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The environment of 5S rRNA in the ribosome: cross-links to the GTPase-associated area of 23S rRNA

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ABSTRACT

Two photoreactive diazirine derivatives of uridine were used to study contacts between 5S rRNA and 23 rRNA *in situ* in *Escherichia coli* ribosomes. 2′-Amino-2′-deoxyuridine or 5-methyleneaminouridine were introduced into 5S rRNA by T7 transcription. After incorporation of these uridine analogues into the transcript their amino groups were modified with 4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]benzyl isothiocyanate or the *N*-hydroxysuccinimide ester of 4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]benzoic acid respectively. 5S rRNA carrying the photoreactive diazirine groups (referred to as the 2′-aminoribose derivative and the 5-methyleneamino derivative respectively) was reconstituted into 50S subunits or 70S ribosomes. After mild UV irradiation cross-links formed to 23S rRNA were analysed by standard procedures. All of the observed cross-links involved residue U89 of the 5S rRNA. Three nucleotides of 23S rRNA were cross-linked to this residue with the 5-methyleneamino derivative, namely U958, G1022 and G1138. With the 2′-aminoribose derivative a single cross-link was found, to U958. The significance of these cross-links for our understanding of the structure and function of 5S rRNA and its environment in the ribosome are discussed.

INTRODUCTION

Since its discovery in 1963 as a component of the *Escherichia coli* ribosome (1), 5S rRNA has been the object of intensive study by many different methods. The structure of the free 5S molecule in solution has been investigated by chemical (2) and enzymatic (3) probing and also by cross-linking techniques (4). Several models for its structure, both free and as a complex with ribosomal proteins L18 and L25, have been proposed (5–7). Within the ribosome 5S rRNA is known from immunoelectron microscopic (IEM) studies to be located on the central protuberance of the large ribosomal subunit (8,9). In agreement with this location, the three proteins that can bind 5S rRNA, namely L5, L18 and L25, have also all been located by IEM on or close to the central protuberance (10,11). The central protuberance of the 50S subunit lies opposite the head of the small 30S subunit in the 70S ribosome and there is evidence that 5S rRNA is in close contact with the 30S subunit. Only two bases of 5S rRNA, G13 and G41, are reactive towards kethoxal in the 50S subunit (12) and one of these (G13) is protected in the 70S ribosome (13). Furthermore, studies on 5S rRNA containing phosphorothioate residues have revealed that the phosphate groups 5′ with respect to positions U48, U55 and U82 are strongly protected against iodine cleavage in the presence of the 30S subunit (O.Shpanchenko *et al.*, submitted for publication).

Less is known concerning the environment of 5S rRNA with respect to 23S rRNA and this is the area which is of primary interest to us. A number of years ago a ribonucleoprotein particle was isolated by mild nuclease digestion from the 50S subunit, comprising 5S rRNA, proteins L5, L18 and L25 and a fragment of 23S rRNA covering residues ~2280–2390 (14) in domain V of the 23S molecule. More recently cross-linking studies have identified contacts from residue U89 at the loop end of helix IV of 5S rRNA to two sites, A960 and C2475, in 23S rRNA (15,16). These sites lie in domains II and V respectively and both are close to functionally important areas of 23S rRNA. In the latter studies (15,16) synthetic 5S rRNA containing randomly distributed 4-thiouridine (4-thioU) residues in place of the normal uridine residues was prepared by T7 transcription and was then reconstituted into 50S subunits or 70S ribosomes. The cross-linking reaction was induced by UV irradiation at 350 nm. 4-ThioU is a ‘zero length’ cross-linking reagent and therefore gives very precise information concerning the neighbourhoods between the two partners involved in each cross-link. On the other hand, 4-thioU can only become cross-linked to single-stranded target RNA regions and, moreover, potential cross-linking targets which are close to a 4-thioU residue, but not in direct contact with it, will not be detected. However, our parallel studies on cross-linking with mRNA (17) have demonstrated that additional information can be obtained using a variety of different photoreactive nucleotide analogues with different spacer distances between the respective photoreactive and nucleotide moieties.

Here we describe a cross-linking study with 5S rRNA containing two different types of photoreactive residue. Modified 5S rRNA was prepared by T7 transcription in the presence of either 5-methyleneamino uridine (17) or 2′-amino-2′-deoxyuridine

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(cf. 18). After transcription the amino groups were derivatized using appropriate diazirine compounds (cf. 19,20), thus giving modified nucleotides carrying a photoreactive group with a cross-link bridging distance of ~10 Å attached either to the uracil or to the ribose moiety. The modified 5S rRNAs were reconstituted into 50S subunits or 70S ribosomes and cross-linking was induced by mild UV irradiation at 350 nm (20). The non-specific photoreaction of the diazirine derivatives allows cross-links to be formed in principle to either single- or double-stranded RNA regions and enables us to 'scan' the space surrounding the modified uridine residues in the 5S molecule out to a distance of ~10 Å. We document analysis of three new cross-links between 5S rRNA and 23S rRNA which serve to define new functional neighbourhoods within the 50S ribosomal subunit.

MATERIALS AND METHODS

Preparation of 2′-amino-2′-deoxyuridine 5′-phosphate

5′-Dimethoxytrityl-2′-acetamido-2′-deoxyuridine was prepared according to Kuznetsova *et al.* (21). The polymer support GPG-500 (Applied Biosystems) was derivatized with a long chain alkylamine carrying a primary amino function and succinic anhydride (22). Derivatization of the carboxyl-containing polymer support with the protected aminouridine compound was carried out in a manner similar to that of Jones (22). After removing the dimethoxytrityl group the 5′-position was phosphorylated with a commercially available 5′-ON reagent (Glen Research). Deprotection and removal of the 2′-amino-2′-deoxyuridine 5′-phosphate from the solid support were achieved by treatment with concentrated ammonia for 6 h at room temperature.

Preparation of 2′-amino-2′-deoxyUTP

2′-Amino-2′-deoxyuridine 5′-monophosphate at a concentration of 1.5 mM was incubated with 1 U nucleotide monophosphate kinase and 10 U nucleotide diphosphate kinase (both from Sigma) in 100 µl buffer containing 25 mM Tris–HCl, pH 8, 5 mM $MgCl_2$, 10 mM KCl, 2.5 mM dithiothreitol and 20 mM ATP (Boehringer Mannheim) for 1 h at room temperature. Subsequently a further 1 U nucleotide monphosphate kinase and 10 U nucleotide diphosphate kinase were added and incubation was continued for 1 h more. The enzymes were inactivated by heating at 95°C for 30 min and removed by centrifugation. Acetone (200 µl) and ethanol (200 µl) were added to the supernatant and the mixture was cooled to –70°C to precipitate 2′-amino-2′-deoxyUTP. After centrifugation the pellet of 2′-amino-2′-deoxyUTP was dried and dissolved in water.

T7 transcription

^{32}P-Labelled 5S rRNA was transcribed using T7 polymerase as in our previous experiments (15,16), where the photoreactive nucleotide to be incorporated was 4-thiouridine. In this case 4-thioUTP was substituted by 2′-amino-2′-deoxyUTP (see above) or by 5-methyleneaminoUTP (17). The molar ratio of 2′-amino-2′-deoxyUTP to 'normal' UTP in the transcription mixture was adjusted to 4:1, so as to give a ratio of ~1 modified uridine residue to 9 unmodified residues in the transcript. This is equivalent to incorporation of an average of 2 modified uridine residues per 5S rRNA molecule. In the case of 5-methyleneaminoUTP a corresponding level of incorporation was achieved with a ratio of modified:unmodified UTP in the transcription mixture of 25:1.

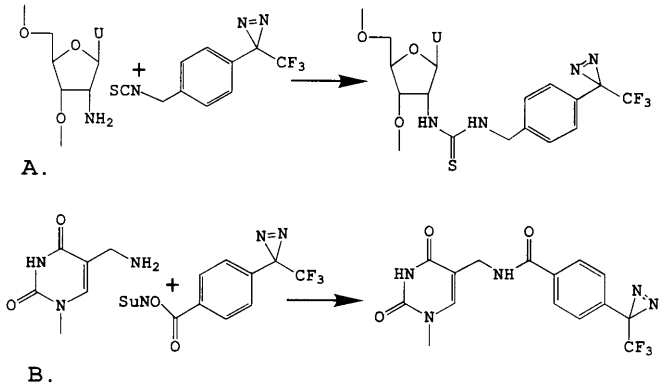

Figure 1. Reactions used for introduction of the photoreactive diazirine groups into the 5S rRNA transcripts. (**A**) Modification of 2′-amino-2′-deoxyuridine residues by 4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]benzyl isothiocyanate. (**B**) Modification of 5-methyleneaminouridine residues by the *N*-hydroxy-succinimide ester of 4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]benzoic acid.

Preparation of 4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]-benzyl isothiocyanate

This reagent (see Fig. 1) was prepared by a two-step synthesis from 4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]benzyl bromide, which in turn was synthesized as described by Nassal (23). In the first step sodium iodide (564 mg, 3.76 mmol) was added to a stirred solution of hexamethylene tetraamine (502 mg, 3.58 mmol) in absolute ethanol (6 ml) and the benzyl bromide precursor (1 g, 3.58 mmol) was then added in portions at room temperature. The reaction mixture was allowed to stand for 48 h, then cooled to 0°C. Dry gaseous HCl was passed through and after 1.5 h the mixture was filtered. The filtrate was diluted with 20 ml 0.2 N HCl and extracted twice with ether (10 ml). The aqueous phase was neutralized with concentrated NaOH to pH 9–10 and the product extracted into 60 ml chloroform. The organic extract was washed with water and dried over $MgSO_4$, followed by evaporation of the solvent. The yield of 4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]benzylamine was 315 mg (41%). The product gave an R_f value of 0.42 in a thin layer chromatography system containing *n*-butanol/water/acetic acid (4:1:1) and was used for the second reaction step without further purification.

The benzylamine derivative from the previous step (300 mg, 1.4 mmol) was taken up in dry pyridine (28 ml) and CS_2 (558 µl, 9 mmol) was added. The mixture was cooled to 0°C and dicyclohexyl carbodiimide (430 mg, 2.09 mmol) was added. The resulting solution was stirred at room temperature overnight. Then the white precipitate that had formed was filtered off and the filtrate evaporated. The residue was subjected to chromatography on silica gel using a chloroform hexane mixture (2:1) as eluant. The product obtained (100 mg, yield 28%) behaved as a pure compound on thin layer chromatography in chloroform/hexane (2:1), with an R_f value of 0.75. IR spectrum: 2080/cm, broad (NCS); 1612/cm (N=N). UV spectrum: λ_{max} = 223, 352 in chloroform. MS: *m/e* 257 (M^+), 229 (M^+-N_2).

Modification of the amino groups of the uridine analogues

The 5-methyleneaminouridine residues in the 5S rRNA T7 transcripts (see above) were modified with the *N*-hydroxysuccinimide ester of 4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]-benzoic acid (see Fig. 1) exactly as previously described (17). Corresponding modification of the 2′-amino-2′-deoxyuridine residues was carried out by a method similar to that described by Sigurdsson *et al.* (18). For this purpose the 5S rRNA transcript was dissolved in 5 µl 100 mM sodium borate buffer, pH 8.6, and mixed with 5 µl 50 mM 4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]benzyl isothiocyanate (above) in dimethyl formamide. The mixture was incubated for 24 h at room temperature. In order to monitor efficiency of the modification reactions, as well as incorporation of the 5-methyleneaminouridine and 2′-amino-2′-deoxyuridine residues into 5S rRNA transcripts, small scale transcriptions were made in the presence of [α-^{32}P]ATP. The transcripts were digested with ribonuclease T2 using the procedure of Zwieb and Brimacombe (24) and the mononucleotide digestion products separated by two-dimensional thin layer chromatography on PEI–cellulose plates, using the 'double-digestion' solvent system of Volkaert and Fiers (25).

Reconstitution, UV irradiation and cross-link site analysis

Modified 5S rRNA was reconstituted into 50S subunits or 70S ribosomes exactly as described previously (15,16). The subsequent steps, including cross-linking by UV irradiation at 350 nm, isolation of 23S rRNA cross-linked to 5S rRNA by sucrose gradient centrifugation and localization of the cross-link sites by ribonuclease H digestion, primer extension analysis and ribonuclease T1 fingerprinting were also carried out exactly as before (15,16).

RESULTS

5-Methyleneaminouridine or 2′-amino-2′-deoxyuridine were randomly incorporated into *E.coli* 5S rRNA by T7 transcription in the presence of the corresponding nucleoside 5′-triphosphates and [α-^{32}P]UTP. As in our previous experiments (15,16), the 5S rRNA sequence was slightly altered in that residue U1 was changed to G, A119 to C and U120 was deleted. These mutations were introduced in order to create a T7 initiation site as well a *Nae*I cleavage site and have no influence on reconstitution or functional activity of the 50S subunits (15,16). After incorporation into 5S rRNA the 5-methyleneaminouridine residues were modified by treatment with the *N*-hydroxysuccinimide ester of 4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]benzoic acid [often called 4-(trifluoromethyl diazirino)benzoic acid (19)] as before (17). In the case of the 2′-amino-2′-deoxyuridine residues the modification was made with 4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]benzyl isothiocyanate (see Materials and Methods), since the 2′-amino derivatives of nucleotides are known (18) to be rather unreactive towards *N*-hydroxysuccinimide esters. Figure 1 shows the two modified uridine compounds together with their diazirine derivatives, which we refer to as the 2′-aminoribose (Fig. 1A) and the 5-methyleneamino (Fig. 1B) derivatives respectively.

In order to determine the ratio of the uridine analogues to normal uridine in the T7 transcripts, as well as the efficiency of subsequent modification of the amino groups, small scale transcriptions were made in the presence of [α-^{32}P]ATP. The transcripts were then digested with ribonuclease T2 and analysed by two-dimensional thin layer chromatography (Materials and Methods); in these chromatograms all residues lying 5′-adjacent to an A residue in the 5S sequence appear as radioactive nucleoside 3′-phosphates. Typical examples are shown in Figure 2. In the control digest of 5S rRNA carrying no modified nucleotides (Fig. 2A) spots can be seen corresponding to Ap, Cp, Gp and Up. In the presence of a 4-fold excess of 2′-amino-2′-deoxyUTP over normal UTP a new spot appears (Fig. 2B), corresponding to 2′-amino-2′-deoxyUp. At this ratio of input nucleotides in the transcription mixture the level of incorporation of the modified uridine nucleotide is ~10% of that of the normal uridine. As noted in Materials and Methods, this corresponds to an average of 2 modified uridine residues per 5S molecule; a similar level of incorporation in the case of 5-methyleneaminoUTP necessitated a 25-fold excess of the modified nucleotide in the transcription mixture. To measure efficiency of the diazirine modification reaction transcription was carried out in the absence of normal UTP (Fig. 2C). In this case the Up spot on the chromatogram was entirely replaced by the spot corresponding to 2′-amino-2′-deoxyUp. After reaction with the diazirine compound a new spot appears (spot 5, Fig. 2D), corresponding to the 2′-aminoribose diazirine derivative (Fig. 1A), and it can be seen that ~90% of the 2′-amino-2′-deoxyUp has been converted to the latter.

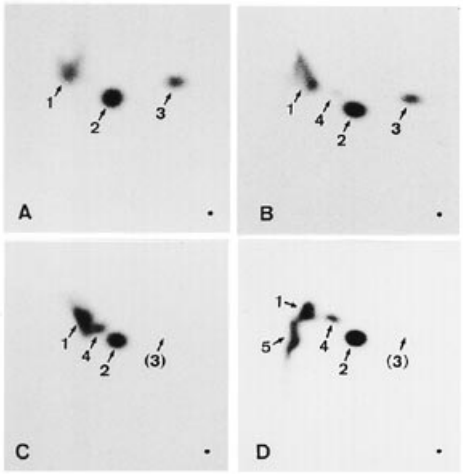

Figure 2. Autoradiograms of ribonuclease T2 digestions of 5S rRNA transcripts labelled with [α-^{32}P]ATP on two-dimensional thin-layer plates (25). Direction of the first dimension is from right to left and that of the second dimension from bottom to top. The black spot in the lower right-hand corner indicates the sample application point. (**A**) Ribonuclease T2 digestion products of a 5S rRNA transcript carrying only naturally occurring nucleotides. (**B**) Digestion products from a transcript prepared in the presence of a 4-fold excess of 2′-amino-2′-deoxyUTP over UTP. (**C**) Digestion products from a transcript where the UTP was completely substituted by 2′-amino-2′-deoxyUTP. (**D**) Digestion products from the same transcript as (C) treated with 4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]benzyl isothiocyanate prior to digestion. The numbered radioactive spots correspond to: 1, Ap and Cp; 2, Gp; 3, Up; 4, 2′-amino-2′-deoxyUp; 5, the diazirine derivative of 2′-amino-2′-deoxyUp (Fig. 1A).

5S rRNA carrying the diazirine derivatives was reconstituted into 50S subunits by the standard procedure (26). For incorporation into 70S ribosomes 50S reconstitution was followed by incubation with a 2-fold molar excess of 30S subunits (15,16). The yields of the reconstitution (20–40%) and subunit association (45–55%) reactions were reduced in comparison with unmodified 5S rRNA. It is known that chemical modification of certain nucleotides in

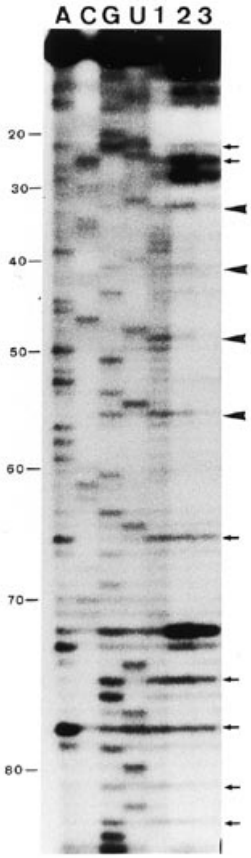

Figure 3. Reverse transcriptase analysis of 5S rRNA carrying the 2′-aminoribose derivative. A, C, G and U denote dideoxy sequencing lanes. Lane 1, primer extension of the T7 transcript after modification with the diazirine derivative; lane 2, primer extension of the same transcript isolated from the reconstituted 50S ribosomal subunits; lane 3, primer extension of the same transcript isolated from reconstituted 50S subunits incorporated into 70S ribosomes. The primer used was complementary to nt 95–119 of the 5S rRNA. The large arrowheads indicate stop signals preceding uridine residues where the modified form was reproducibly under-represented in the 50S subunits or 70S ribosomes. Small arrows indicate signals preceding uridine residues where this discrimination was not observed.

large rRNA molecules can inhibit ribosome–ligand interactions (see for example 27), and the distribution of diazirine-modified residues in 5S rRNA incorporated into 50S or 70S ribosomes was therefore investigated by primer extension (28). This can in fact only be done in the case of the 2′-aminoribose derivative, since the presence of the corresponding 5-methyleneamino derivative in the sequence does not cause reverse transcriptase to pause or stop. A typical primer extension gel for the 2′-aminoribose derivative is illustrated in Figure 3. It can be seen that the pattern of reverse transcriptase stop signals in 5S rRNA re-isolated from the reconstituted 50S subunits or 70S ribosomes (lanes 2 and 3 respectively) differs from the pattern in the original 5S rRNA transcript (lane 1). In particular, 5S rRNA from the 70S ribosomes shows a greatly reduced level of modified nucleotides at uridine positions 32, 40, 48 and 55, a result which correlates well with protection of phosphorothioates at positions U48 and U55 by the presence of 30S subunits, as noted in the Introduction.

50S subunits or 70S ribosomes containing 5S rRNA carrying the 5-methyleneamino or 2′-aminoribose derivatives were separated by sucrose gradient centrifugation and irradiated with 350 nm UV light (20). After formation of cross-links in this manner the rRNAs were separated by further sucrose gradient centrifugation in the presence of SDS and EDTA, as before (15–17). Approximately 2% of 5S rRNA became cross-linked to 23S rRNA, a value which is typical for the highly reactive diazirine derivatives (cf. 17,20). No cross-linking to 16S rRNA was observed.

As usual (15,17), a preliminary localization of the cross-linked regions within 23S rRNA was made by digestion with ribonuclease H in the presence of pairs of oligodeoxynucleotides (10–17 bases long) complementary to selected sequences in the 23S molecule. This analysis showed that for both the 2′-aminoribose derivative and the 5-methyleneamino derivative the cross-links lay between nt 940 and 1250. Further ribonuclease digestions revealed that in the case of the 5-methyleneamino derivative there were three distinct cross-links within this region, and a typical set of results is illustrated in Figure 4. Lanes 1–3 show digests releasing three contiguous fragments of 23S rRNA and in each case a band can be seen with the expected mobility containing radioactive 5S rRNA cross-linked to the 23S fragment concerned. Corresponding analysis with the 2′-aminoribose derivative indicated that only the first of the three cross-links (that in lane 1, Fig. 4) was present. No differences were observed in the cross-link patterns of 23S rRNA derived from 50S subunits and those from 70S ribosomes.

Further localization of the cross-link sites was made by the primer extension method (28), and examples for the 5-methyleneamino derivative are given in Figure 5. Figure 5A shows the cross-link site from the 1045–1250 region of 23S rRNA (cf. Fig. 4) and here prominent reverse transcriptase stop signals are present at positions 1139 and 1140, indicating a cross-link site at nt G1138. Similarly, Figure 5B shows the cross-link site from the 1010–1045 region (Fig. 4) and reveals a stop signal (from two independent sets of samples) at position 1023, indicative of a cross-link site at nt G1022. Primer extension analysis of the cross-link from the 940–1010 region (Fig. 4) gave a stop signal at position 959 (data not shown) and the cross-link site is thus at U958, 2 nt away from the site (at A960) found in our previous series of experiments (16) with 5S rRNA containing 4-thioU. The cross-link site in this region from the 2′-aminoribose derivative was reproducibly to the identical nucleotide, U958.

The residue in 5S rRNA participating in each of the cross-links was determined by ribonuclease T1 digestion of cross-linked complexes released by ribonuclease H digestion (such as those illustrated in Fig. 4). As in both our previous sets of experiments (15,16), these analyses showed that the cross-linked T1 oligonucleotide was the UCUCCCCAUGp sequence at positions 87–96 of 5S rRNA and the corresponding secondary digestion analysis (cf. 15,16) indicated that again U89 was the nucleotide involved in every case (data not shown).

DISCUSSION

The 5S–23S rRNA cross-linking data are summarized in Figure 6. Figure 6 also includes the positions of cross-links within the same area of the 23S molecule from the elbow region of A-site bound tRNA (31) and an internal cross-link within 23S rRNA connecting nt 993 and 1045 in helices 41 and 42 respectively (48). It is noteworthy that we have now observed three independent cross-links from U89 of 5S rRNA to the almost universally

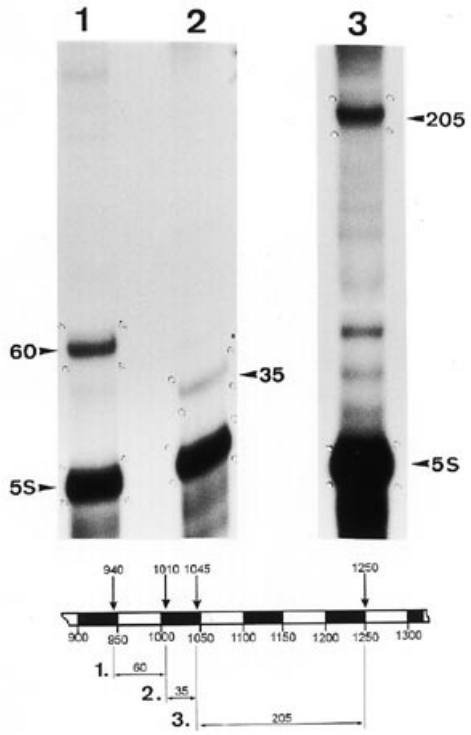

Figure 4. Autoradiograms of ribonuclease H digests on 4% polyacrylamide gels of 23S rRNA cross-linked to ^{32}P-labelled 5S rRNA carrying the 5-methyleneamino derivative. Lane 1, products of ribonuclease H digestion in the presence of oligodeoxynucleotides complementary to 15 nt sequences centred on positions 940 and 1010 of the 23S rRNA; lane 2, digestion products in the presence of oligodeoxynucleotides centred on 23S positions 1010 and 1045; lane 3, digestion products in the presence of oligodeoxynucleotides centred on 23S positions 1045 and 1250. The approximate length of the excised 23S rRNA fragment cross-linked to the 5S rRNA is indicated in each case, as well as the position of free 5S rRNA. The cleavage sites within the 23S rRNA for each gel lane are summarized in the sketch at the bottom of the Figure.

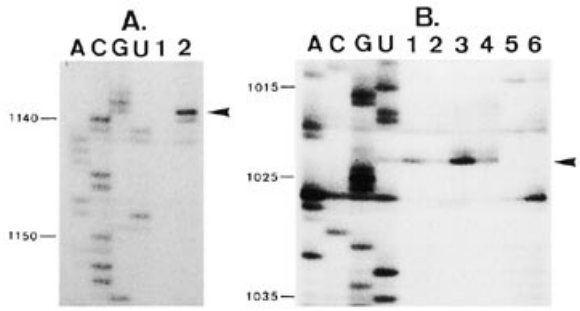

Figure 5. Primer extension analysis of the 23S rRNA fragments cross-linked to 5S rRNA carrying the 5-methyleneamino derivative. In (A) and (B) the lanes are primer extension reactions of cross-linked and non-cross-linked 23S rRNA fragments, isolated in each case from a single ribonuclease H digest (cf. Fig. 4). (**A**) Primer extension showing the cross-link site corresponding to lane 3 of Figure 4. Lane 1, control (non-cross-linked) fragment; lane 2, cross-linked fragment. The primer was complementary to nt 1170–1189 of the 23S rRNA and the excised ribonuclease H fragment covered nt 1045–1250. The principle reverse transcriptase stop at nt 1139 is marked by the arrowhead. (**B**) Primer extension analysis showing the cross-link site corresponding to lane 2 of Figure 4. Lanes 5 and 6, control reactions from two independent experiments; lanes 1–4, the corresponding cross-linked fragments (at two different gel loadings) respectively. The primer was complementary to nt 1062–1079 of the 23S rRNA and the excised ribonuclease H fragment covered nt 970–1110. The principle reverse transcriptase stop specific to the cross-linked sample at nt 1023 is marked.

conserved UAA motif at nt 958–960 in helix 39; these cross-links occur from 4-thioU (16) and from the 2′-aminoribose and 5-methyleneamino derivatives described here. The other two new cross-links from the 5-methyleneamino derivative are to nt G1022 and G1138, positions which lie opposite one another in the secondary structure of 23S rRNA at the end of helix 41 and which were previously shown to be neighbours by *in vivo* intra-RNA cross-linking studies (32). The remaining cross-link, from 5S rRNA containing 4-thioU (15), is to nt C2475 in helix 89, close to the peptidyl transferase region of the 23S molecule (33). It is perhaps surprising that all of the cross-links from 5S rRNA involve the same nucleotide, U89, but this phenomenon is at least partially explained by the observed discrimination against incorporation of 5S molecules carrying modified residues at positions 32, 40, 48 and 55 (Fig. 3) in helices II and III. Other modified uridine positions may be shielded within the 5S rRNA structure or may simply not be sufficiently close to any part of the 23S rRNA to form cross-links. In this context it is to be expected that each of the three different cross-linking agents, 4-thioU and the 2′-aminoribose and 5-methyleneamino derivatives, would show an individual spectrum of cross-linking targets on 23S rRNA, as we have indeed observed (Fig. 6).

Nucleotide G1138, the target of cross-link 5 in Figure 6, is also a component of the pseudoknot involving nt 1137–1138 and 1005–1006 in helix 41 (34). Disruption of this pseudoknot has been shown to inactivate the 50S subunit by abolishing its ability to associate with the 30S subunit (35). Taken together with cross-link 6 (Fig. 6), which connects nt 993 and 1045 in helices 41 and 42 as noted above, there is clearly a sharp bend in 23S rRNA within the 50S subunit between helices 41 and 42, which has the effect of bringing helices 43 and 44 back towards helices 40 and 45. Helices 43 and 44 are well known as being the GTPase-associated region of 23S rRNA, by virtue of their interactions with EF-G (36,37) and the antibiotic thiostrepton (38). The network of cross-links shown in Figure 6 thus begins to establish a pattern of interconnected neighbourhoods between 5S rRNA and the GTPase region. The cross-link from 5S rRNA to the loop end of helix 89 adds the peptidyl transferase area to this network and the latter loop end is, furthermore, a target of cross-linking from the elbow region of A-site bound tRNA (31), consistent with the observation that A-site function is abolished in the absence of 5S rRNA (26). The elbow region of A-site bound tRNA has in addition been cross-linked to a site near to the loop end of helix 38 (31) and this site (Fig. 6) is also relevant to the arrangement of 5S rRNA in the 50S subunit.

In our previous publication (15) we proposed an arrangement for 5S rRNA in which helices I–III lie within the central protuberance of the 50S subunit and helix IV is directed downwards into the body of the subunit. The new data are consistent with this arrangement, but it is important to note that in the latter publication (15) A- and P-site bound tRNAs were modelled in the ‘R’ configuration. In contrast, recent high resolution cryoelectron microscopic reconstructions of the *E.coli* 70S ribosome, in which A- and P-site bound tRNA molecules were directly visualized (39), have clearly demonstrated that the tRNAs are in fact in the ‘S’ configuration. This has the

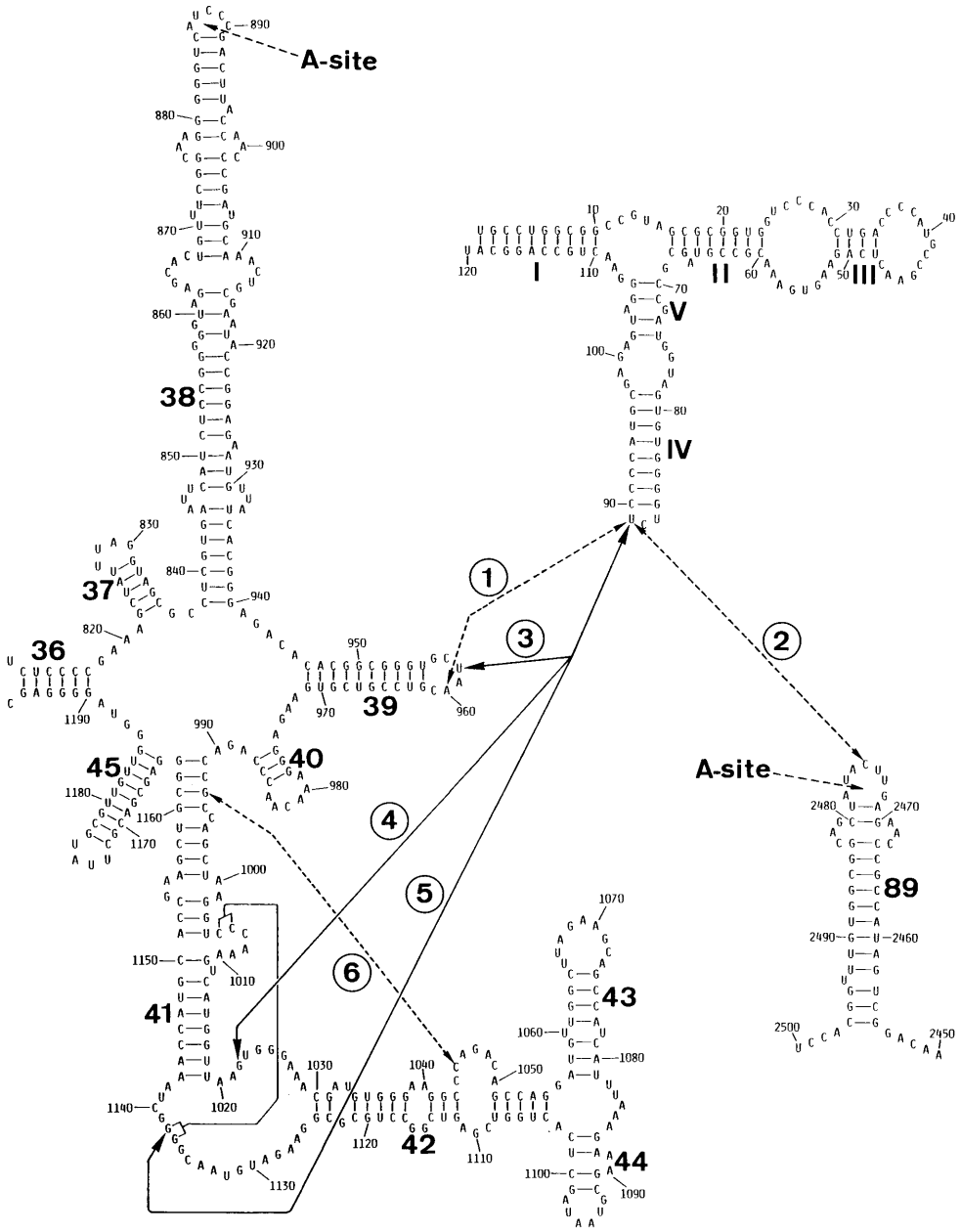

Figure 6. The secondary structure of 5S rRNA (helices numbered I–V; 29) and of parts of the 23S rRNA (helices numbered as in 30), showing the cross-linking data. Cross-links 1 and 2, indicated by dotted lines, are those previously observed (15,16). Cross-links 3–5, indicated by solid lines, are those described in this study. Cross-link 6 (dotted line) is an internal cross-link in 23S rRNA recently determined by Baranov *et al*. (48). Cross-links to A-site tRNA (31) are also indicated. See text for further details and correlations.

consequence that the peptidyl transferase centre (which must *a priori* coincide with the CCA ends of the tRNAs) lies much lower down in the 50S subunit (39) than the position which was deduced from older electron microscopic studies (10,11). As a result, in combination with RNA–protein cross-linking (40) and binding site (41) data and the position of the ribosomal proteins within the 50S subunit (42), the elements of 23S rRNA shown in Figure 6 can be placed in a consistent arrangement relative to helix IV of 5S rRNA, which can be briefly summarized as follows.

Helix 89 points upwards from the peptidyl transferase centre, to contact helix IV of 5S rRNA pointing downwards from the central protuberance of the 50S subunit, as in Dontsova *et al.* (15). The central ring in domain II connecting helices 36–41 and 45 (Fig. 6) also occupies a position low down in the 50S subunit, as evidenced by a cross-link (40) to protein L30 from a site near to the base of helix 38; L30 is located towards the bottom of the subunit (42). Helices 39 and 41 both point upwards from the central ring, roughly parallel to helix 89, so as to account for

cross-links 1, 3 and 5 (Fig. 6) to helix IV of 5S rRNA. The long helix 38 also points upwards, curving across the 50S subunit to contact the elbow of A-site bound tRNA (31) at its upper end; helix 38 is the prime candidate for the 'A-site finger' observed by electron microscopy (39), just below the central protuberance. As already noted above, there is a sharp bend between helices 41 and 42, which serves to bring helices 42–44 (containing the binding sites for proteins L10, L11 and L7/L12; 41) back towards the central ring of helices 36–41 and 45, at a location close to the loop end of helix 89. The latter loop end has a cross-link to protein L6 (40) and all these proteins, namely L6, L10 and L11, form a cluster just below L7/L12 in the 50S subunit (42).

These arguments do not alter the arrangement of helices I–III of 5S rRNA in the central protuberance of the 50S subunit, as already proposed (15). The arrangement of these helices was consistent with the immunoelectron microscopic locations of the 3′-end of 5S rRNA (8) and of residues A39–U40 (9), as well as with the footprinting data for proteins L5, L18 and L25 (43–45) and the locations of these proteins in the central protuberance (42). It is noteworthy that L25 lies below L5 and L18, closer to L6, L10 and L11 (42), and a recent chemical probing study (46) has indicated that L25 interacts with helices I, II, IV and V of the 5S rRNA (cf. 45), which is again consistent with a downwards orientation of helix IV.

Detailed modelling studies on 23S rRNA are currently in progress, analogous to those recently made with 16S rRNA (47), in which the rRNA molecule was fitted to the cryoelectron microscopic contour of Stark *et al.* (39). It remains to be seen how well all of the constraints discussed above can be accomodated within the electron microscopic structure. Nonetheless, it is clear that 5S rRNA, which is now topographically implicated in both the peptidyl transferase and GTPase-associated regions, as well as in A-site tRNA binding and 30S–50S subunit association, plays an increasingly central role in the ribosome. It could well act as a mediator of allosteric signal transmission between the various functional components.

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