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Interaction of tetracycline with RNA: photoincorporation into ribosomal RNA of *Escherichia coli*

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ABSTRACT

Photolysis of [^{3}H]tetracycline in the presence of *Escherichia coli* ribosomes results in an approximately 1:1 ratio of labelling ribosomal proteins and RNAs. In this work we characterize crosslinks to both 16S and 23S RNAs. Previously, the main target of photoincorporation of [^{3}H]tetracycline into ribosomal proteins was shown to be S7, which is also part of the one strong binding site of tetracycline on the 30S subunit. The crosslinks on 23S RNA map exclusively to the central loop of domain V (G2505, G2576 and G2608) which is part of the peptidyl transferase region. However, experiments performed with chimeric ribosomal subunits demonstrate that peptidyltransferase activity is not affected by tetracycline crosslinked solely to the 50S subunits. Three different positions are labelled on the 16S RNA, G693, G1300 and G1338. The positions of these crosslinked nucleotides correlate well with footprints on the 16S RNA produced either by tRNA or the protein S7. This suggests that the nucleotides are labelled by tetracycline bound to the strong binding site on the 30S subunit. In addition, our results demonstrate that the well known inhibition of tRNA binding to the A-site is solely due to tetracycline crosslinked to 30S subunits and furthermore suggest that interactions of the antibiotic with 16S RNA might be involved in its mode of action.

INTRODUCTION

The antibiotic tetracycline inhibits binding of tRNA to ribosomes (1). Specifically, it mainly influences binding to the A-site although some effects on the binding constant of Ac-Phe-tRNA to the P-site have also been observed (2,3). Tetracycline binds to a single strong binding site on the 30S ribosomal subunit as well as to a number of weaker sites on both, the 30S and 50S subunits (2,4–6). The precise mechanism of tetracycline inhibition is not known, but it is generally assumed that inhibition is caused by binding of tetracycline to the strong binding site on the 30S subunit (2,5,6). In a series of experiments where single proteins were omitted from the 30S subunit it has been established that the high affinity site is dependent on the presence of 16S RNA and the proteins S3, S7, S8, S14 and S19 (7). Of these proteins, S7 was the main target in experiments using [^{3}H]tetracycline as a photoaffinity reagent (5).

In previous experiments, we have used a photoreactive benzophenone derivative of tRNA [3-(4′-benzoylphenyl)propionyl-phenylalanine transfer RNA (BP-Phe-tRNA)] to characterize the peptidyltransferase region on the 50S subunit (8,9). The photoreaction with the 23S RNA was completely inhibited by tetracycline, and tetracycline itself crosslinked efficiently to the loop V region of 23S RNA (9,10). This was somewhat surprising since the data from several investigations suggested that tetracycline might incorporate mainly into ribosomal proteins (5,11,12). We therefore undertook a thorough analysis of the photoincorporation of tetracycline into ribosomal RNAs under conditions optimized to avoid non-specific binding and labelling due to tetracycline photoproducts. We show that tetracycline can be crosslinked to 16S RNA as well as to 23S RNA but not to 5S RNA. Activity data from the crosslinked subunits show that the inhibitory effect results solely from the interaction of tetracycline with the small subunit. This suggests that tetracycline crosslinks to 16S RNA from the strong binding site and that it might act via interaction with the 16S RNA.

MATERIALS AND METHODS

Materials

Tetracycline hydrocloride was purchased from Sigma, highly purified tetracycline was a present from Dr George Ellestad (Wyeth-Ayerst, Pearl River, NY). [^{3}H]Tetracycline was purchased from New England Nuclear (0.5 mCi/μmol). All tetracycline solutions were stored frozen in the dark and replaced frequently because the drug undergoes both thermal and photochemical degradation. 70S Ribosomes were prepared from *Escherichia coli* MRE600 as described (13). 30S and 50S ribosomal subunits were isolated as in (14).

Photocrosslink experiments

Photolysis experiments were performed using a short arc mercury lamp (HBO 500 W/2 from OSRAM) having an output concentrated at 366 nm. Samples were irradiated in vertical tubes at a distance of ~200 mm from the lamp in the outer focal point (average luminance 3000 cd/cm^2). Filters were chosen in such a way that any light below 300 nm was completely eliminated. All photolyses were performed in standard TMK buffer (20 mM Tris–HCl, pH

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7.4, 100 mM KCl, 6 mM $MgCl_2$, 0.4 mM EDTA and 2 mM DTE) at 0°C. For the identification of the labelled nucleotides highly purified tetracycline was used for crosslinking.

Distribution of [^{3}H]tetracycline photocrosslinked to ribosomal proteins and ribosomal RNA

After photolysis, ribosome samples were separated into two equal parts. In one of them ribosomal RNA was degraded by RNase T_1. The ribosomal proteins were precipitated by addition of 0.1 vol 100 g/l BSA and 1 vol 10% TCA, redissolved in 10 M urea and TCA precipitated again. The precipitate was filtered through a GF/C (Millipore) filter and washed several times with diethyl-ether/ethanol (10:1) to remove unbound [^{3}H]tetracycline. For determination of the amount of [^{3}H]tetracycline photoincorporated into ribosomal RNA, the RNA was isolated by phenol/chloroform extraction and precipitated. The pellet was dissolved in water and the radioactivity was measured. Virtually no background of [^{3}H]tetracycline was detectable in non-irradiated control samples.

Reverse transcriptase analysis

RNA isolated from the ribosomes as described above was used for reverse transcriptase analysis according to (15). Primers used to investigate crosslinks on 16S and on 23S rRNA were the same as used in (16).

Synthesis of Ac-[^{3}H]Phe-tRNA

tRNA was charged and acetylated as described (17). Ac-[^{3}H]Phe-tRNA was purified by reversed phase high performance liquid chromatography on nucleosil 300-5-C4-column (4 × 250 mm). Up to 30 nmol was typically applied to the column. The eluting solvent had constant 400 mM NaCl, 10 mM $Mg(CH_3COO)_2$, 20 mM NH_4-acetate, pH 5.0. The gradient steps had the following percentages of methanol: 0%, 5 min; 0–9% in 5 min; 9–25% in 50 min; 25%, 5 min. The different tRNA species were separated in the linear gradient from 9 to 25% of methanol. The fractions containing Ac-[^{3}H]Phe-tRNA were collected, desalted using an Econopac P6 desalting column (BioRad) and dried in a speedvac.

Preparation of chimeric ribosomes

Isolated 30S and 50S subunits were irradiated in the presence of tetracycline as described above. To remove unbound tetracycline, the subunits were pelleted twice (first 8 h at 31 000 r.p.m. for 30S or 5 h at 28 000 r.p.m. for 50S, then 18 h at 21 000 r.p.m. for 30S or 18 h at 15 000 r.p.m. for 50S) in a Beckman ultracentrifuge using the SW50.1 rotor. The pellet was dissolved in $T_{20}M_{20}N_{400}$ buffer (20 mM Tris–HCl pH 7.5, 20 mM $MgCl_2$, 400 mM NH_4Cl, 4 mM β-mercaptoethanol). After addition of an equimolar amount of the complementary untreated ribosomal subunit the samples were incubated for 10 min at 37°C. The samples were then centrifuged in a 10–30% sucrose gradient in $T_{20}M_{10}N_{100}$ buffer (20 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$, 100 mM NH_4Cl, 6 mM β-mercaptoethanol, 0.5 mM EDTA) for 18 h at 18 000 r.p.m. (Beckman SW28 rotor). The fractions containing 70S chimeric ribosomes were collected and centrifuged for 24 h at 24 000 r.p.m. (Beckman 45Ti rotor). The pellet was resuspended in $T_{20}M_{10}N_{100}$ (containing 50% glycerol) and stored at –70°C.

Determination of Ac-[^{3}H]Phe-tRNA binding to the ribosomal A- and P-site

For P-site binding, 0.2 pmol/µl 70S ribosomes were incubated in $T_{20}K_{100}M_6$ buffer (20 mM Tris–HCl pH 7.4, 100 mM KCl, 6 mM $MgCl_2$, 0.4 mM EDTA and 2 mM DTE) for 10 min at room temperature in the presence of 0.2 pmol/µl Ac-[^{3}H]Phe-tRNA and 0.1 µg/µl poly(U). For A-site binding 0.2 pmol/µl 70S ribosomes were pre-incubated in $T_{20}K_{100}M_{12}$ buffer (20 mM Tris–HCl pH 7.4, 100 mM KCl, 12 mM $MgCl_2$, 0.4 mM EDTA and 2 mM DTE) for 3 min at 37°C in the presence of 0.2 pmol/µl uncharged $tRNA^{Phe}$ and 0.1 µg/µl poly(U). Then 0.2 pmol/µl Ac-[^{3}H]Phe-tRNA was added and the sample was incubated for 10 min at room temperature. To remove unbound Ac-[^{3}H]Phe-tRNA the samples were filtered through a nitrocellulose filter (NC-Filter, 45µm, Milipore, Molsheim, France). The filter was washed several times with $T_{20}K_{100}M_6$ or $T_{20}K_{100}M_{12}$, respectively. The radioactivity of the filter corresponded to the bound Ac-[^{3}H]Phe-tRNA.

Determination of peptidyltransferase activity

The puromycin reaction, the formation of Ac-[^{3}H]Phe-puromycin from Ac-[^{3}H]Phe-tRNA and puromycin, was used to measure peptidyltransferase activity. Ac-[^{3}H]Phe-tRNA was bound to the P-site as described above and incubated with 1 mM puromycin for 10 min at room temperature. After addition of 1 vol 0.3 M Na-acetate (pH 5.5) in saturated $MgSO_4$ the Ac-[^{3}H]Phe-puromycin was extracted with ethyl acetate and the radioactivity measured in a scintillation counter.

RESULTS

Photoincorporation of [^{3}H]tetracycline into *E.coli* ribosomes

In the present work we reinvestigated the interaction of tetracycline with ribosomal RNA. First, we determined the distribution of tetracycline photocrosslinked to ribosomal RNA and proteins using [^{3}H]tetracycline as photoaffinity label. 70S ribosomes were irradiated in the presence of 100 µM [^{3}H]tetracycline for different periods of time. As shown in Figure 1, the amount of tetracycline photocrosslinked to RNA and proteins increased with increasing irradiation time. Tetracycline was photocrosslinked to both, RNA and proteins, to about the same extent (Fig. 1). This is in contrast to the results of Goldman and co-workers (5,11) who only found up to 10% of the tetracycline label incorporated into rRNA (see Discussion). Using 25 s irradiation time, tetracycline was already incorporated into 70S ribosomes at an approximate ratio of 1:1. Therefore, and to avoid secondary reactions of tetracycline photoproducts we used an irradiation time of just 30 s in all following experiments.

Localization of tetracycline–rRNA photocrosslinks

The analysis of the ribosomal proteins photolabelled by [^{3}H]tetracycline confirmed the results of Goldman *et al.* (5), as we could also identify protein S7 as the major labelled protein (data not shown). We therefore concentrated on the analysis of the sites of tetracycline–rRNA interactions using the primer extension method. The primers chosen were spaced every ~200 nucleotides on the 16S, 23S and 5S RNA, so we were able to scan the entire RNAs except the 3′-ends. The RNAs used for the templates were from 70S ribosomes irradiated in the presence of different

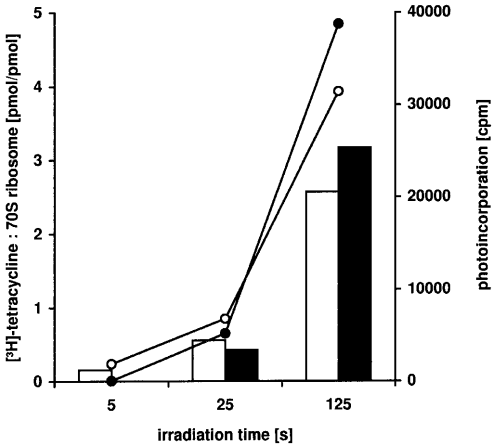

Figure 1. Photoincorporation of [^{3}H]tetracycline into *E.coli* ribosome. 70S Ribosomes (36 pmol) were irradiated in presence of 100 µM [^{3}H]tetracycline in 100 µl TMK buffer for the indicated period of time. Lines indicate radioactivity found upon irradiation in ribosomal RNA (filled circles) and in ribosomal proteins (open circles). The boxes indicate pmoles of [^{3}H]tetracycline photoincorporated per pmole ribosomal RNA (black boxes) and ribosomal proteins (open boxes). Values are given as mean of four parallel experiments.

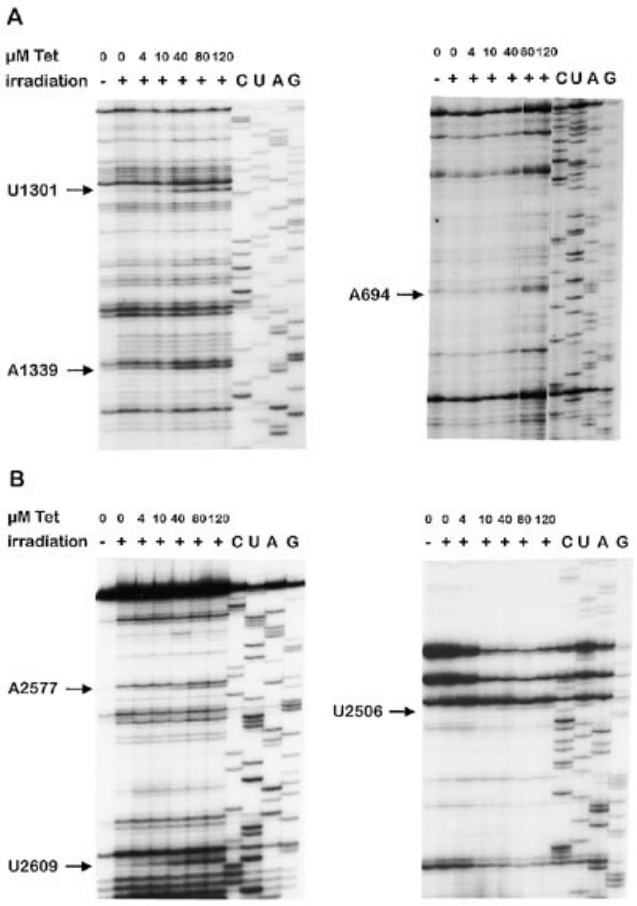

Figure 2. Primer extension experiments showing reverse transcriptase elongation stops caused by photocrosslinks of tetracycline to ribosomal RNAs. A suitable set of oligonucleotide primers were used for analysing 16S RNA (**A**) and 23S RNA (**B**). Only those crosslinks are indicated which are already visible at 40 µM tetracycline.

amounts of tetracycline (Fig. 2). RNAs from non-irradiated 70S ribosomes and from ribosomes irradiated in the absence of tetracycline were used as controls for random stops on the RNA template and possible UV-induced internal RNA–RNA crosslinks. When a stop was observed, the crosslinked nucleotide was taken to be the following nucleotide in the rRNA template (i.e. the preceding one in the rRNA sequence). The numbers of photoaffinity labelled nucleotides increased with rising concentrations of tetracycline. The half maximal inhibition of Ac-Phe-tRNA binding to the ribosome by tetracycline was reported to be 40 (18) or 4 µM (3), respectively, whereas under our incubation conditions we observed a value of ~10 µM. Therefore, only those nucleotides were considered to correlate well with the inhibitory action of tetracycline which were labelled in the presence of 40 µM or lower concentrations of the antibiotic. Under these conditions, three sites on the 16S RNA (Fig. 2A; G693, G1300 and G1338) and three sites on the 23S RNA (Fig. 2B; G2505, G2576 and G2608) were photoaffinity labelled by tetracycline. No incorporation of tetracycline into 5S RNA could be detected (data not shown). Several additional labelled nucleotides on 16S and 23S RNA could be identified when 80 or 120 µM tetracycline were used and some of them are discussed later.

The positions of the labelled nucleotides are shown in Figure 3A in a two dimensional model of the 16S RNA. In addition, we have indicated nucleotides which produce footprints with tRNA (19), protein S7 (20) and tetracycline (21). As can be seen in Figure 3A, the labelled nucleotides are close to nucleotides either involved in binding of tRNA or protein S7.

On the 23S RNA the labelled nucleotides were located exclusively in the central loop region of domain V (Fig. 3B). This loop has been identified as an essential part of the peptidyltransferase region on the 50S subunit [for reviews see 10, 22)]. As the main binding site of tetracycline had been located on the 30S subunit we wondered if the photocrosslinks to the 50S subunit were derived from tetracycline bound to the main binding site on the 30S subunit which might be located at the interface between the 30S and 50S subunit. However, irradiation of isolated 50S subunits in the presence of 40 µM tetracycline produced the same RNA labelling pattern as irradiation of 70S ribosomes (data not shown). This indicated that the 50S crosslinks originated from a tetracycline binding site on the 50S subunit.

Effect of photoincorporated tetracycline on ribosomal function

Next we investigated the effect of tetracycline photocrosslinks on ribosomal function using chimeric ribosomes. To distinguish the effects of tetracycline crosslinked to the 30S subunit from those of tetracycline crosslinked to the 50S subunit we performed the following experiments. Isolated 30S or 50S ribosomal subunits were irradiated in the presence of tetracycline. Then we immediately removed the unbound tetracycline by centrifugation. The ribosomal subunits were reconstituted to 70S particles with the complementary untreated subunit. The chimeric ribosomes were then isolated by density gradient centrifugation and the effects of the photomodified subunits on binding of peptidyl-tRNA to the ribosome and on peptidyltranferase activity were investigated. As shown in Figure 4, the photocrosslinked 50S ribosomal subunit had no effect on binding of Ac-[^{3}H]Phe-tRNA either to

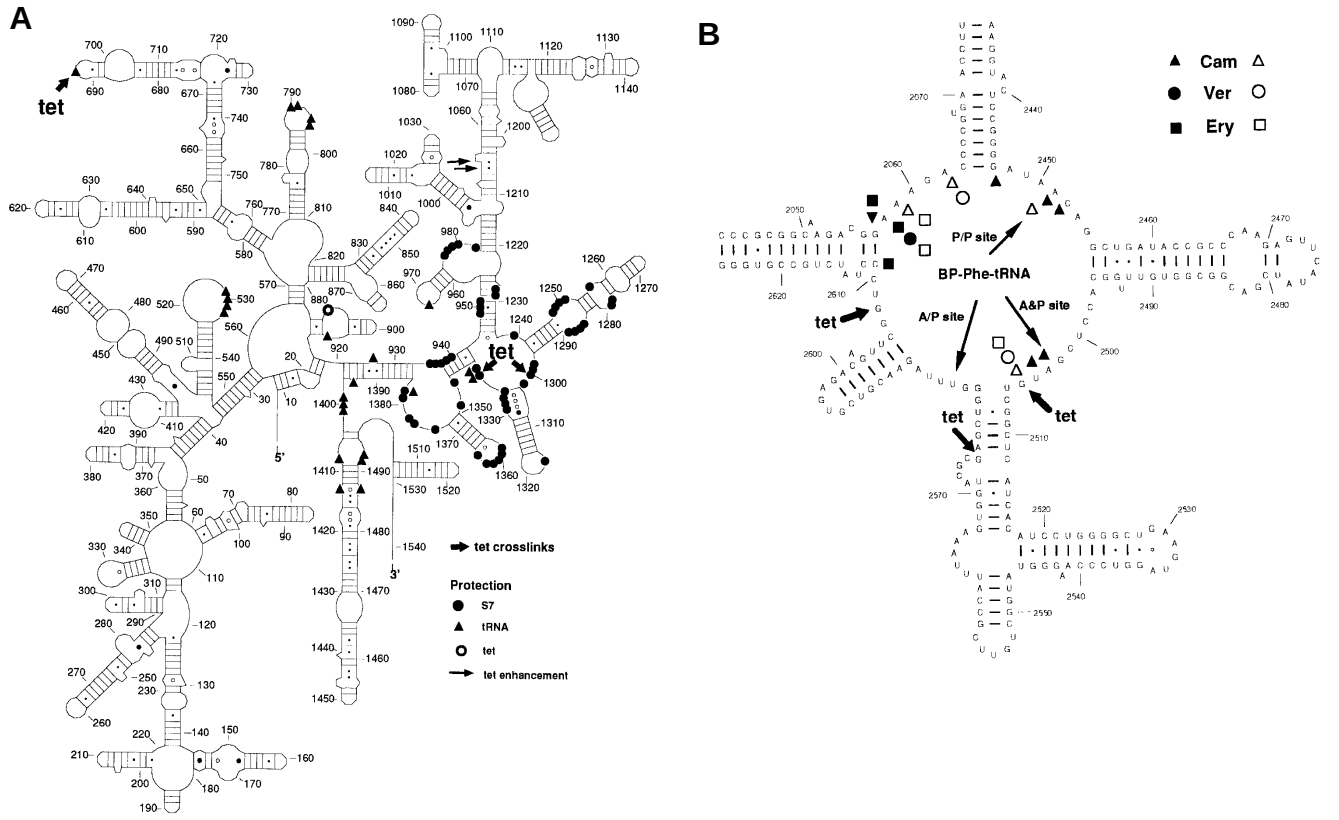

Figure 3. **(A)** Secondary structure diagram of 16S rRNA. Positions of nucleotides photoaffinity labelled by 40μM tetracycline are indicated by thick arrows. On the 16S rRNA diagram bases protected from chemical modification by binding of protein S7 (filled circles) (30), of tRNA (triangles) (19) and of tetracycline (open circle) (21) are indicated in addition to nucleotides which have enhanced reactivities towards chemical probing when tetracycline is bound to the ribosome (thin arrows) (21). **(B)** Secondary structure of the central loop region of domain V of 23S rRNA. Positions of nucleotides photoaffinity labelled by 40μM tetracycline are indicated by thick arrows. The nucleotides specifically labelled by A- and P-site bound BP-Phe-tRNA are indicated by the thin arrows (9). Cam, chloramphenicol; Ery, erythromycin; Ver, vernamycin. Filled symbols indicate nucleotides whose mutation confers resistance to the respective antibiotic (31–34) open symbols designate antibiotics whose binding to the ribosome causes an alteration of reactivity of the respective nucleotide toward chemical modification (35).

the ribosomal A- or to the P-site when compared with untreated 70S ribosomes.

The Ac-[^{3}H]Phe-puromycin formation, which measures peptidyl-tranferase activity, was also unaffected by tetracycline photocrosslinked to the 50S subunit. In contrast, chimeric ribosomes with tetracycline photocrosslinked to the 30S subunit had diminished ability of binding Ac-[^{3}H]Phe-tRNA to the ribosomal A-site compared with untreated 70S ribosomes, whereas the P-site binding and the Ac-[^{3}H]Phe-puromycin formation remained unaffected. These results correlate well with the published data on the inhibition of A-site binding of tRNA by tetracycline (2,3).

DISCUSSION

The experiments described in this paper show that tetracycline can be photocrosslinked not only to ribosomal proteins, but also to rRNA. We found an approximately 1:1 incorporation of radioactivity in ribosomal proteins and RNA, respectively. [^{3}H]Tetracycline has been previously used in extensive studies to characterize ribosomal binding sites for this antibiotic (5,11,12). In these experiments up to 90% of the radioactivity was found to be incorporated in ribosomal proteins with S7 being the main protein labelled (5). The difference in the distribution of the label may result from the different irradiation conditions used. We used ~500 W for only 30 s (15 kJ) whereas Cooperman's group used 30 W and irradiation times between 60 and 90 min (108–162 kJ) (11). These workers also showed that upon irradiation tetracycline photoproducts were generated which could further react with the ribosome (5). Therefore, we took care to avoid long irradiation times. Furthermore, as our results did not change upon addition of β-mercaptoethanol which has been used to avoid light independent incorporation of tetracycline photoproducts and as our protein labelling pattern conforms to the one published previously (5), we are confident that the crosslinks observed derive from genuine tetracycline.

The experiments were performed with increasing concentrations of tetracycline; however, only those crosslinks have been described which appear ≤40 μM tetracycline. The number of crosslink sites increased with higher concentrations (e.g. on 23S RNA three more at 80 μM, and additional seven at 120 μM tetracycline), in accordance with a large number of low affinity binding sites for tetracycline observed on both the 30S and 50S subunits (4–6).

Previously, the strong binding site of tetracycline was localized to the 30S ribosomal subunit (4–6). There are several results supporting the idea that the inhibitory effect of tetracycline on protein synthesis, i.e. the blocking of aminoacyl-tRNA binding to the A-site, is a direct consequence of its binding to the strong binding site (4–6,23). In addition, it has been shown that 16S

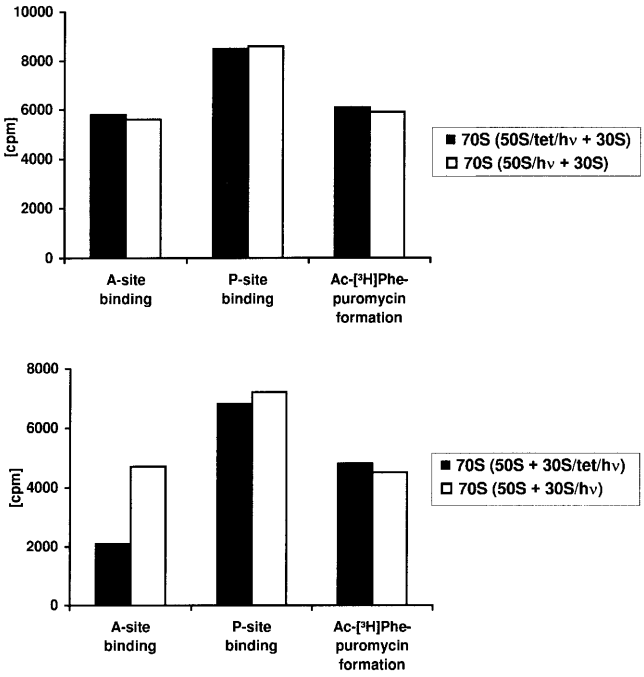

Figure 4. Effects of photocrosslinked tetracycline on ribosomal function. Tetracycline was photocrosslinked to isolated 30S and 50S ribosomal subunits. After removing the unbound tetracycline the subunits were reconstituted to 70S ribosomes by addition of untreated 50S subunits and 30S subunits, respectively. The diagrams compare these chimeric ribosomes with ribosomes, which were reconstituted from a subunit irradiated in the absence of tetracycline and an untreated subunit. Ac-[^{3}H]Phe-tRNA binding to the A-site and P-site and Ac-[^{3}H]Phe-puromycin formation are shown.

RNA together with the proteins S3, S7, S8, S14 and S19 are essential for providing the binding domain for tetracycline on the 30S subunit and that within this domain S7 is the major labelled protein (5,7). Although we observed an RNA to protein labelling ratio different from that seen by other investigators, we too found the same protein labelling pattern with protein S7 being the main protein labelled (data not shown). Tetracycline has been found to bind either to proteins, such as the Tet repressor (24), or to RNA as was demonstrated for group I and group II introns (25). As no binding to ribosomal proteins free in solution occurs (26) there is the possibility of a mixed RNA–protein binding site on the ribosome. In order to characterize this site, the crosslinked nucleotides on the ribosomal RNAs were analysed.

Both 16S and 23S rRNAs were photoaffinity labelled by tetracycline. Interestingly, 23S RNA was labelled exclusively in the central loop of domain V, the peptidyltransferase centre (Fig. 3B), but this labelling did not affect tRNA binding and peptidyl transfer. These crosslinks originate from a binding site on the 50S subunit as the same crosslinks were observed when merely 50S subunits were used. At 80 µM tetracycline two additional crosslinks were found in this region; some of them have previously been identified in crosslink experiments where 100 µM tetracycline has been used (9). Therefore, on the 50S subunit tetracycline seems to bind solely to the peptidyl transferase region at least at lower concentrations. It is interesting and probably not by chance that tetracycline crosslinks mainly to the peptidyl transferase region on 23S RNA where various other antibiotics like chloramphenicol and erythromycin bind (Fig. 3B). This region is mainly composed of RNA (8,27,28) and located at the interface between the two ribosomal subunits. Given the location of the crosslinks, we were surprised not to find any effect on peptidyltransferase activity. In our previous experiments using BP-Phe-tRNA as affinity label for the peptidyltransferase region, we observed an inhibition of the BP-Phe-tRNA crosslinks, but not of its binding, by several antibiotics including tetracycline and chloramphenicol (9). One explanation of this observation would be that tetracycline might bind to the peptidyltransferase region and distort the peptidyl end of tRNA thus inhibiting the BP-Phe-tRNA crosslink to the 23S RNA. Binding of tetracycline to this region is similar but not identical to chloramphenicol (an inhibitor of peptidyltransferase activity) as peptidyltransferase activity *in vitro* is not inhibited. Although, an effect on ribosomal function *in vivo* cannot be excluded, this effect would be overruled by the effect of tetracycline on binding of tRNA to the A-site.

The labelled nucleotides of the 16S rRNA are shown in Figure 3A together with footprints created by binding of tRNA, protein S7, and tetracycline. The distribution of all these nucleotides demonstrates that the photoaffinity labelled nucleotides (G693, G1300 and G1338) on the 16S RNA are close to footprint sites of tRNA and protein S7. Furthermore, G890 which is labelled by 120 µM tetracycline (data not shown) is adjacent to nucleotide A892 which was protected upon binding of 100 µM tetracycline to chemical probing (Figure 3A; 21). It is interesting to note that several of the other nucleotides labelled additionally at higher concentrations of tetracycline are also located near tRNA footprint sites (data not shown). As 16S RNA and the protein S7 are essential components of the strong binding site of tetracycline on the 30S subunit, it is likely that the labelling of the nucleotides on the 16S RNA occurs from the main binding site. It is not known how tetracycline inhibits binding of tRNA to the A-site but our data reveal that the close proximity of tetracycline to the 16S RNA may be one of the determinants of its mode of action. Thus tetracycline might act by interfering with the tRNA/16S RNA interaction directly or via a structural distortion of the 16S RNA brought about by binding of tetracycline to its high affinity binding site with its main contacts to S7 and 16S RNA. Distortion of 16S RNA was also implicated by the enhancement of the reactivities of U1052 and C1054 to chemical probing upon binding of tetracycline (21). A thorough analysis of the action of streptomycin and neomycin has recently led to a model putting forward the idea that these antibiotics act by distortion of 16S RNA structures (29). These antibiotics which give different footprints to 16S RNA than tetracycline have an influence on the decoding fidelity. Tetracycline, on the other hand, might have an effect on the high affinity binding of tRNA after the decoding process.

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