The localization of multiple sites on 16S RNA which are cross-linked to proteins S7 and S8 in Escherichia coli 30S ribosomal subunits by treatment with 2-iminothiolane

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SUMMARY
RNA-protein cross-links were introduced into E. coli 30S ribosomal subunits by reaction with 2-iminothiolane followed by a mild ultraviolet irradiation treatment. After removal of non-reacted protein and partial nuclease digestion of the cross-linked 16S RNA-protein moiety, a number of individual cross-linked complexes could be isolated and the sites of attachment of the proteins to the RNA determined. Protein S8 was cross-linked to the RNA at three different positions, within oligonucleotides encompassing positions 629-633, 651-654, and (tentatively) 593-597 in the 16S sequence. Protein S7 was cross-linked within two oligonucleotides encompassing positions 1238-1240, and 1377-1378. In addition, a site at position 723-724 was observed, cross-linked to protein S19, S20 or S21.

INTRODUCTION
In a recent paper (1) we described how the reagent 2-iminothiolane, which has been widely used as a protein-protein cross-linking agent for the E. coli ribosome (2,3), can also be used under appropriate conditions to generate RNA-protein cross-links. For this purpose, the compound is first allowed to react with the protein moiety of the ribosomal subunits in the usual manner (2,3), and the modified subunits are then subjected to a mild ultraviolet irradiation treatment. This leads to a cross-linking reaction with the RNA moiety, which was presumed (1) to involve a coupling between an excited pyrimidine residue and the thiol group of the reagent (cf. 4).

The application of a bifunctional reagent of this type inevitably leads to a complex mixture of reaction products, but in the same publication (1) we were able to demonstrate that the reaction products could be separated and analysed with the help
of specially developed electrophoretic techniques (5), and we described the simultaneous localization of a number of RNA-protein cross-link sites on the *E. coli* 23S RNA involving six different 50S ribosomal proteins. These experiments opened the way for a detailed study by cross-linking of the topology of RNA and protein within the ribosomal subunits. As a result, the rapid accumulation of a catalogue of RNA-protein cross-link sites should now be possible, and it would be expected that such a catalogue would include multiple sites on the RNA for any given protein. The nature of the secondary structures which have already been established for the ribosomal RNA molecules (6-11) suggests that individual proteins are likely to interact with several different regions or loops of the RNA, and this property should be reflected in the spectrum of cross-link sites. The data should therefore begin to define three-dimensional "pockets" in the RNA structure, into which the individual proteins can be fitted.

In this paper, we describe for the first time the analysis of such multiple cross-link sites on the ribosomal RNA, for proteins S7 and S8. The cross-links were induced by the action of 2-iminothiolane on intact 30S particles, and were localised on the 16S RNA using our established methodology (1,5). In addition, we describe a further cross-link site in the centre of the 16S RNA molecule, involving either protein S19, S20 or S21.

**MATERIALS AND METHODS**

$^{32}$P-labelled 30S ribosomal subunits from *E. coli* strain MRE 600 were prepared by the rapid method of Stiege et al. (12). Cross-linking with 2-iminothiolane, followed by separation of non-cross-linked protein on sucrose gradients containing dodecyl sulphate was carried out as previously described (1), using 25 $A_{260}$ unit aliquots of $^{32}$P-labelled 30S subunits (0.5 - 1.0 x $10^9$ counts/min total). The RNA plus RNA-protein cross-linked complex isolated from the gradient was digested for 15 min at 37° with 3 - 6 units of ribonuclease T1 per $A_{260}$ unit RNA, and applied to the Triton X100/dodecyl sulphate two-dimensional gel system, as described (1,5). After electrophoresis, the RNA-pro-
tein complexes (each containing $10 - 100 \times 10^3$ counts/min) were located by autoradiography, extracted from the gel, and submitted to protein and oligonucleotide analysis as before (1), with the following modifications:

For analysis of the protein moiety, an aliquot of each complex was subjected to total digestion with ribonucleases A and T$_1$, and during the incubation with the enzymes the temperature was slowly raised from 37° to 60° as described in ref. 13, to ensure complete digestion. The sample was then mixed with unlabelled 30S protein, and applied to the two-dimensional gel system described in ref. 14, in which the first gel dimension was that of Mets and Bogorad (15), and the second dimension a 15% gel in the system of Laemmli and Favre (16), 1 mm thick slab gels being used for both dimensions (14). After electrophoresis the gels were first stained, then dried and subjected to autoradiography to locate the protein-oligonucleotide complex.

For the oligonucleotide analysis, aliquots of each cross-linked complex were subjected to digestion by either ribonuclease T$_1$ or ribonuclease A, with heating to 60° as above (cf. ref. 13), and the digestion products were separated on the two-dimensional mini-fingerprint system of Volckaert and Fiers (17), as before (1, 5). In some cases the nuclease digestion was followed by an incubation for 15 min at 37° with proteinase K (0.1 mg/ml) in 0.1% sodium dodecyl sulphate, before applying the sample to the fingerprint (cf. ref. 5, 18). Secondary digestions of individual oligonucleotides from the fingerprints were made with ribonuclease A or T$_1$, as previously described (5), and the oligonucleotide data were fitted to the 16S RNA sequence of Brosius et al. (19).

**RESULTS**

The properties of the RNA-protein cross-linking reaction induced by 2-iminothiolane are very similar for both 30S and 50S ribosomal subunits (1). To recapitulate them very briefly, a short incubation with the reagent at room temperature is sufficient, and the level of reaction reaches a plateau at a reagent concentration of approximately 20 mM, corresponding to a
cross-linking to RNA of 5 - 6% of the total protein after the ultraviolet irradiation. The irradiation treatment (which is the second step in the cross-linking process) leads in the absence of reagent to a cross-linking of approximately 1.5% of the total protein. In the case of the 30S subunit, the proteins predominantly involved in the cross-linking reaction with the complete system are S3, S4 and S7, although most of the other proteins are also cross-linked to some extent (1). In contrast, the irradiation treatment alone (without iminothiolane) leads solely to cross-linking of protein S7 (cf. 20). As already noted (1), the irradiation conditions in this step are particularly mild, the irradiation time (3 min) being only one-fifth to one-tenth of that used in our previous experiments (20,18) to study the highly specific cross-link between protein S7 and the 16S RNA.

In order to analyse the sites of cross-linking to protein on the RNA, $^{32}$P-labelled 30S subunits were treated with iminothiolane and irradiated. Non-cross-linked protein was removed by sucrose density gradient centrifugation in the presence of dodecyl sulphate, and the isolated RNA fraction (which contains the RNA-protein cross-linked complexes) was subjected to a partial digestion with ribonuclease T$_1$ as described in Materials and Methods and ref. 1. The digested material was applied to the Triton X-100/dodecyl sulphate two-dimensional polyacrylamide gel system (5) as before (1). This gel system has the property of separating fragments of free RNA from fragments cross-linked to protein, the cross-linked complexes appearing as "reverse diagonals" on the gel (5), and a typical example is shown in Fig. 1. The left-hand side of the diagram is the autoradiogram of the gel, and the key diagram on the right indicates which 30S proteins and which cross-link sites on the 16S RNA were contained in the various individual complexes. The pattern in Fig. 1 was extremely reproducible, and the ensuing discussion is concerned with the analysis of the products separated on this gel. Protein analysis of the cross-linked complexes (see below) showed the presence of "reverse diagonals" corresponding to proteins S7, S8 and S19/20/21; the spots containing S19/20/21 (and the lowest spot containing S7) are rather faint in the autoradio-
Figure 1: Triton X-100/dodecyl sulphate two-dimensional gel of partially digested 32P-labelled RNA-protein complexes from 30S subunits cross-linked with 2-iminothiolane. The left-hand side of the diagram shows the relevant part of the autoradiogram of the gel (cf. 1, 5) and the key diagram on the right indicates which spots were cut out from the gel for further analysis; the shading indicates which groups of spots form "reverse diagonals" corresponding to a particular protein. The small letters (a - d) show the distribution of the different cross-link sites in the cases of proteins S7 and S8 (see text). Direction of electrophoresis was from left to right (first dimension), and top to bottom (second dimension).

graphic exposure reproduced in Fig. 1, but could clearly be seen at longer exposure times. Other diagonals can also be seen, corresponding to S9/11 (also faint in Fig. 1) and to S15/16/17, but the oligonucleotide analyses in these latter cases did not give unequivocal results, and will not be considered further. Similar gels from 30S samples subjected to the irradiation treatment alone (without iminothiolane) showed a single weak reverse diagonal corresponding to protein S7. As was also observed in the case of the 50S subunit (1), the spectrum of proteins contained in the cross-linked complexes on the two-dimensional gel (Fig. 1) does not correspond to the overall pattern of cross-linking (as revealed by gel electrophoresis of the proteins released by total nuclease digestion of the 16S RNA-protein cross-linked...
Figure 2: Two-dimensional protein gel analysis of oligonucleotide-protein complexes containing (A) S7, and (B) S8. The Figures are autoradiograms of the gels, with the positions of the stained protein spots indicated by the circles. Arrows denote the direction in which the cross-linked radioactive complex is displaced from the corresponding free protein. Direction of electrophoresis is from left to right (first dimension) and top to bottom (second dimension).

complex). For example, no reverse diagonals containing proteins S3 or S4 were found. This apparent discrepancy is a consequence of the highly selective nature of the partial digestion with ribonuclease T1, and will be discussed further below.

Each individual RNA-protein complex was extracted from the gel (Fig. 1) and subjected to both protein and oligonucleotide analysis, as described in Materials and Methods. In contrast to our previous experiments (1), no further purification of the complexes on 17% gels was undertaken, as this procedure led to unacceptably high losses of material. Typical protein analyses on two-dimensional polyacrylamide gels from complexes containing proteins S7 (cross-link "a" (Fig. 1)) and S8 (cross-link "c") are illustrated in Fig. 2. The use of large thin gel slabs for this procedure (14) gives a good resolution of the proteins, and it can be seen from Fig. 2 that in each case the gel showed a single radioactive spot, moving to the "north-west" of the corresponding unlabelled protein. This mobility shift is due to the oligonucleotide remaining covalently attached to the protein
after complete digestion with ribonucleases A and T₁, the shift being proportional to the length of this oligonucleotide. In the case of protein S7 (cross-link "b"), for example, a weaker spot moving closer to the unlabelled S7 position was observed. The S7 complex shown in Fig. 2A could be construed as arising from protein S5, and not S7, but this possibility can be discounted for two reasons. Firstly, the S7-oligonucleotide complex from subunits subjected to ultraviolet irradiation alone (where S7 is the only cross-linked protein) runs at precisely the same position in the gel (cf. 20). Secondly, in mixtures of cross-linked complexes where a number of proteins are present (see e.g. ref. 21), a spot corresponding to cross-linked S5 can be seen in addition to the S7-oligonucleotide complex, moving in the expected position below and to the left of the latter. In the case of protein S8 (Fig. 2B), the identity of the protein was further confirmed by an Ouchterlony double diffusion test with an S8-specific antibody (cf. ref. 1, data not shown). Proteins S19, S20 and S21 are not resolved in the gel system of Fig. 2, and a definitive identification of the protein contained in the cross-linked complexes corresponding to one of these three proteins (Fig. 1, and see below) has not yet been possible, since this protein-oligonucleotide complex tends to run as a smear in those gel systems so far tried which do resolve the three proteins.

The oligonucleotide analyses of the complexes containing proteins S7, S8 and S19/20/21 (Fig. 1) were made on polyethylene-imine thin-layer plates (17), after digestion with ribonuclease T₁ (or ribonuclease A), and some typical examples of the ribonuclease T₁ fingerprints obtained are shown in Fig. 3. The individual oligonucleotides were further characterized by secondary digestions with ribonuclease A (or ribonuclease T₁ in the case of ribonuclease A fingerprints) and the data were fitted to the sequence of 16S RNA (19). Each RNA-protein complex showed a characteristic region of the RNA sequence, with one oligonucleotide missing, and the results from all the complexes analysed are summarized in Fig. 4. As before (1), the missing oligonucleotide contains the cross-link site, and the positions of these oligonucleotides are indicated in the examples in Fig. 3. In
Figure 3: Examples of ribonuclease T₁ fingerprints of cross-linked RNA-protein complexes on polyethyleneimine plates (17). The first dimension ran from right to left, the second from bottom to top, arrows denoting the point of sample application. Identities of the principal oligonucleotides are shown, those in brackets giving the positions of the "missing oligonucleotides" (see text). The fingerprints should be compared with the sequences in Fig. 4. (A) An S8-RNA complex (cross-link "c"; cf. Fig. 1). (B) An S8-RNA complex (cross-link "d"). (C) An S19/20/21-RNA complex. (D) An S7-RNA complex (cross-link "a"), the fingerprint in this case being from a proteinase K-treated sample (see text). (E) An S7-RNA complex (cross-link "b"). (The oligonucleotide spots in some of the fingerprints are ringed, as a result of being photographed subsequent to the routine processing for secondary digestion and analysis).

In some cases a digestion with proteinase K was made prior to running the fingerprint (see Materials and Methods), and in such cases a new spot or spots appeared on the fingerprint, corresponding to the residual cross-linked peptide-oligonucleotide (cf. 5,18). In the absence of proteinase K digestion, the ribonuclease T₁-resistant protein-oligonucleotide complex either remained at the origin of the fingerprint, or (since these products are often rather insoluble) did not appear on the fingerprint.
Figure 4: Summary of the sequence data for the cross-linked complexes. The complexes are given in the order in which they appear in the 16S RNA sequence (19). The black bars denote the sequences found, with shaded regions indicating oligonucleotides whose presence or absence was either variable or uncertain. Other minor variations were also found, with slightly longer or shorter sequences at either end; these have been omitted for clarity. The white boxes indicate the missing oligonucleotides, which are shown in large letters in the sequence (cf. Fig. 3). The bracketed nucleotides and arrows show the location of the cross-link site within the missing oligonucleotide (see text). The fingerprints shown in Fig. 3 are those of complexes 2 and 4 (S8), complex 2 (S19/20/21), and complexes 2 and 3 (S7), respectively.

The fingerprint in Fig. 3A shows an example of an S8-RNA cross-linked complex, containing cross-link "c" (Fig. 1). In this case the sequence runs from position 582 to position 664 of the 16S RNA (S8 complex 2 in Fig. 4), and the oligonucleotide A-A-C-U-G at positions 629-633 was clearly missing. (The faint spot in the position of A-A-C-U-G in Fig. 3A is a contaminant, and did not give A-A-C in the secondary digest with ribonuclease A). This cross-link was the most commonly observed site with protein S8 (cf. Fig. 4) and appeared in association with RNA.
fragments of varying length in the two-dimensional gels (Fig. 1). In one instance the oligonucleotide U-U-A-A-G (positions 593-597) was also clearly absent, and this was tentatively assigned as an additional cross-link site.

Fig. 3B gives an example of an S8-RNA complex containing cross-link "d" (Fig. 1). Here the sequence runs from positions 589-666 of the RNA (S8 complex 4 in Fig. 4), and A-A-C-U-G was present. Instead, the oligonucleotide C-U-U-G (651-654) was absent. Thus, protein S8 is cross-linked to two distinct sites (plus a tentative third site) within the same region of the 16S RNA. The distribution of the two cross-links "c" and "d" between the various RNA-protein complexes is indicated in Fig. 1. Attempts to pinpoint the cross-link sites further to a single nucleotide within oligonucleotides 629-633 and 651-654 (from fingerprints of proteinase K-treated samples) did not give definitive results.

Fig. 3C shows the fingerprint obtained from the shortest cross-linked complex containing protein S19/20/21 (Fig. 1). This fingerprint also arises from the central region of the 16S RNA (positions 715-727, S19/20/21 complex 2, Fig. 4), and the cross-link site is clearly defined by the absence of the U-G sequence at positions 723-724. The same site was observed with longer attached RNA fragments, (e.g. S19/20/21 complex 1, Fig. 4, cf. Fig. 1).

Fig. 3D is an example of an S7-RNA complex containing cross-link "a" (Fig. 1), and in this case the fingerprint was from a proteinase K-treated sample. Although the fingerprint shows a non-specific background of faint contaminating spots, the sequence data were unambiguous and correspond to S7 complex 2 (Fig. 4). This fragment occupies positions 1221-1255 in the 16S RNA, and the oligonucleotide C-U-A-C-A-A-U-G (1234-1241) was absent. Two anomalous spots (marked X and Y in Fig. 3D) were observed on the fingerprint, corresponding to ribonuclease T₁/proteinase K-resistant peptide-oligonucleotides. Both these spots gave similar rather complex patterns on digestion with ribonuclease A, but the oligonucleotide A-C was clearly discernible. Further, a ribonuclease A fingerprint of the same S7-RNA
complex showed no trace of the oligonucleotide A-A-U (expected at positions 1238-1240), and the cross-link site must therefore lie within this A-A-U sequence.

In our previous experiments with simple ultraviolet-induced cross-linking, the uridine at position 1240 was found to be cross-linked to the methionine at position 114 of the S7 sequence (18,22), and the iminothiolane-induced cross-link is at the same position on the RNA. As already mentioned above, control experiments with ultraviolet irradiation alone showed only a weak reverse diagonal corresponding to protein S7 on the two-dimensional gel (cf. Fig. 1), and it follows that the cross-link observed at positions 1238-1240 is not merely this "background" cross-link, unless the iminothiolane treatment is in some way able to enhance the formation of the simple ultraviolet cross-link. More likely is the possibility that the cross-link occurs to the lysine residue at position 112 in the protein S7 sequence (23); this lysine is very close to the methionine residue (position 114), and could be cross-linked via the iminothiolane molecule to the same reactive uridine residue at position 1240.

More important in the context of this article is the finding of a second, totally distinct cross-link site (site "b", Fig. 1) to protein S7. This is illustrated in Fig. 3E, the sequence concerned being from positions 1366-1417 in the 16S RNA (S7 complex 3, Fig. 4), with the hexanucleotide A-A-U-A-C-G (1374-1379) absent. The fingerprint in Fig. 3E was made from a sample without proteinase K treatment; analysis of a similar fragment with proteinase K treatment showed a new spot close to the oligonucleotide mC₃-C-C-G (cf. Fig. 3E), corresponding to the cross-linked peptide-oligonucleotide. This released A-A-U and G on secondary digestion with ribonuclease A (in addition to the residual peptide-oligonucleotide material), thereby establishing the cross-link site as being within the dinucleotide 1377-1378. Thus, as with protein S8, S7 also shows two cross-link sites, but, in contrast to S8, the two sites are relatively widely separated in the RNA sequence. The distribution of the two sites ("a" and "b") between the various RNA-protein complexes is indicated in Fig. 1.
Figure 5: Location of the RNA-protein cross-link sites to proteins S8 and S19/20/21 in the secondary structure of the central domain of the 16S RNA (8). The oligonucleotides encompassing the cross-link sites are ringed. The S8 site at positions 593-597 is tentative (see text).

DISCUSSION

The sites of RNA-protein cross-linking established in this set of experiments are shown in Figs. 5 and 6, incorporated into our secondary structure model (8) for the 16S RNA. In Fig. 7 we have made a first step towards building the sites for proteins S7 and S8 into a three-dimensional structure, simply by arranging the double-stranded regions of the secondary structure in appropriate helical conformations. The situations of the two proteins with respect to the RNA look very different. In the case of protein S8, all three cross-link sites lie within the
Figure 6: Location of the RNA-protein cross-link sites to protein S7, in the secondary structure of the 3'-domain of the 16S RNA (8). The cross-link sites are ringed as in Fig. 5.

"binding site" for S8, which was established from nuclease digestion studies with S8-16S RNA complexes (reviewed in 24). All the sites are in the region of the hairpin loop encompassing bases 588-651, and it can be seen from Fig. 7 that all the sites are on one side of the helix. There are some trivial differences between the various models which have been proposed for this region (6-8), but in all cases the helix is essentially the same rather rigid structure. It should be noted here that the nucleotides actually concerned in the cross-links may be looped-out bases, or they may be within the double helix (Fig. 5). Since the cross-links are likely to involve the 5-6 positions of pyrimidine residues (cf. 4), there is no reason to expect that single-stranded regions of the RNA would be preferred as targets for the cross-linking, although the majority of the cross-links found in the 50S subunit (1) were in fact in single-stranded loops.

The distance between the two principal cross-link sites for protein S8 is of the order of 60 Å (Fig. 7). Taking into account
Figure 7: A three-dimensional representation of the phosphate backbones of the RNA regions containing the cross-link sites to proteins S7 and S8 (cf. Figs. 5 and 6). A scale in Angstrom units is given. In the right-hand half of the diagram, the hairpin loop 1301-1339 should be imagined as running back "into the paper", and the loop 1347-1378 as "coming out of the paper".
the bridging length of the iminothiolane reagent (ca. 5 Å), and a possible de-stacking of the RNA helix at bases 596/642, this means that the two cross-linked lysine residues in the protein are separated by a minimum distance of about 50 Å, which is compatible with a relatively compact conformation of the protein. S8 has a molecular weight of 14,000 (25), and various physicochemical estimates of its shape have been made. Hydrodynamic studies for example have given a value of 2.3:1 for the axial ratio of the protein (26), which would fit well to the cross-linking data, whereas other experiments have suggested either a slightly more extended (27) or a more globular (28) conformation.

In contrast to those of S8, the cross-link sites for protein S7 lie in a rather flexible region of the RNA structure. Both sites are on the same side of the structure ("coming out of the paper", Fig. 7), as defined by the helical element comprising bases 938-943/1340-1345 in the middle of the region; this element introduces approximately one-half of a helix turn into the structure. As drawn in Fig. 7, the two cross-link sites are about 80 Å apart, but they can be brought very close (to within 30 Å) by "lifting" the two loops (1239-1298 and 1347-1378) "out of the paper" and pulling them together. Again, small discrepancies between the various secondary structure models (6-8) do not affect the overall situation here, which, as suggested in the Introduction, begins to define a three-dimensional "pocket" for protein S7 on the RNA. In this context it should be noted that a further cross-link site for S7 has been reported in the literature (29), within the oligonucleotide A-C-C-U-C-G (positions 1261-1266). This was claimed to be the site of ultraviolet-induced cross-linking of S7 to the 16S RNA in 30S subunits, which we had previously definitively established as uridine-1240 (18). The identification of A-C-C-U-C-G was based solely on an analysis of the oligonucleotide products released by ribonuclease A from a ribonuclease T₁-resistant cross-linked protein-oligonucleotide complex, these products being identical to those which we found to be released from the octanucleotide C-U-A-C-A-A-U-G (1234-1241) in our own experiments (18). This discrepancy has been discussed in a recent review article (30), and underscores the importance of determining cross-link sites within fragments of RNA which are
sufficiently long to be located unequivocally in the sequence.

Our cross-linking data for protein S7 also show why no clear "binding site" has ever been found for this protein, in contrast to S8 (24). If S7 interacts with several flexible regions of the RNA (as opposed to the single rigid helix found with S8), then one would not expect the "binding site" approach to yield a clearcut result; the whole S7-RNA complex would be likely to disintegrate when subjected to even a mild nuclease digestion. From the point of view of topographical and model-building studies on the other hand, a protein such as S7 is likely to be more informative than one such as S8. In this sense the cross-link to S19/20/21 (Fig. 5) is also particularly interesting. Although we have not yet been able to determine which of the three proteins was actually involved in the cross-link, none of the three candidates has so far been implicated with this region of the RNA. Protein S19 has been found in ribonucleoprotein fragments containing the 3'-region of the RNA (reviewed in 24), whereas S20 has a binding site in the 5'-region (24). S21 has been implicated in interaction with the extreme 3'-terminus of 16S RNA (31, 32). Regardless of which of the three proteins is involved, the cross-link at position 723-724 is therefore of considerable topographical importance. An analogous situation has been found with protein S1, which has been reported to be cross-linked both to the 3'-terminus of the 16S RNA (31), and to an RNA fragment encompassing positions 860-890 (33).

The results described here show for the first time that multiple cross-link sites for individual ribosomal proteins can be found in a single cross-linking experiment, and this finding has some further consequences, apart from the topographical considerations just discussed. Firstly, our original strategy (e.g. 5) of defining RNA-protein cross-link sites on both protein and RNA moieties now appears considerably more complex. The identification of cross-link sites on ribosomal proteins induced by bifunctional reagents has proved to be technically difficult (P. Maly and J. Wower, unpublished results), and the additional requirement of determining which of several sites on the RNA corresponds to which site on the protein makes this problem even more difficult. However, since many of the ribosomal pro-
teins are globular or only slightly elongated (see ref. 34 for a summary), the localization of RNA-protein cross-link sites on the RNA alone should fortunately yield enough topographical information to locate the protein in the RNA structure, and subsequently to correlate its position with other data, such as models derived from immune electron microscopical (35,36) or neutron scattering studies (37).

The second point to be made concerns the nature of the cross-link sites and the identities of the cross-linked proteins which we have been able to analyse. It has already been noted that the spectrum of proteins for which cross-link sites on both 23S RNA (1) and 16S RNA have been found does not correspond to the overall pattern of iminothiolane-induced cross-linking. No sites have been found for the most strongly cross-linked proteins (e.g. S3, S4, or L2), and we have suggested (1,13) that this reflects the highly selective character of the partial digestion reaction with ribonuclease T1. This is particularly noticeable in this series of experiments, in which the most predominant cross-links found were to the region of RNA from positions 580-760. In our previous studies on base-paired interactions in 16S RNA (38), and on the localisation of intra-RNA cross-links (13), this same region also occurred most frequently in the partial ribonuclease T1 digests. Our recent experiments on the intra-RNA cross-linking reaction induced by nitrogen mustard (12) have shown clearly that, if a double-strand specific nuclease is used for the partial digestions, a completely different spectrum of cross-links can be observed. The implication here is that a large number of iminothiolane-induced RNA-protein cross-links in both 30S and 50S subunits still remain to be discovered.

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