend these earlier observations by describing the optimization of conditions for binding the BP-Phe-tRNA to the A- or P-site and by identifying the affinity-labelled nucleotides at each site. Crosslinking at the A-site occurred primarily to nucleotides U-2584 and U-2585 whereas A-2451 and C-2452 were crosslinked at the P-site. Although the labelling patterns are different at each site, all labelled nucleotides are located within the highly conserved central loop of domain V (Noller *et al.*, 1981; Barta *et al.*, 1984). In addition, the influence of several antibiotics on the binding and on the photo-reaction of BP-Phe-tRNA was investigated. The experiments demonstrate that antibiotics known to inhibit peptidyl transferase activity have a pronounced effect on photo-crosslinking. The data presented in this paper confirm that domain V of the 23S rRNA is spatially close to the aminoacyl moieties of ribosome-bound tRNAs and extend our knowledge of the topography of the A- and P-site at the peptidyl transferase region.

©IRL Press Limited, Oxford, England

Table I. Binding of BP-[^{3}H]Phe-tRNA to ribosomes and photo-crosslinking to 23S RNA[a]

	Binding (c.p.m.)	Photo-crosslinking (c.p.m.)
P-site		
− Poly(U), − UV	21.000	ND[b]
+ Poly(U), − UV	183.000	13.000
+ Poly(U), + UV	196.000	152.000
+ Poly(U), + Puromycin, + UV	35.000	5.000
Puromycin sensitivity[c]	82%	95%
A-site		
− Poly(U), − UV	52.000	ND[b]
+ Poly(U), − UV	149.000	9.000
+ Poly(U), + UV	168.000	150.000
+ Poly(U), + Puromycin, + UV	135.000	42.000
Puromycin sensitivity[c]	20%	72%

[a]The values are normalized to 10 pmol of ribosomes or 23S RNA respectively.
[b]Not determined.
[c]In the case of the puromycin controls, the antibiotic was added after binding of BP-Phe-tRNA but prior to irradiation.

Table II. Translocation of A-site bound BP-[^{3}H]Phe-tRNA by EF-G and GTP to the P-site[a]

	A-site (c.p.m.)	+EF-G/GTP (c.p.m.)
− Poly(U)	8.500	ND
+ Poly(U)	69.000	46.000
+ Poly(U), + Puromycin	51.000	8.500
Puromycin sensitivity	26%	82%

[a]Binding of BP-[^{3}H]Phe-tRNA to ribosomes was measured as described in Table I.

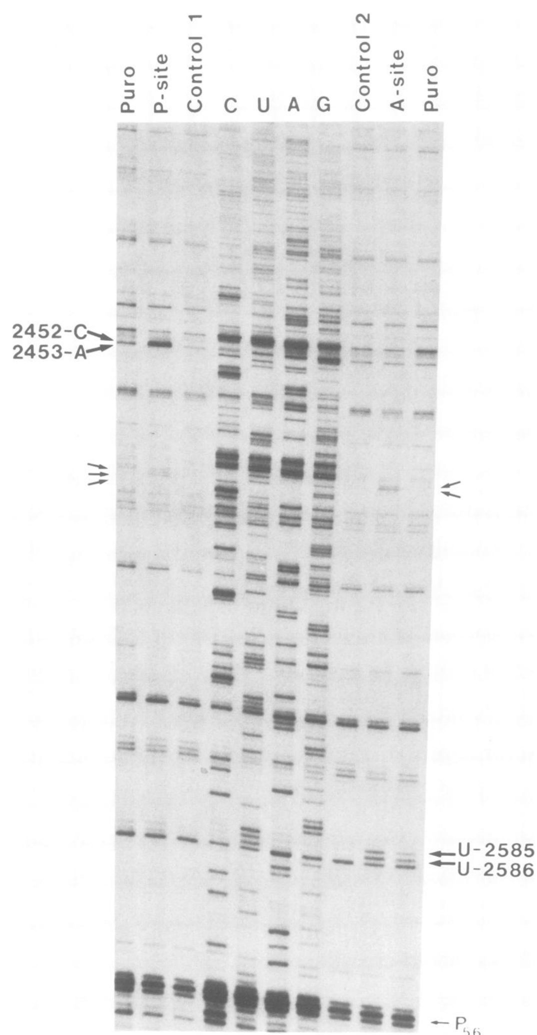

Fig. 1. Autoradiograph showing the distinct photo-crosslinking pattern of P- and of A-site bound BP-[^{3}H]Phe-tRNA to 23S rRNA. Specific binding and photo-crosslinking was performed as described in Materials and methods. The isolated RNAs were used as templates for reverse transcriptase reactions primed with a 56 bp *Ava*II DNA fragment corresponding to positions 2663–2607 on 23S RNA. **Lanes C, U, A** and **G** are dideoxy sequencing lanes and refer to the nucleotide sequence of 23S rRNA. rRNA from non-irradiated ribosomes (**lane: control 1**) and from ribosomes irradiated in the absence of BP-[^{3}H]Phe-tRNA (**lane: control 2**) served as controls. **Lane Puro:** 1 mM puromycin was added after binding but before irradiaton. Stops at the designated nucleotides are due to photo-crosslinking to the ensuing nucleotide. Little arrows on both sides represent minor crosslinking sites.

Results

Binding of BP-Phe-tRNA to the P- and A-sites and photo-reaction with 23S RNA

In order to obtain specific binding of BP-Phe-tRNA to either the A- or the P-site, the conditions had to be optimized. In all experiments ribosomes were present in a 2.5-fold molar excess over BP-Phe-tRNA. For specific P-site binding, it was necessary to purify BP-Phe-tRNA by removing uncharged tRNA; optimal P-site binding was obtained at 6 mM Mg^{2+}. The specificity of P-site binding was checked by addition of puromycin before irradiation. In order to place BP-Phe-tRNA into the A-site, ribosomes were preincubated with uncharged tRNA and the Mg^{2+} concentration was raised to 10 mM. Table I shows the results obtained for binding and photo-reaction under P- and A-site conditions.

The extent of photo-crosslinking was nearly identical under both binding conditions, >70% of bound BP-Phe-tRNA reacting with the 23S RNA. Binding of BP-Phe-tRNA under P-site conditions was suppressed >80% by puromycin indicating that this amount was functionally bound at the P-site. No photo-reaction could be detected in the presence of puromycin. BP-Phe-tRNA bound under A-site conditions showed only 20% puromycin sensitivity. Furthermore, A-site bound BP-Phe-tRNA could be translocated to the P-site by the addition of EF-G and GTP as determined by the increase of puromycin sensitivity to 82% (Table II). Although binding of BP-Phe-tRNA to the A-site was only

Table III. Binding and photo-crosslinking of BP-[^{3}H]Phe-tRNA in the presence of antibiotics[a]

Antibiotic	P-site		A-site	
	Binding (%)	Crosslink (%)	Binding (%)	Crosslink (%)
None (control)	100	100	100	100
Puromycin	25	3	80	29
Chloramphenicol	93	38	74	35
Erythromycin	90	68	91	54
Tiamulin	89	5	89	4
Tetracyclin	90	3	100	7
Ampicillin	100	100	100	100

[a]The values are indicated as percent of the control incubations which are taken as 100%. Antibiotics were added prior to irradiation.

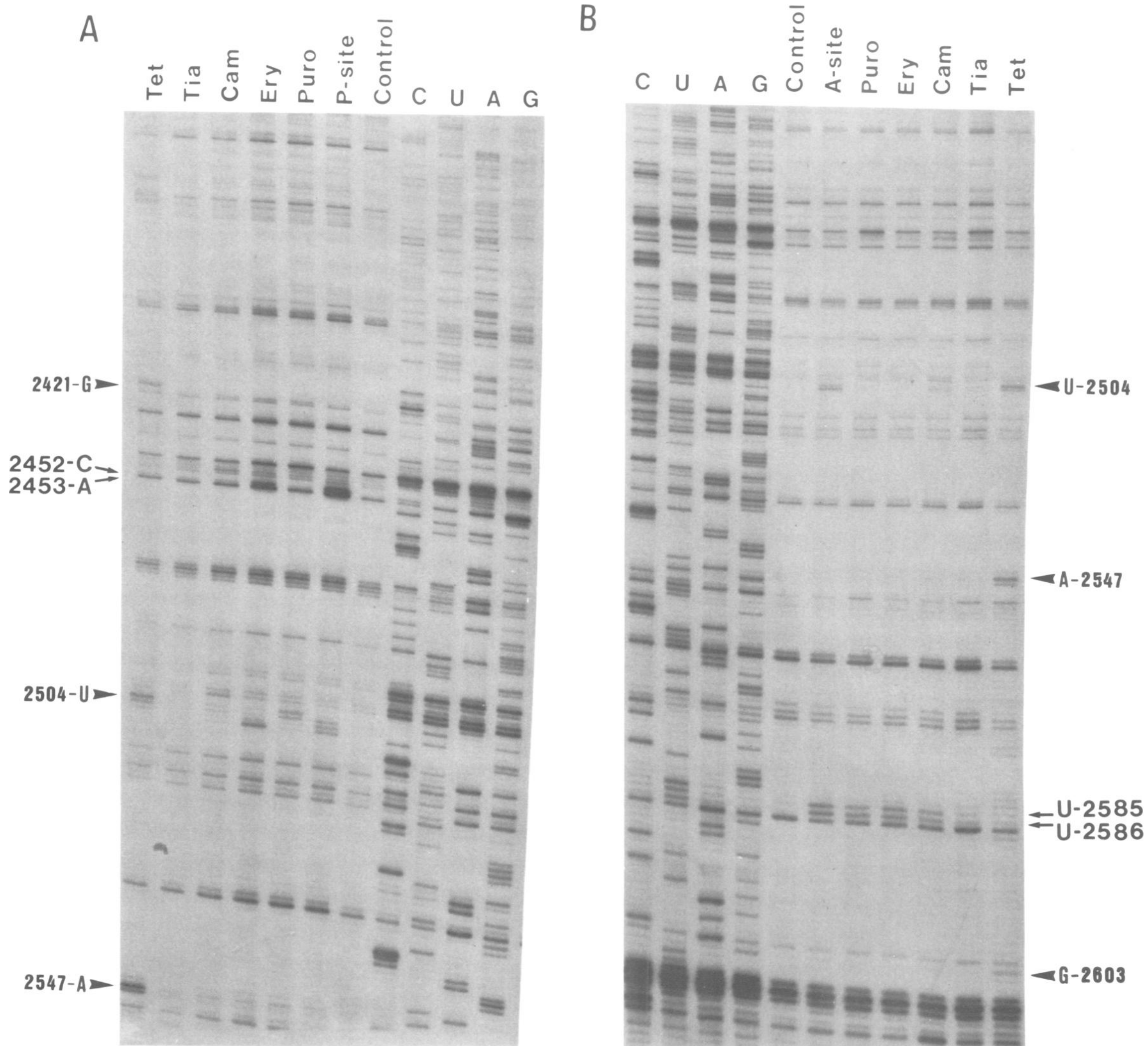

Fig. 2. Influence of antibiotics on specific photo-crosslinking of BP-[^{3}H]Phe-tRNA to 23S rRNA. Autoradiographs show the reverse transcriptase products of rRNA obtained from irradiated ribosomes with BP-[^{3}H]Phe-tRNA bound either to P-site (**A**) or to A-site (**B**). **Lanes C, U, A** and **G** are dideoxy sequencing lanes and refer to the nucleotide sequence of 23S rRNA. rRNA from non-irradiated ribosomes served as controls (**lane: control**). All antibiotics were added prior to irradiation: **lane: Puro**, 1 mM puromycin; **lane: Ery**, 0.1 mM erythromycin; **lane: Cam**, 0.1 mM chloramphenicol; **lane: Tia**, 0.1 mM tiamulin; **lane: Tet**, 0.1 mM tetracycline. Nucleotides before the specifically crosslinked bases are indicated by arrows. The arrowheads point to stops brought about by photo-crosslinking of tetracycline with ribosomes.

slightly affected by puromycin, photo-crosslinking was inhibited by 72% (Table I). Interestingly, this inhibition could be reversed by removal of the antibiotic prior to irradiation (data not shown). The fact that the photo-crosslinking but not the binding of BP-Phe-tRNA was strongly reduced by puromycin can be explained best by competition between the 3′-end of BP-Phe-tRNA and puromycin for a common binding site. This phenomenon shows that puromycin inhibition of the photo-crosslinking is not necessarily a valid criterion for specific binding to the A- and/or the P-site.

Identification of photo-affinity-labelled nucleotides on the 23S RNA

In previous experiments reverse transcriptase (Barta *et al.*, 1984) was used to identify the affinity-labelled nucleotides. This assay is based on the propensity of reverse transcriptase to stop at the nucleotide preceding the site of modification (Youvan and Hearst, 1979). By applying this technique the localization of nucleotides crosslinked to 23S RNA obtained upon irradiation of BP-Phe-tRNA present at the A- or the P-site was determined. A primer corresponding to positions 2663−2607 was employed. rRNA from non-irradiated ribosomes (Figure 1, lane: control 1) and from ribosomes irradiated in the absence of BP-Phe-tRNA (lane: control 2) served as controls. Primer extension products from reaction mixtures using dideoxynucleotide sequencing reactions were run in parallel. The autoradiograph of the gel shows the same distinctive pattern of 'background' bands in all lanes. Additional bands appear in the lanes designated A- and P-site. When 23S RNA crosslinked under P-site conditions was used as a template, reverse transcription stopped at nucleotides C-2452 and A-2453, thus identifying A-2451 and C-2452 as affinity-labelled bases. In addition, three minor bands corresponding to modifications at positions U-2504, G-2505 and U-2506 could be detected (see small arrows at left side, Figure 1). No photo-crosslinking to these sites occurred when puromycin was added prior to irradiation (lane: Puro, at left side, Figure 1). As expected, removal of puromycin prior to irradiation did not restore affinity labelling under P-site binding conditions (data not shown). Some crosslinking at

Table IV. Inhibition of photo-crosslinking of BP-[^{3}H]Phe-tRNA to 23S RNA by antibiotics

	A-site	P-site
Inhibitors of peptidyl transferase and/or translocation		
Chloramphenicol	+++	++++
Lincomycin	−	+++
Tiamulin	++++	++++
Sparsomycin	++	+++
Vernamycin B	++++	++++
Erythromycin	++	++
Inhibitors of tRNA-binding		
Fusidic acid	−	−
Gentamycin	−	+
Kanamycin	+	−
Streptomycin	−	−
Thiostrepton	−	−
Tetracycline	++++	++++
Inhibitor of cell wall synthesis		
Ampicillin	−	−

(++++) complete, (+++) strong, (++) medium, (+) weak, (−) no inhibition.

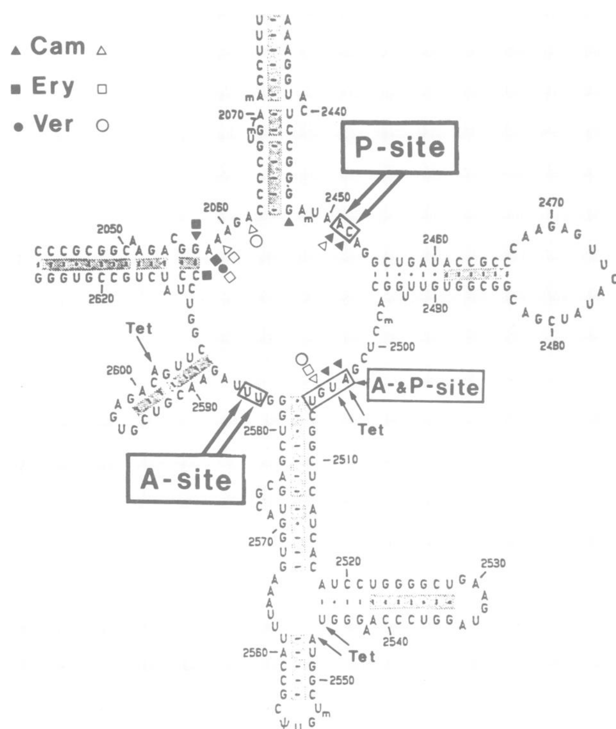

Fig. 3. A schematic diagram of the secondary structure of the central loop region of domain V of 23S rRNA. The nucleotides specifically affinity labelled by A- and P-site bound BP-[^{3}H]Phe-tRNA identified in this paper are boxed and designated. Arrows point to nucleotides modified by irradiating ribosomes with tetracycline alone (this paper). Cam, chloramphenicol; Ery, erythromycin; Ver, vernamycin. Filled in symbols indicate nucleotides whose mutations confer resistance to the respective antibiotic (reviewed in Noller, 1984; Sor and Fukuhara, 1984; Douthwaite *et al.*, 1985; Ettayebi *et al.*, 1985); open symbols designate antibiotics whose binding to the ribosome causes an alteration of reactivity of the respective nucleotide towards chemical modification (Moazed and Noller, 1987).

U-2585 and U-2586 resulting in faint bands was observed under these conditions probably reflecting some BP-Phe-tRNA bound at the A-site. This interpretation is strengthened by the finding that similar bands are also found in the presence of puromycin (see below).

Use of 23S RNA crosslinked under A-site conditions as a template resulted in strong stops at U-2585 and U-2586. Therefore U-2584 and U-2585 must have been labelled by BP-Phe-tRNA. In addition two weak bands corresponding to modifications at A-2503 and U-2504 could also be detected in this lane (small arrows at right side). When crosslinking was carried out in the presence of puromycin, the intensity of the two main bands (U-2585 and U-2586) was decreased (see lane: Puro, right side). However, removal of puromycin prior to irradiation restored affinity labelling completely (data not shown) confirming the A-site location as discussed above.

Influence of antibiotics on photo-crosslinking

The observed effect of puromycin on photo-crosslinking led us to investigate the influence of other antibiotics known to inhibit ribosomal functions. If photo-crosslinking indeed occurs at or near the peptidyl transferase site, antibiotics known to inhibit peptide bond formation should affect affinity labelling with BP-Phe-tRNA. In a first series of experiments the following antibiotics were tested: chloramphenicol (Vazquez, 1979) and tiamulin (Hoegenauer, 1974; Hodgin and Hoegenauer, 1975) as well defined inhibitors of peptidyl transferase activity, erythromycin considered to be an inhibitor of translocation (see Menninger and Otto, 1982) and tetracycline (Cundliffe, 1979) acting on EF-Tu dependent binding of aminoacyl-tRNA. Ampicillin, an inhibitor of bacterial cell wall synthesis, served as a control. The samples were irradiated and aliquots were taken to determine bound BP-Phe-tRNA. The extent of photo-crosslinking was measured by column chromatography (Table III). A slight inhibition of binding could be observed in all cases with the exception of ampicillin and tetracycline. The observed 100% binding at the A-site in the presence of tetracycline is in contrast to published experiments with Ac-Phe-tRNA, where A-site binding is substantially inhibited (Geigenmüller and Nierhaus, 1986). This difference in tetracycline sensitivity is probably due to the different binding properties of the BP-Phe-tRNA as compared to Ac-Phe-tRNA. However, the effects on photo-crosslinking were much more pronounced. Almost complete inhibition of photo-crosslinking was exerted by tiamulin both in A- and P-site, the effect of tetracycline being similar. Considerable inhibition was achieved by chloramphenicol, whereas erythromycin inhibited only weakly under P-site and by ~50% under A-site conditions. Ampicillin which does not bind to ribosomes had no effect on binding or photoreaction.

RNAs were isolated by phenol extraction and used as templates for reverse transcription. The effect of the antibiotics chloramphenicol, tiamulin, erythromycin and tetracycline on affinity labelling of individual nucleotides is demonstrated in Figure 2 using P-site (Figure 2A) and A-site (Figure 2B) binding conditions. In agreement with data in Table III the intensity of the bands corresponding to crosslinked nucleotides in both A- and P-sites is reduced in all lanes, reaching background levels for tiamulin and tetracycline. However, upon irradiation in presence of tetracycline additional bands can be observed corresponding to G-2421, U-2504, A-2547 and G-2603. These bands appeared also when ribosomes were irradiated in the presence of this antibiotic without BP-Phe-tRNA (data not shown). It is therefore clear that these bands result from a

photo-reaction between tetracycline and 23S rRNA. Further experiments should clarify whether other sites on rRNA are also involved in photo-crosslinking with tetracycline.

In a second series of experiments several additional antibiotics acting on translocation (vernamycin) and on EF-Tu dependent aminoacyl-tRNA binding (fusidic acid, gentamycin, kanamycin, streptomycin and thiostrepton), were tested and the effects compared with those of peptidyl transferase inhibitors such as lincomycin and sparsomycin (Cundliffe, 1979, 1986). Under the conditions employed none of these antibiotics had a pronounced effect on binding of BP-Phe-tRNA either to the A- or to the P-site (data not shown). This is not unexpected since binding of BP-Phe-tRNA was carried out under non-enzymatic conditions. Using the reverse transcription assay the influence of the antibiotics on the affinity-labelling of the specific nucleotides on 23S RNA was tested. The extent of inhibition was estimated visually from the intensity of the bands. Table IV shows the combined results from several experiments. Significantly, inhibitors of peptidyl transferase (chloramphenicol, tiamulin, sparsomycin, lincomycin) have a pronounced effect on crosslinking at the P-site. Under A-site binding conditions all peptidyl transferase inhibitors tested exhibited strong effects except for lincomycin. Erythromycin and vernamycin B (generally considered to be inhibitors of translocation) showed inhibition both at the A- and the P-site. In contrast, inhibitors of EF-Tu dependent aminoacyl-tRNA binding in general have no or very small effects except for tetracycline (see below).

Discussion

The results presented in this paper clearly show that photo-crosslinking of BP-Phe-tRNA to 23S rRNA occurred both under P- and A-site binding conditions. In both cases the reaction was complete within 20 min of irradiation and ~70% of the bound BP-Phe-tRNA reacted with the rRNA. As previously demonstrated, the benzophenone moiety can react with proteins as well as with nucleic acids (Galardy *et al.*, 1973; Barta and Kuechler, 1983). It is therefore likely that the labelling pattern obtained reflects the actual topography of the peptidyl transferase region. The nucleotides involved in the crosslink were A-2451 and C-2452 when BP-Phe-tRNA was bound to the P-site and U-2584 and U-2585 when bound to the A-site. In addition weak labelling could be detected at nucleotides 2503-AUGU-2506 under both A- and P-site conditions (Figure 3).

Evidence for the specificity of the photo-affinity labelling is provided by inhibition studies using antibiotics known to act on protein biosynthesis. Puromycin which resembles the 3′-end of a charged tRNA was used to distinguish between A- and P-site binding. As puromycin binds to the A-site and releases P-site bound BP-[^{3}H]Phe-tRNA, photo-crosslinking under P-site conditions was strongly and irreversibly inhibited. To our surprise, affinity labelling of 23S RNA by A-site bound BP-[^{3}H]Phe-tRNA was also considerably inhibited although binding was only slightly affected. However, in contrast to the P-site, removal of puromycin almost completely restored photo-crosslinking at the A-site. These observations could be interpreted as a competition between the aminoacyl-terminus of A-site bound BP-Phe-tRNA and puromycin for a common binding site on 23S RNA. In a previous paper the effect of puromycin on crosslinking of U-2584 and U-2585 was taken as tentative evidence for labelling from the P-site (Barta *et al.*, 1984). However, the results described in this paper indicate that inhibition of U-2584 and U-2585 crosslinking observed earlier was due to competition at the A-site between puromycin and the 3′-terminus of BP-Phe-tRNA rather than release of the affinity label by the puromycin reaction at the P-site.

Our data strongly suggest that the central loop region of domain V is an integral part of the peptidyl transferase site as it is photo-affinity-labelled by both A- and P-site bound BP-[^{3}H]Phe-tRNA (Figure 3). The sites identified are localized within a stretch of nucleotides between bases 2442 and 2625 previously identified by hybridization to contain the crosslink. All crosslinked nucleotides are within a phylogenetically highly conserved structure (Barta *et al.*, 1984; Noller, 1984). Although these sites appear rather distant in the primary structure, they are brought into close proximity by the secondary folding of the RNA molecule (Branlant *et al.*, 1981; Glotz *et al.*, 1981; Noller *et al.*, 1981). Moreover, the labelled nucleotides are unpaired and are highly conserved in a wide variety of species supporting the notion that this region is involved in an essential ribosomal function (Noller, 1984).

There is also accumulating evidence that sites on rRNA act as targets for antibiotics (Cundliffe, 1986). In particular, single base changes in the central-loop region of domain V confer resistance to chloramphenicol and erythromycin (Sor and Fukuhara, 1984; Ettayebi *et al.*, 1985). As these mutations are close to or at the nucleotides identified in our experiments (Figure 3), it was of considerable interest to investigate the effects of antibiotics on both the binding and crosslinking of BP-[^{3}H]Phe-tRNA. Several antibiotics known to inhibit various steps in ribosomal function were used, with ampicillin which inhibits cell wall formation serving as control. As shown in Table IV, all inhibitors of peptidyl transferase tested had a strong effect on photo-crosslinking. Tiamulin which acts similarly to and has overlapping binding sites with chloramphenicol (Hoegenauer, 1974) strongly inhibits photo-affinity labelling at both sites although tRNA binding is hardly affected. Data on the effect of chloramphenicol are particularly clear as both strong P-site crosslinks (A-2451 and C-2452) and two of the weak crosslinks (A-2503 and U-2504) are also sites of point mutations conferring resistance to chloramphenicol (Sor and Fukuhara, 1984). In addition, A-2451 and G-2505 are protected by chloramphenicol against chemical modifications with kethoxal and dimethylsulphate (Moazed and Noller, 1987). Thus three completely independent experimental approaches suggest that the region around A-2451 is involved in peptidyl transferase activity. It can therefore be concluded that chloramphenicol acts by binding at or near A-2451 and thus inhibits peptidyl transfer. The observed inhibitions on the photo-affinity labelling with BP-Phe-tRNA can be best explained by assuming that the various antibiotics act by interfering with the proper sterical arrangement of 23S RNA and the 3′-terminus of tRNA.

Erythromycin and vernamycin B have overlapping binding sites on the ribosome and compete with chloramphenicol for binding to the 50S subunit (Celma *et al.*, 1970; Fernandez-Munoz *et al.*, 1971). Neither of these compounds inhibit peptidyl transferase *per se*, as measured by the fragment reaction (Celma *et al.*, 1970). They are believed to inhibit

translocation, possibly by preventing the release of deacylated tRNA or inducing conformational changes in the 50S subunit (Vazquez, 1979; Andersson and Kurland, 1987; Vester and Garrett, 1987). Mutational studies, both *in vivo* and *in vitro*, as well as protection against chemical modifications indicate primary binding sites for these antibiotics on 23S RNA (Sigmund and Morgan, 1984; Sor and Fukuhara, 1984; Ettayebi *et al.*, 1985; Moazed and Noller, 1987). For both erythromycin and vernamycin B there is evidence for an involvement of the central loop of domain V and of regions in domain II (Douthwaite *et al.*, 1985; Moazed and Noller, 1987). The conserved region from 2057-GAAAGA-2062 seems to be an important site for binding of these antibiotics. According to the secondary structure model this region is sterically close to the nucleotides crosslinked by BP-Phe-tRNA (Figure 3). Vernamycin inhibits photo-crosslinking considerably whereas the effect of erythromycin is less pronounced. Only the minor crosslinks are totally inhibited which is in agreement with protection of G-2505 from chemical modifications by both antibiotics (Moazed and Noller, 1987).

With the exception of tetracycline, none of the antibiotics known to inhibit EF-Tu dependent aminoacyl-tRNA binding had a pronounced effect on photo-affinity-labelling of BP-Phe-tRNA. In photo-crosslinking studies, using light of 254 nm, binding sites for tetracycline were identified mainly on proteins of the small subunit and no crosslinking to 23S RNA was reported (Goldman *et al.*, 1983). However, due to its intrinsic photo-reactivity at 320 nm, several cross-linking sites for tetracycline in the central loop of domain V of 23S RNA could be detected (Figure 3). This photo-reactivity may be in part responsible for the observed inhibition of the BP-Phe-tRNA crosslinking. In addition, photo-occlusion due to the high absorbance of tetracycline at 320 nm might have contributed to this inhibition.

Taken together, the results of the experiments described in this paper confirm and extend our previous hypothesis that the central loop of domain V is an integral and functionally important part of the peptidyl transferase. Our data allow for the first time the A- and P-sites to be localized at specific loci on the 23S RNA. These sites are brought into close proximity by the secondary structure folding of the 23S RNA chain. Dissection of the peptidyl transferase centre into two specific regions on 23S RNA provides important information on the topography of the two ribosomal tRNA binding sites. Knowledge of the mutual arrangement of the -CCA termini of the tRNAs lays the basis for an understanding of the mechanism underlying the vectorial transport process responsible for the peptidyl transfer reaction. The results on the inhibition of the photo-affinity labelling by antibiotics are in line with the idea that certain antibiotics act by disturbing the sterical arrangement within the peptidyl transferase centre. Finally, the demonstration of specific, high yield crosslinks at the level of 23S RNA strongly supports the current idea of an RNA-catalysed enzymatic activity promoting the peptidyl transferase reaction.

Materials and methods

Derivatization of the Phe-tRNA from Escherichia coli

Charged [^{3}H]Phe-tRNA from *E.coli* ([^{3}H]Phe, sp. act. 70–110 Ci/mmol from The Radiochemical Centre, Amersham, UK; uncharged tRNA^{Phe} from Boehringer Mannheim, FRG) was modified by 3-(4′-benzoylphenyl) propionyl-*N*-hydroxysuccinimidester as described (Kuechler and Barta, 1977). Non-derivatized tRNA was removed by adsorption of BP-Phe-tRNA to BD-cellulose (Gillam and Tener, 1971). BP-Phe-tRNA was eluted with buffer (10 mM Na-acetate, pH 5, 10 mM $MgCl_2$, 1 M NaCl, 30% ethanol). It was virtually free of Phe-tRNA and uncharged tRNA as assessed by TLC (Kuechler and Barta, 1977) and as calculated from the specific activity.

Binding of BP-Phe-tRNA to the ribosomal P- and A-site (Kuechler et al., 1988)

P-site: 250 pmol *E.coli* MRE600 ribosomes (Kuechler and Barta, 1977), 0.1 mg poly(U) and 100 pmol BP-[^{3}H]Phe-tRNA were incubated in 1 ml binding-buffer [100 mM KCl, 20 mM Tris–HCl, pH 7.4, 1 mM dithiothreitol (DTT), 0.2 mM EDTA] containing 6 mM $MgCl_2$ for 10 min at 25°C.

A-site: 250 pmol *E.coli* ribosomes, 0.1 mg poly(U) and 750 pmol uncharged tRNA^{Phe} were incubated in 1 ml binding buffer containing 10 mM $MgCl_2$ for 3 min at 37°C. Subsequently 100 pmol BP-[^{3}H]Phe-tRNA were added and the incubation continued for 10 min at 25°C. Translocation of BP-[^{3}H]Phe-tRNA to the P-site was achieved by the addition of 0.1 mg/ml EF-G (kind gift of K.Nierhaus, MPI fuer Molekulare Genetik, Berlin, FRG) and 1 mM GTP. Correct binding to either site was checked by the addition of 1 mM puromycin and subsequent incubation for 20 min at 25°C. Incubations with other antibiotics were performed at 0.1 mM. The extent of binding was assessed by determination of the radioactivity profile following column chromatography of the ribosomal complexes on Sepharose 6B minicolumns (80 × 5 mm) (Kuechler *et al.*, 1988).

Photo-crosslinking

Irradiation at 320 nm has been described in detail before (Kuechler and Barta, 1977). Irradiation time was reduced to 20 min. The yield of the crosslinking reaction was measured by determination of the radioactivity incorporated into 23S RNA. RNA was extracted with phenol/chloroform and chromatographed on Sepharose 6B minicolumns in buffer (100 mM KCl, 20 mM Tris–HCl, pH 7.4, 10 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTE; Kuechler *et al.*, 1988).

Primer extension with reverse transcriptase

A 56 bp *Ava*II fragment of pKK123 corresponding to position 2663–2607 on 23S RNA was used for priming. Assay conditions for reverse transcription and dideoxy-sequencing were essentially as described (Barta *et al.*, 1984; Kuechler *et al.*, 1988).

Acknowledgements

This work was supported by a grant from the 'Anton Dreher-Gedaecht-nisschenkung fuer Medizinische Forschung'. We thank G.Hoegenauer (Universitaet Graz) for a gift of tiamulin, K.Nierhaus (MPI fuer Molekulare Genetik, Berlin, FRG) for EF-G. We also thank Tim Skern for critical reading of the manuscript and K.Hartmuth and Z.Rattler for encouraging and stimulating discussions.

References

Andersson,S. and Kurland,C.G. (1987) *Biochimie*, **69**, 901–904.
Barta,A. and Kuechler,E. (1983) *FEBS Lett.*, **163**, 319–323.
Barta,A., Kuechler,E., Branlant,C., Widada,J.S., Krol,A. and Ebel,J.P. (1975) *FEBS Lett.*, **56**, 170–174.
Barta,A., Steiner,G, Brosius,J., Noller,H.F. and Kuechler,E. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 3607–3611.
Branlant,C., Krol,A., Machatt,M.A., Pouyet,J., Ebel,J.P., Edwards,K. and Koessel,H. (1981) *Nucleic Acids Res.*, **9**, 4303–4324.
Brimacombe,R. and Stiege,W. (1985) *Biochem. J.*, **229**, 1–17.
Brimacombe,R., Atmadja,J., Kyriatsoulis,A. and Stiege,W. (1986) In Hardesty,B. and Kramer,G. (eds), *Structure, Function and Genetics of Ribosomes*. Springer-Verlag, New York, pp. 184–202.
Celma,M.L., Monro,R.E. and Vazquez,D. (1970) *FEBS Lett.*, **6**, 273–277.
Cundliffe,E. (1979) In Chambliss,G., Craven,G.R., Davies,J., Davis,K., Kahan,L. and Nomura,M. (eds), *Ribosomes: Structure, Function and Genetics*. University Park Press, Baltimore, pp. 555–581.
Cundliffe,E. (1986) In Hardesty,B. and Kramer,G. (eds), *Structure Function and Genetics of Ribosomes*. Springer-Verlag, New York, pp. 586–604.
Douthwaite,St., Prince,J.B. and Noller,H.F. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 8330–8334.
Ebel,J.-P., Ehresmann,B., Ehresmann,C., Mougel,M., Giege,R. and Moras,D. (1986) In Hardesty,B. and Kramer,G. (eds), *Structure, Function*

and Genetics of Ribosomes. Springer-Verlag, New York, pp. 270–285.
Ettayebi,M., Pasad,S.M. and Morgan,E.A. (1985) *J. Bacteriol.*, **162**, 551–557.
Fernandez-Munoz,R., Monro,R.E., Torres-Pinedo,R. and Vazquez,D. (1971) *Eur. J. Biochem.*, **23**, 185–193.
Galardy,R.E., Craig,L.C. and Printz,M.P. (1973) *Nature*, **242**, 127–128.
Geigenmüller,U. and Nierhaus,K.H. (1986) *Eur. J. Biochem.*, **161**, 723–726.
Gillam,I.C. and Tener,G.M. (1971) *Methods Enzymol.*, **20**, 55–70.
Glotz,C., Zwieb,C., Brimacombe,R., Edwards,K. and Koessel,H. (1981) *Nucleic Acid Res.*, **9**, 3287–3330.
Goldman,R.A., Hasan,T., Hall,C.C., Strycharz,W.A. and Cooperman,B.S. (1983) *Biochemistry*, **22**, 359–368.
Guerrier-Takada,C., Gardiner,K., Marsh,T., Pace,N. and Altman,S. (1983) *Cell*, **35**, 849–857.
Hall,C.C., Johnson,D. and Cooperman,B.S. (1988) *Biochemistry*, **27**, 3983–3990.
Hodgin,L.A. and Hoegenauer,G. (1975) *Eur. J. Biochem.*, **52**, 93–98.
Hoegenauer,G. (1974) *Eur. J. Biochem.*, **47**, 527–533.
Kuechler,E. and Barta,A. (1977) *Methods Enzymol.*, **46**, 676–683.
Kuechler,E., Steiner,G. and Barta,A. (1988) *Methods Enzymol.*, **164**, 361–372.
Menninger,J.R. and Otto,D.P. (1982) *Antimicrob. Ag. Chemother.*, **21**, 811–818.
Moazed,D. and Noller,H.F. (1987) *Biochimie*, **69**, 879–884.
Noller,H.F. (1984) *Annu. Rev. Biochem.*, **53**, 119–162.
Noller,H.F., Kop,J., Wheaton,V., Brosius,J.,Gutell,R.R., Kopylov,A.M., Dohme,F., Herr,W., Stahl,D.A., Gupta,R. and Woese,C.R. (1981) *Nucleic Acids Res.*, **9**, 6167–6189.
Noller,H.F., Asire,M., Barta,A., Douthwaite,S., Goldstein,T., Gutell,R.R., Moazed,D., Normanly,J., Prince,J.B., Stern,S., Triman,K.,Turner,S., Van Stolk,B., Wheaton,V., Weiser,B. and Woese,C.R. (1986) In Hardesty,B. and Kramer,G. (eds), *Structure, Function and Genetics of Ribosomes*. Springer-Verlag, New York, pp. 143–163.
Ofengand,J., Ciesiolka,J., Denman,R. and Nurse,K. (1986) Hardesty,B. and Kramer,G. (eds), *Structure, Function and Genetics of Ribosomes*. Springer-Verlag, New York, pp. 473–494.
Prince,J.B., Taylor,B.H., Thurlow,D.L., Ofengand,J. and Zimmerman,R.A. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 5450–5454.
Schulze,H. and Nierhaus,K.H. (1982) *EMBO J.*, **1**, 609–613.
Sharp,P.A. and Eisenberg,D. (1987) *Science*, **238**, 729–731.
Shine,J. and Dalgarno,L. (1974) *Proc. Natl. Acad. Sci. USA*, **71**, 1342–1346.
Sigmund,C.D. and Morgan,E.A. (1984) *Nucleic Acids Res.*, **12**, 4653–4662.
Sor,F., Fukuhara,H. (1984) *Nucleic Acids Res.*, **12**, 8313–8318.
Steitz,J.A. (1979) In Chambliss,G., Craven,G.R., Davies,J., Davis,K., Kahan,L. and Nomura,M. (eds), *Ribosomes: Structure, Function and Genetics*. University Park Press, Baltimore, pp. 479–495.
Uhlenbeck,O.C. (1987) *Nature*, **328**, 596–600.
Vazquez,D. (1979) In Kleinzeller,H. (ed.), *Molecular Biology, Biochemistry and Biophysics*. Springer Verlag, Berlin, Vol. 30.
Vester,B. and Garrett,R.A. (1987) *Biochimie*, **69**, 891–900.
Youvan,P.C. and Hearst,J.E. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 3751–3754.
Zaug,A.J. and Cech,T.R. (1986) *Science*, **231**, 470–475.

Received on July 25, 1988; revised on August 29, 1988

Note added in proof

A recent paper by Hall *et al.* (1988) localizes an affinity site of a puromycin analogue to G-2502 and U-2504 which is in good agreement with our localization of the minor crosslinks obtained from the A- and the P-site.