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The ribosomal neighbourhood of the central fold of tRNA: cross-links from position 47 of tRNA located at the A, P or E site

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ABSTRACT

The naturally occurring nucleotide 3-(3-amino-3-carboxy-propyl)uridine (acp^{3}U) at position 47 of tRNA-^{Phe} from *Escherichia coli* was modified with a diazirine derivative and bound to ribosomes in the presence of suitable mRNA analogues under conditions specific for the ribosomal A, P or E sites. After photo-activation at 350 nm the cross-links to ribosomal proteins and RNA were identified by our standard procedures. In the 30S subunit protein S19 (and weakly S9 and S13) was the target of cross-linking from tRNA at the A site, S7, S9 and S13 from the P site and S7 from the E site. Similarly, in the 50S subunit L16 and L27 were cross-linked from the A site, L1, L5, L16, L27 and L33 from the P site and L1 and L33 from the E site. Corresponding cross-links to rRNA were localized by RNase H digestion to the following areas: in 16S rRNA between positions 687 and 727 from the P and E sites, positions 1318 and 1350 (P site) and 1350 and 1387 (E site); in the 23S rRNA between positions 865 and 910 from the A site, 1845 and 1892 (P site), 1892 and 1945 (A site), 2282 and 2358 (P site), 2242 and 2461 (P and E sites), 2461 and 2488 (A site), 2488 and 2539 (all three sites) and 2572 and 2603 (A and P sites). In most (but not all) cases, more precise localizations of the cross-link sites could be made by primer extension analysis.

INTRODUCTION

The most recent high-resolution electron microscopic reconstructions of *Escherichia coli* 70S ribosomes, derived from micrographs in vitreous ice (1,2), show that the interface cavity between the 30S and 50S ribosomal subunits is perfectly shaped to accommodate L-shaped tRNA molecules. It follows that a precise analysis of contact sites between tRNAs and the ribosome should be very useful for determining the topography of the ribosomal components at the 30S–50S interface area. This is particularly true for the central fold or 'elbow' region of the tRNA, because from here contacts from the A, P or E site would be expected to the 'head' of the 30S subunit, to the 'central protuberance' of the 50S subunit and to the interface 'bridge' (3), as well as possibly to other regions such as the 'side lobe' of the 30S subunit or the 'L1 protuberance' and the 'L7/L12 stalk' of the 50S subunit (see 4,5 for a description of these terms).

A number of cross-linking studies have been made with a view to identifying the ribosomal proteins located in the neighbourhood of the central fold. These studies include cross-links from azido derivatives attached to naturally occurring modified nucleotides at tRNA positions 8 (6), 47 (7) or 20:1 (8), as well as direct ultraviolet-induced cross-links from several positions in the elbow region (9,10). However, with the exception of a cross-link identified from position 47 of a P site-bound tRNA to nt 2309 of the 23S rRNA (11), there is so far no detailed information relating to tRNA–rRNA contacts from the central fold of the tRNA. This is in contrast to the situation with the anticodon or CCA extremities of the tRNA molecules, where a number of rRNA–tRNA contacts have been established, either from the tRNA itself (12–14) or, in the case of the CCA end, from the immediately adjacent amino acid residue in aminoacyl tRNA (11,15,16).

The study mentioned above involving a cross-link from tRNA to nt 2309 of the 23S rRNA was made with an azido derivative attached to the 3-(3-amino-3-carboxy-propyl)uridine residue (acp^{3}U) at position 47 of *E.coli* tRNA^{Phe} (11, cf. 7). In our more recent investigations (14,16) we have used diazirine derivatives rather than azides, as these compounds are extremely reactive (17) and, although the cross-linking yields are in general quite low, they yield a rich spectrum of information. In particular, we were able to identify a series of cross-link sites to 16S rRNA with the help of a diazirine derivative attached to the modified cytidine residue at position 32 in the anticodon loop of tRNA$^{Arg}_I$ (14). In this paper we describe the analysis of an analogous series of cross-links to ribosomal proteins and rRNA from a diazirine derivative attached to the same acp^{3}U residue at position 47 of tRNA^{Phe} which we had previously exploited (11), the modified tRNA being bound to the ribosomal A, P or E sites in appropriate experiments. Similar studies, to be published elsewhere, are underway with tRNAs correspondingly modified at positions 8 (cf. 6) or 20:1 (cf. 8), so as to collect information from sites on the 'inside' and 'outside' of the central fold. The results from tRNA^{Phe} position 47 which we present here show highly differentiated patterns of cross-linking for the A, P and E sites

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involving ribosomal proteins from both subunits, as well as both the 16S and 23S rRNA molecules.

MATERIALS AND METHODS

Derivatization and charging of tRNA^{Phe}

Escherichia coli tRNA^{Phe} (from Boehringer, Mannheim or Biogenes, Berlin) was dephosphorylated with alkaline phosphatase by the procedure of Gnirke *et al.* (18) and then purified by electrophoresis on a 15% polyacrylamide gel. After extraction from the gel the tRNA was labelled with ^{32}P at its 5′-end to a specific activity of ~40 000 c.p.m./pmol as described (18). Derivatization with the *N*-hydroxysuccinimide ester of 4-(3-trifluoromethyl diazirino)benzoic acid (19) was carried out in DMSO under the conditions of Bochkariov and Kogon (20), excess reagent being removed by passing the reaction mixture over a NAP column (Pharmacia) followed by ethanol precipitation of the modified tRNA. This uncharged derivatized tRNA^{Phe} was used for binding to the ribosomal P or E sites. For A site binding the derivatized tRNA^{Phe} was charged with phenylalanine by the method of Rheinberger *et al.* (21), followed by purification over a NICK column (Pharmacia) as previously described (14). The level of charging was ~40% (compared with ~70% for non-derivatized tRNA), as determined by parallel experiments using [^{3}H]phenylalanine and unlabelled derivatized tRNA^{Phe}.

Binding of derivatized tRNA^{Phe} to the ribosomal A, P or E sites

mRNA analogues were prepared by T7 transcription from synthetic DNA templates (custom synthesized by TIB, Berlin) by our usual procedure (22,23). For A and P site binding an mRNA (mRNA-1) was used with the sequence GGGAGAAGGAGAAAAGAAG**AUGUUC**AAAAGAAAA; the two codons in bold type are for fMet and Phe respectively and the Shine–Dalgarno sequence is underlined. For E site binding the AUGUUC sequence was changed to UUCAUG (mRNA-2). Binding of the derivatized Phe–tRNA^{Phe} to the A site was in the presence of EF-Tu using 70S tight coupled ribosomes from *E.coli* MRE600 and of mRNA-1 and tRNA^{fMet} (Boehringer, Mannheim) under conditions described previously (14,16). E site binding of uncharged derivatized tRNA^{Phe} was also made with 70S tight couples, using in this case mRNA-2 and tRNA^{fMet}, again as described (14). For P site binding either tight couples or 70S ribosomes reconstituted from 30S and 50S subunits were used in the presence or absence of mRNA-1, also using the conditions previously described (14). Reaction mixtures contained 250 pmol ribosomes, as before (14).

Isolation and analysis of cross-linked products

The tRNA–mRNA ribosome complexes were irradiated in the cold for 10 min at 350 nm, as in Tate *et al.* (24), and were then applied to a series of sucrose gradients (22) in order to isolate ribosomal proteins or rRNA cross-linked to the derivatized tRNA^{Phe}. The cross-linked ribosomal protein–tRNA complexes were separated by electrophoresis on 10% polyacrylamide gels containing SDS and urea (25). Radioactive bands were excised from the gels and the proteins identified by the method of Gulle *et al.* (26), using antibodies immobilized on agarose. The cross-link sites on the rRNA were localized by treatment with RNase H in the presence of selected pairs of oligodeoxynucleotides (14,23), followed by primer extension analysis (23,27) according to our usual procedures.

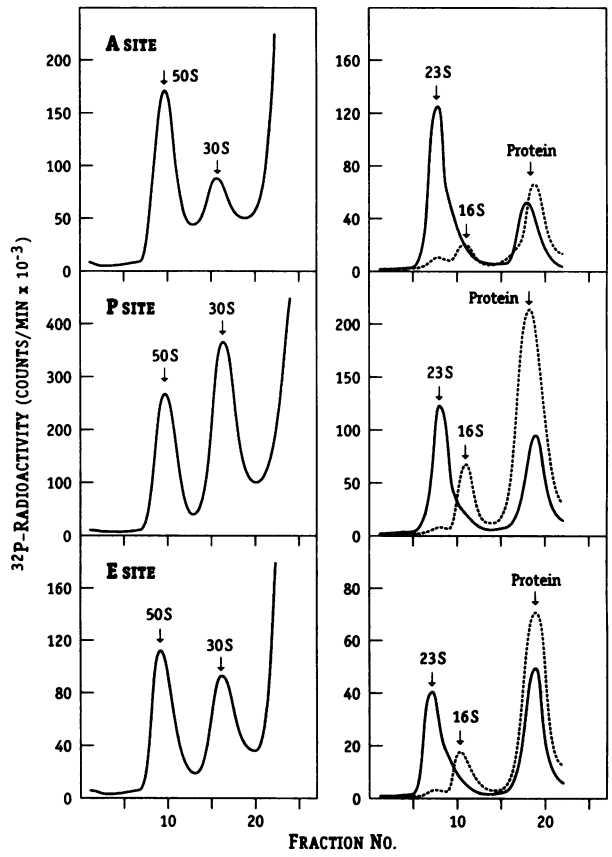

Figure 1. Sucrose gradient centrifugation of cross-linked ^{32}P-labelled TDB–tRNA^{Phe}–ribosome complexes from the A, P and E sites. In each case the left-hand panels show the distribution of tRNA radioactivity between the 50S and 30S subunits on gradients at low magnesium concentration. The right-hand panels show the subsequent separations into cross-linked rRNA and protein fractions on SDS-containing gradients from the 50S (solid lines) and 30S subunits (dotted lines). The direction of sedimentation is from right to left.

RESULTS AND DISCUSSION

The acp^{3}U residue at position 47 of *E.coli* tRNA^{Phe} reacts with *N*-hydroxysuccinimide esters via its primary amine group and this property has been made use of in cross-linking studies with azido reagents (7,11). Here we have used the *N*-hydroxysuccinimide ester of 4-(3-trifluoromethyl diazirino)benzoic acid (19,20) in a similar way, as described in Materials and Methods. For binding to the ribosomal A site the tRNA^{Phe} was modified with the diazirine reagent (TDB) prior to charging with phenylalanine, so as to avoid confusion by concomitant modification of the α-amino group of the phenylalanine residue. EF-Tu-dependent binding to the A site was carried out by first programming the ribosomes with tRNA^{fMet} and mRNA-1 (which contains the coding sequence AUG UUC; see Materials and Methods), followed by addition of the charged derivatized Phe–tRNA^{Phe}. For E site binding mRNA-2 was used, in which the order of the AUG and UUC codons is reversed, and uncharged derivatized tRNA^{Phe} (TDB–tRNA^{Phe}) was added after tRNA^{fMet}; although

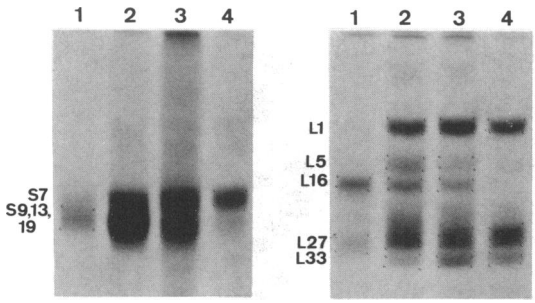

Figure 2. Autoradiogram of ^{32}P-labelled tRNA–protein cross-linked complexes from the SDS–sucrose gradients (Fig. 1) separated on 10% polyacrylamide gels in SDS and urea. 30S protein samples are on the left, 50S samples on the right. In each gel lane 1 is an A site sample, lane 2 a P site sample (with tRNA bound in the presence of mRNA; see Materials and Methods; 14), lane 3 a P site sample (in the absence of mRNA) and lane 4 an E site sample. Radioactive bands were extracted from the gels and the proteins identified immunologically (26).

binding tRNA to a codon 'upstream' of the initiator codon represents a non-physiological situation, our previous experiments (14) have shown this to be an effective way of binding a modified tRNA to the E site. In the case of the P site the same series of experiments (14) had indicated a certain variability in the cross-linking patterns dependent on whether 70S tight couples or 70S ribosomes reconstituted from 30S and 50S subunits were used or whether the tRNA binding was conducted in the presence or absence of mRNA. Accordingly, all these possibilities were tested with TDB–tRNA^{Phe} in the current series of experiments, but in fact no significant differences between the various P site binding conditions were observed (cf. Fig. 3 below).

Table 1. Summary of ribosomal proteins cross-linked to tRNA^{Phe} at the A, P and E sites (cf. Fig. 2)

30S subunit			50S subunit		
A	P	E	A	P	E
	S7	S7		L1	L1
(S9)	S9			L5	
(S13)	S13		L16	L16	
S19			L27	L27	
				L33	L33

Parentheses indicate that the proteins concerned were only weakly or occasionally present and may just represent cross-contamination with P site-bound TDB–tRNA^{Phe}.

Reaction mixtures containing ^{32}P-labelled TDB–tRNA^{Phe} at the A, P or E sites were irradiated at 350 nm (24) and applied to sucrose gradients in high magnesium (22), so as to separate 70S ribosomal complexes from unbound tRNA and mRNA. The 70S complexes were then applied to a second sucrose gradient at low magnesium, to separate the ribosomal subunits, followed by a third gradient in SDS to dissociate the 30S and 50S subunits into rRNA and protein fractions respectively containing the cross-linked TDB–tRNA^{Phe}. Examples of the two latter types of gradient are illustrated in Figure 1 (cf. 22). It can be seen that the distribution of cross-linked radioactivity between the 30S and

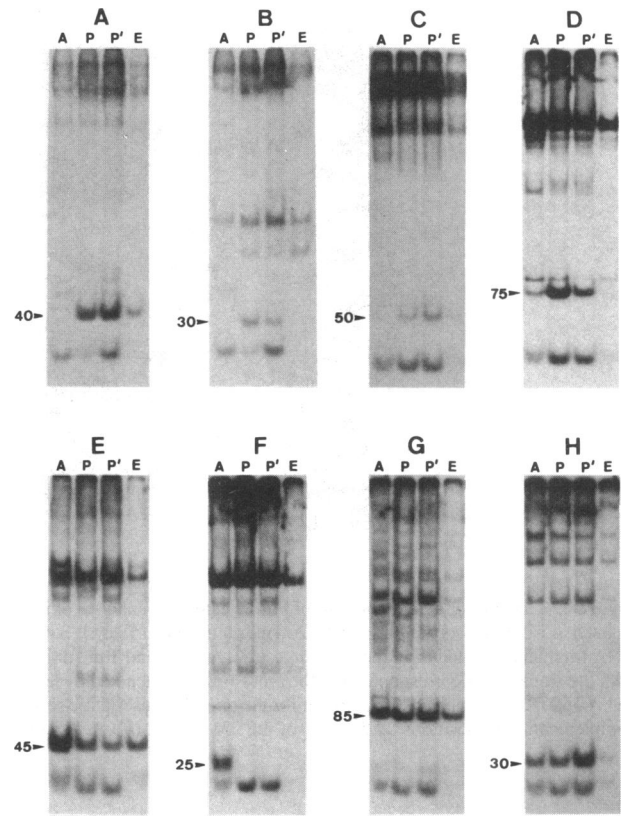

Figure 3. Autoradiograms of RNase H digests of cross-linked A, P or E site tRNA–16S or tRNA–23S complexes containing ^{32}P-labelled tRNA. The lanes marked P are from P site samples prepared with 70S tight couples in the presence of mRNA, those marked P' from samples in the absence of mRNA (see Materials and Methods; 14). The digestions were made in the presence of pairs of oligodeoxynucleotides centred on two 16S or 23S rRNA positions respectively as follows. (**A**) 16S positions 687 and 727, releasing a fragment of ~40 nt. (**B**) 16S positions 1318 and 1350 (an ~30 nt fragment). (**C**) 23S positions 1845 and 1892 (~50 nt). (**D**) 23S positions 2282 and 2358 (~75 nt). (**E**) 23S positions 2442 and 2488 (~45 nt). (**F**) 23S positions 2461 and 2488 (~25 nt). (**G**) 23S positions 2488 and 2572 (~85 nt). (**H**) 23S positions 2572 and 2603 (~30 nt). The fast moving band in each gel slot corresponds to free tRNA (cf. 14).

50S subunits is distinctly different for TDB–tRNA^{Phe} bound at the three ribosomal sites, as is also the distribution of radioactivity between the rRNA and protein peaks. In the typical experiment illustrated in Figure 1 the radioactivity associated with rRNA varies from ~40 000 c.p.m. (to 16S rRNA) in the case of A site binding up to ~300 000 c.p.m. (to 23S rRNA) for P site binding. Similarly, the radioactivity associated with ribosomal protein varies from 120 000 c.p.m. (for 50S proteins cross-linked at the E site) up to ~750 000 (for 30S proteins cross-linked at the P site). A cross-linking yield of 1% corresponds to ~150 000 c.p.m. and these yields are thus in the usual range for diazirine derivatives (14,16), the relatively low values reflecting the extremely high reactivity of the reagent; the short-lived free radical (20) generated during the irradiation process reacts to a large extent with the solvent.

The proteins cross-linked to TDB–tRNA^{Phe} were identified by gel electrophoresis followed by immunological analysis, as described in Materials and Methods. Examples of the gels obtained are shown in Figure 2 and the immunological results for the A, P and E sites are summarized in Table 1. The most important feature

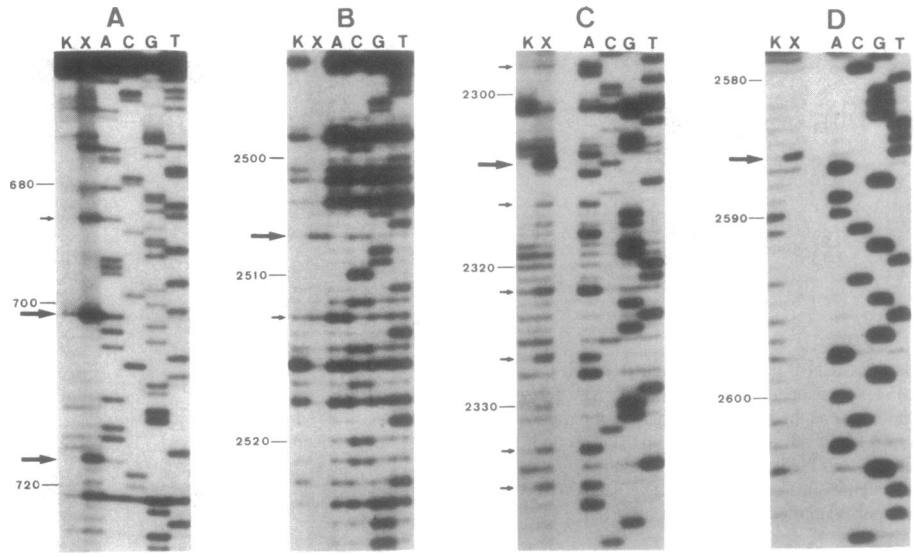

Figure 4. Examples of autoradiograms of primer extension analyses of individual cross-linked complexes. In each case lane X is a cross-linked sample from the P site, isolated as a complex containing a 100–200 nt fragment of 16S or 23S rRNA by RNase H digestion (see text). Lane K is the corresponding free (i.e. non-cross-linked) RNA fragment, isolated from the same digestion. A, C, G and T are dideoxy sequencing lanes. In each panel an appropriate part of the 10% sequencing gel is shown, with the stop signals corresponding to the cross-link sites denoted by large arrows (principal stop signals) or small arrows (weaker signals or those not always seen). (**A**) A 16S rRNA sample, using a primer for the reverse transcriptase reaction complementary to positions 760–776 of the 16S rRNA. (**B**) A 23S sample, with primer complementary to positions 2529–2251 of the 23S rRNA. (**C**) A 23S sample, primer complementary to 2354–2370. (**D**) A 23S sample, primer complementary to 2620–2638.

of this table is that the pattern of cross-linking is clearly specific for each of the three ribosomal sites, although there is not unexpectedly some overlap between the P and the A site and the P and the E site patterns. Taken as a group, both the 30S and 50S proteins found (Table 1) are among those that have previously been identified in cross-linking studies with tRNA derivatives (see 28 for a review). If one divides the tRNA molecule crudely into three regions, the acceptor stem–loop, the anticodon stem–loop and the central fold, then the previous data can be categorized as follows. Cross-links from the P site involving the proteins in Table 1 have been observed to L16 and L27 from the acceptor region (13), to S7, S13 and S19 (29–31) and to L1, L5, L27 and L33 (10,29) from the anticodon region and to S7, S9 and S19 (7,9,10,32) and to L1, L5, L27 and L33 (7,8,10,32) from the central region. From the A site, corresponding cross-links have been found to L27 from the acceptor region (28), to S7 from the anticodon region (10,31) and to S19 and L27 (6,10) from the central region. An E site cross-link to protein L33 has been observed to the acceptor region (33). There is thus a considerable degree of inter-relationship and overlap between the cross-linked proteins from the different ribosomal sites and from the different regions of the tRNA.

More interesting to us are the cross-link sites on the 16S and 23S rRNA. To identify these sites the isolated cross-linked 16S– or 23S–TDB–tRNA^{Phe} complexes (Fig. 1) were subjected to a series of digestions by RNase H in the presence of pairs of oligodeoxynucleotides (10mers or 17mers) complementary to selected sequences on the rRNA (14,23). First, the whole 16S or 23S molecule was 'scanned' in the usual way (14,16) using pairs of oligodeoxynucleotides spanning 100–200 nt segments of the rRNA. Then those segments found to contain cross-links were subjected to further digestions using different pairs of oligodeoxynucleotides, so as to narrow down the localization of the cross-linked regions as far as possible (14,16). In each experiment samples from the A, P and E sites were run in parallel and examples of the gels obtained are illustrated in Figure 3. Finally, 100–200 nt fragments of the 16S or 23S rRNA encompassing the cross-linked regions were isolated, again by RNase H digestion, and the sites of cross-linking were further localized by primer extension analysis (14,27). Examples of these gels are given in Figure 4.

The combined results of the RNase H digestions and primer extension analyses from a large number of experiments are summarized in Table 2 and the locations of the cross-link sites within elements of the 16S or 23S rRNA are illustrated in Figure 5.

Cross-links to 16S rRNA

Both P site- and E site-bound TDB–tRNA^{Phe} showed cross-links to 16S rRNA between positions 687 and 727 (Fig. 3A and Table 2), although the level of P site cross-linking was markedly higher. In both cases the primer extension analysis revealed stop signals at nt 702 and 718 (Fig. 4A), indicative of cross-link sites at positions 701 and 717 respectively. A further weak signal was occasionally seen at nt 687, at the extreme limit of the cross-linked region defined by the RNase H digestions, indicating a possible additional cross-link at position 686; the latter position lies 'opposite' 701 in the internal loop of helix 23 in the secondary structure of the 16S rRNA (Fig. 5). In our previous studies using tRNA$^{Arg}_{I}$ derivatized at position 32 in the anticodon loop (14) both P site- and E site-bound tRNA, as here, gave identical cross-links in helix 23, but in that case they were to position 693 at the loop end of the helix (cf. Fig. 5). Thus the loop end of helix 23 lies adjacent to the anticodon loop of the tRNA, whereas the

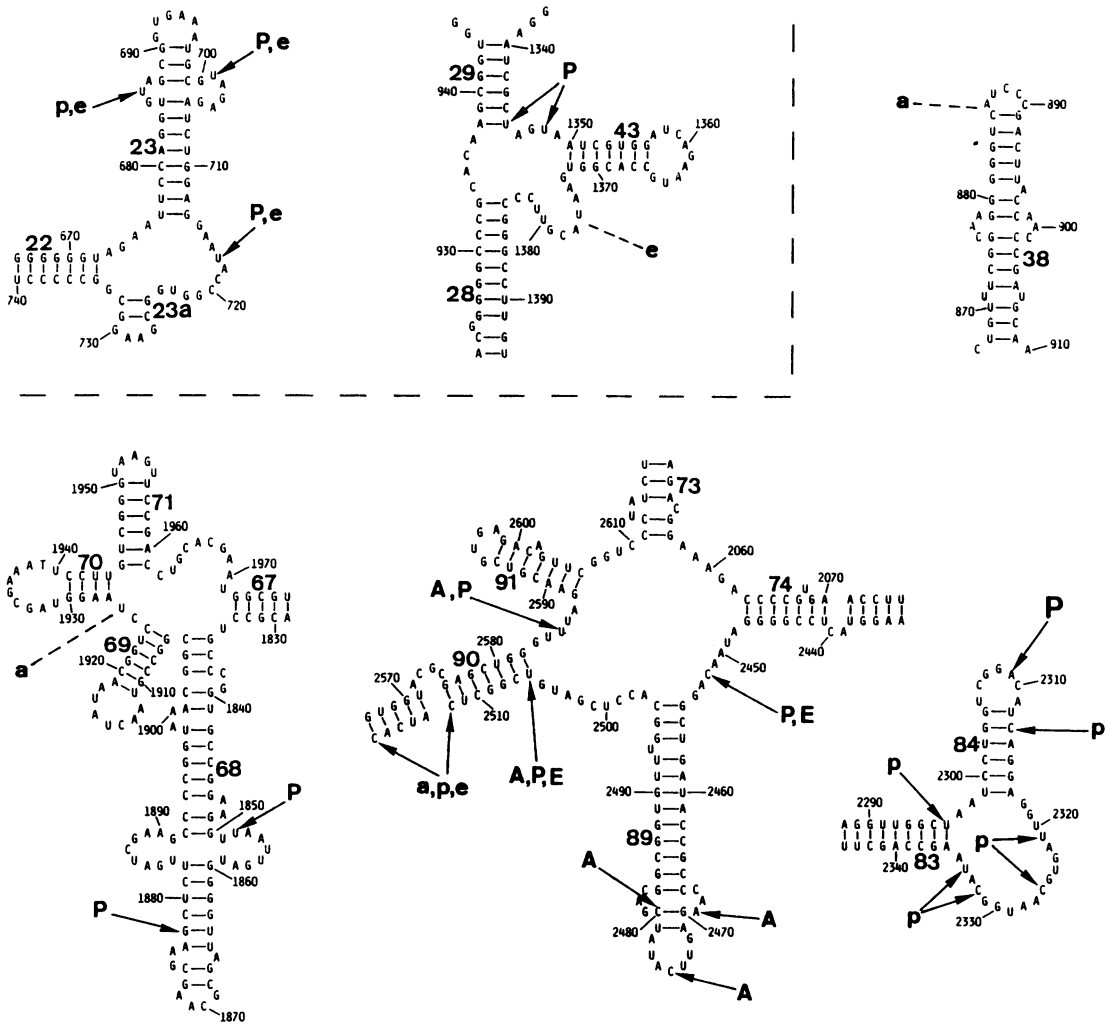

Figure 5. Locations of the cross-link sites (Table 2) in the corresponding regions of the secondary structures of 16S and 23S rRNA. The 16S rRNA regions are those in the upper left-hand corner, enclosed by the dotted line. The helices are numbered as in Brimacombe (34) and Leffers *et al.* (35) for 16S and 23S rRNA respectively. The A, P and E site cross-links are denoted by corresponding capital letters (major cross-links) or small letters (minor cross-links or those not always seen). Cross-links indicated by dotted lines (as opposed to arrows) are those where no reverse transcriptase stop signal could be detected in the cross-linked region identified by RNase H digestion (see Table 2).

central fold lies adjacent to positions 686 and 701, suggesting that helix 23 and the anticodon stem–loop must be arranged in a roughly 'parallel' configuration in the ribosome. This is in contrast to the arrangement proposed in older models of 16S rRNA (36,37), where the corresponding configuration was 'anti-parallel'. The data suggest further that the single strand encompassing position 717 must be folded back towards helix 33 (Fig. 5), to allow nt 701 and 717 to be cross-linked at the same time.

A cross-link that was entirely specific for the P site (with no concomitant E site cross-linking) was observed between positions 1318 and 1350 of the 16S rRNA (Fig. 3B) and here the primer extension analysis indicated cross-link sites at nt 1345 and 1348 (Table 2). Here again, an analogous situation had been seen with $tRNA^{Arg}_{I}$, where a P site-specific cross-link from position 32 of the tRNA to nt 1338 was found (14) at the other end of helix 29 (Fig. 5). As with helix 23 just discussed, these results suggest how helix 29 (Fig. 5) must be oriented relative to the anticodon stem–loop of the P site tRNA, namely with the anticodon loop adjacent to the 'upper' end of helix 29 (as drawn in Fig. 5) and the central fold adjacent to the 'lower' end of this helix.

The RNase H digestion data showed an E site-specific cross-link between nt 1350 and 1387 of the 16S rRNA (Table 2). In this case, however, no reproducible reverse transcriptase stop signal was observed, a phenomenon that we have observed on previous occasions (38,39), possibly indicating a cross-link to (for example) a ribose moiety in the 16S rRNA which does not give rise to a pause signal in the primer extension analysis. This cross-link could therefore not be finally localized, although it should be noted that analogous E site cross-linking to positions 1376 and 1378 (cf. Fig. 5) was observed from position 32 of $tRNA^{Arg}_{I}$ (14). Additional cross-links from the P and E sites were seen further towards the 3′-end of the 16S rRNA; they are visible in Figure 3B as the slower moving digestion products, representing 16S fragments between nt 1318 or 1350 and the 3′-terminus, but the precise location of these cross-links appeared to be rather variable from one experiment to another and they were accordingly

not analyzed in detail. Similarly, the A site tRNA, which in any event only showed very low levels of cross-linking to 16S rRNA (Fig. 1), did not reveal any specific cross-link sites, although, as already noted (Table 1), the corresponding cross-linking to ribosomal protein was highly specific for S19.

Table 2. Summary of cross-links to 16S and 23S rRNA from $tRNA^{Phe}$ at the ribosomal A, P and E sites

RNA	Localization by RNAse H	Cross-link site(s) by primer extension	A	P	E
16S	687–727	(686), 701, 717	–	++	+
	1318–1350	1345, 1348	–	++	–
	1350–1387	–	–	–	+
23S	865–910	–	+	–	–
	1845–1892	1852, 1878	–	++	–
	1892–1945	–	+	–	–
	2282–2358	(2296), 2309, (2313, 2321, 2326, 2332, 2334)	(+)	++	–
	2242–2461	(2452)	(+)	++	++
	2461–2488	2469, 2475, 2480	++	–	–
	2488–2539	2506, (2512–2517)	++	++	++
	2572–2603	2585	++	++	–

The Table shows the shortest RNA region encompassing each cross-link site as determined by RNase H digestion (cf. Fig. 3) and the corresponding cross-linked positions as determined by primer extension analysis (cf. Fig. 4). In the primer extension column – indicates that no reproducible stop signal was observed; minor stop sites or those not always observed are in parentheses. Distribution of the cross-links between the A, P and E sites is denoted by ++ (major) or + (minor). Here the symbol – means that the cross-link concerned was either absent or drastically reduced in relation to the corresponding cross-link from one of the other two tRNA sites and in the latter case is thus regarded as cross-contamination; (+) means that it was unclear whether there was a genuine contribution from the tRNA site concerned. (Note: The limits of the RNA regions defined by the RNase H digestions are given in each case to the mid-point of the sequence complementary to the oligodeoxynucleotide concerned. Depending on the precise sites of RNase H cleavage, these regions may of course be extended by up to two or three nucleotides in either direction.)

Cross-links to 23S rRNA

In contrast to the 16S rRNA, a number of A site-specific cross-links were observed to 23S rRNA. The first of these (Table 2) is a weak but reproducible cross-link between nt 865 and 910; no reverse transcriptase stop signal was found in this case (cf. the E site cross-link to the 1350–1387 region of 16S rRNA described above). The 23S rRNA region concerned (helix 38, Fig. 5) has not up to now been implicated in either tRNA foot-printing (40) or cross-linking studies and this cross-link might be dismissed as an aberration, were it not for the fact that our corresponding investigation (J. Rinke-Appel, N. Jünke and R. Brimacombe, manuscript in preparation) of cross-links from position 20:1 of lupin $tRNA^{Met}$ (cf. 8) has shown a major A site-specific cross-link to precisely the same short segment of the 23S rRNA.

Another A site-specific cross-link, also relatively weak but reproducible and again with no reverse transcriptase stop signal, was found between positions 1892 and 1945 (Table 2). This 23S rRNA region encompasses both the inter-RNA cross-link site to 16S rRNA at the loop end of helix 69 (Fig. 5), which we have described (41), and the zero-length cross-link reported by Wower *et al.* (13) to nt 1945 from position 76 of P site-bound tRNA. A P site-specific cross-link was observed to a neighbouring region within this domain of the 23S rRNA, namely between positions 1845 and 1892 (Fig. 3C), with primer extension signals indicative of cross-links at nt 1852 and 1878 in helix 68 (Table 2 and Fig. 5). The 11 nt pitch of the RNA A-helix would serve to bring these two sites onto the same side of the helix and it is furthermore noteworthy that a cross-link to protein L1 was found (cf. Table 1) from a site close to the loop end of helix 68 (34).

A cluster of P site-specific cross-links was observed between positions 2282 and 2358 (Fig. 3D) showing a number of reverse transcriptase stop signals (Fig. 4C), of which the most prominent was at nt 2310, indicating a cross-link to position 2309; this is the same site that we defined in earlier experiments (11) using $tRNA^{Phe}$ modified at position 47 with an azidohippuric acid derivative. These cross-link sites all lie in the region of helices 83 and 84 (Fig. 5) and are close to sites that have been cross-linked to proteins L5 and L27 (34; cf. Table 1). Very weak cross-linking from the A site was also observed in this area of the RNA (Table 2), but this was most probably due to small amounts of P site-bound tRNA contaminating the A site samples.

The remaining cross-link sites are in the vicinity of the peptidyl transferase ring (42), enclosed by helices 73, 74, 89, 90 and 91 (Fig. 5). An RNase H digest between positions 2442 and 2488 (Fig. 3E) showed cross-linking from all three tRNA sites, but a further digest (Fig. 3F) indicated that there was an A site-specific component between positions 2461 and 2488. By inference, the P and E site contributions must therefore lie between positions 2442 and 2461 (although we cannot exclude the possibility that there was a second A site cross-link in the latter segment; cf. Table 2). Primer extension analysis showed reproducible stop signals, indicating cross-links at nt 2469, 2475 and 2480 in helix 89 (Table 2) for the A site cross-link in the 2461–2488 region and a rather tentative site at nt 2452 for the P and E site cross-links in the 2442–2461 region. Position 2452 is notably the identical site to which Steiner *et al.* (15) observed a cross-link from an aminoacyl derivative attached to P site-bound tRNA. Position 47 of the tRNA is ~50 Å away from the aminoacyl residue; since the lengths of the respective cross-linking reagents are of the order of 10 Å, a cross-linking from both the aminoacyl residue and position 47 to the same nucleotide on 23S rRNA can only be explained in terms of a certain degree of flexibility either in tRNA binding or within the structures of the tRNA and 23S rRNA molecules themselves. The A site-specific cross-links in helix 89 (Fig. 5) are also noteworthy, since nt 2477 has been cross-linked in high yield to a thiouridine residue at position 89 of 5S rRNA (43); 50S subunits lacking 5S rRNA show a drastic reduction in elongation factor Tu-dependent binding of tRNA to the A site (44).

Cross-linking from all three tRNA sites was located between nt 2488 and 2539 (cf. the corresponding digest giving a somewhat longer fragment in Fig. 3G). The major cross-link site here was to nt 2506 (Fig. 4B), although minor sites were observed at variable positions between nt 2512 and 2517 (Fig. 4B and Table 2). Nucleotide 2506 in helix 90 (Fig. 5) is again a position that has been cross-linked from derivatized amino acids in aminoacyl tRNA at both the A and P sites (15,16). The final cross-link was observed from the A and P sites (Table 2) at nt 2585 (Fig. 4) within the RNase H region encompassing positions 2572–2603

(Fig. 3H). This site was found by Steiner *et al.* (15) to be cross-linked from their aminoacyl tRNA derivative at the A site and we have also observed cross-links to this position from diazirine derivatives of aminoacyl tRNA (16). Both positions 2506 and 2585 are among the nucleotides that have been allocated to the P site in tRNA footprinting experiments (40).

CONCLUSIONS

The use of the diazirine reagent (19,20), as opposed to the azido compound applied in our earlier study (11), has enabled us to detect a number of new cross-links to both rRNA and ribosomal protein from the acp^3U residue at position 47 in the central fold of $tRNA^{Phe}$. In the 30S subunit the cross-linked proteins identified all lie within the head of the 30S subunit (4), whereas the cross-links to 16S rRNA are to sites both in the head and on the side lobe (36,37). In the 50S subunit the proteins found are all believed to be located in the general area of the central and L1 protuberances (4,45). The cross-links to 23S rRNA are to sites in the area of the central protuberance, the peptidyl transferase ring and the interface bridge (3), as indicated by current model building studies (46). We have postponed a detailed discussion of the relevance of these cross-links to the orientation of tRNA on the ribosome until the corresponding data sets from positions 8 and 20:1 in the central fold of the tRNA (mentioned in the Introduction) are complete; these investigations are currently in their final stages in our laboratory. Taking all the results together, we will then have a considerable body of cross-linking information from the anticodon stem–loop, the acceptor stem and the central fold regions of tRNA at the A, P and E sites; this information will be incorporated into our three-dimensional models for *E.coli* 16S and 23S rRNA (cf. 2,46).

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