Dependence of the 16S rRNA Decoding Region Structure on Mg^{2+}, Subunit Association, and Temperature†

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ABSTRACT: The effects of Mg^{2+} concentration, subunit association, and temperature on the structure of 16S rRNA in the *Escherichia coli* ribosome were investigated using UV cross-linking and gel electrophoresis analysis. Mg^{2+} concentrations between 1 and 20 mM and temperatures between 5 and 55 °C had little effect on the frequency of 12 of the 14 cross-links in 30S subunits and modest effects on the same cross-links in 70S ribosomes. In contrast, two cross-links, C967 × C1400 and C1402 × C1501, involving rRNA in the decoding region are present in 30S subunits only above 3 mM Mg^{2+}, increase in frequency at higher Mg^{2+} concentration, and are both more frequent when 50S subunits are included in the reactions. In 70S ribosomes, the cross-link C1402 × C1501 increases but the cross-link C967 × C1400 decreases at higher Mg^{2+} concentrations. One cross-link, C1397 × U1495, is detected only in 70S ribosomes and decreases in frequency as Mg^{2+} concentration is increased. An additional cross-link, A1093 × C1182, decreases upon subunit association. The cross-link frequency differences indicate that the arrangement of the decoding region of the 16S rRNA, but not in the rest of the subunit, is readily altered by Mg^{2+} ions and subunit association.

The size, structural complexity and subunit organization of the ribosome must somehow be related to the processes it carries out, in conjunction with multiple factors, during protein synthesis. The fact that distinctly different functions of initiation, elongation, and termination need to be performed by the ribosome during each round of protein synthesis may have necessitated its evolution and present structure; alternatively, the ribosome may need to undergo significant structural changes during the elongation cycle (*1, 2*).

As part of the approaches to understand how the ribosome functions, there has been a significant effort to determine how ribosome activity is affected in in vitro experiments by the ionic environment. A ratio of $[Mg^{2+}]/[M^+] = 0.1$, where M^+ is K^+ or NH_4^+ in the range 100−200 mM, is important for efficient subunit activation and poly (U)-directed nonenzymatic Phe-$tRNA^{Phe}$ binding (*3*−*5*). At ratios less than this, reversible inactivation of 30S subunits occurs (*4*). Optimal translational accuracy and efficiency were also achieved at Mg^{2+} concentrations around 5 mM in the presence of 100 mM NH^+ or K^+, by the addition of polycations such as spermine and spermidine (*6, 7*) and by the addition of Ca^{2+} (*7*). These counterion conditions are thought to more closely mimic in vivo conditions. These experiments designate optimum in vitro translation conditions that are markedly different from the optimum tRNA-binding conditions. The connection between the ribosome functional properties and the ionic environment is likely to lie in the ribosome structure itself since even the simplest of the processes (i.e., tRNA binding to the P-site) show dependencies that are not predicted by electrostatic considerations. It has been suggested that the higher Mg^{2+} concentrations that favor tRNA binding do not favor the necessary ribosome conformation or the correct flexible interaction between subunits needed during translation (*6, 8*).

UV cross-linking allows the identification of nucleotides in proximity to one another in compactly folded RNA molecules, and we have used this to identify 13 intramolecular RNA cross-links in 16S rRNA in the isolated 30S subunit (*9*). These reflect both details of the geometrical structure around secondary structure regions as well as the tertiary folding of 16S rRNA. The presence of the cross-links was determined by a gel electrophoresis technique that allows an estimate of their frequency as well as allowing their separation for subsequent sequence analysis by primer extension experiments. Importantly, the methodology provides a means of measuring the structural stability of specific regions in 16S rRNA because changes in the frequency of a particular cross-link must reflect underlying structural changes.

In the present experiments, the pattern and frequency of cross-links in 16S rRNA in 30S and 70S ribosomes was determined under different Mg^{2+} concentrations and temperatures to determine the general flexibility of the rRNA and to determine whether subunit association alters the 16S rRNA structure. We find that most of the 16S rRNA is insensitive to changes in Mg^{2+}, temperature, or subunit association. However, there are significant changes in the conformation of the 16S rRNA decoding region under these same conditions, that may indicate a fundamental property of the 16S rRNA three-dimensional arrangement.

EXPERIMENTAL PROCEDURES

Preparation of Ribosomes and UV-Cross-Linking Procedures. Escherichia coli 70S ribosomes and ribosomal

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subunits were prepared according to Makhno et al. (*10*) and were stored at −85 °C in activation buffer [20 mM Tris-HCl, pH 7.5, 200 mM NH_4OAc, 20 mM $Mg(OAc)_2$, and 4 mM β-mercaptoethanol]. 30S subunits were prepared in solutions containing from 0.5 to 50 mM Mg^{2+} with 20 mM Tris-HCl, pH 7.5, 100 mM NH_4Cl and 4 mM β-mercaptoethanol. 30S and 50S subunits (at equal molarity) were mixed in solutions containing similar Mg^{2+} concentrations; these are referred to as "70S ribosomes" in all cases, even though subunit association is incomplete below 5 mM Mg^{2+} (*5*). Samples for the Mg^{2+} study were incubated for 30 min in the appropriate Mg^{2+} concentration at 37 °C, placed on ice for 10 min, and irradiated at 4 °C. Irradiation was with a 312 nm trans illuminator for 20 min in a quartz cuvette with continuous stirring, as previously described (*9*). Sample concentrations were usually 1 μg/μL, except samples for preparative separation were irradiated at 6 μg/μL. Samples for the temperature study were prepared in 20 mM Tris-HCl, pH 7.5, 10 mM Mg^{2+}, 100 mM NH_4Cl, and 4 mM β-mercaptoethanol, activated for 30 min at 37 °C, and placed on ice. Temperature samples were then preincubated (5 min) at the chosen temperatures (4−55 °C) and irradiated at the chosen temperature. After irradiation, RNA was recovered from the samples by proteinase K digestion (1 mg/mL final concentration for 30 min at 37 °C) in the presence of 2% SDS and 20 mM EDTA, phenol extraction, and ethanol precipitation. The RNA was dephosphorylated with shrimp intestinal phosphatase, purified by proteinase K digestion, phenol extraction, and ethanol precipitation. 16S rRNA was then isolated on a 1% agarose gel before 5′-end-labeling with [γ-^{32}P]ATP by T4 polynucleotide kinase.

Cross-linked 16S rRNA was separated by gel electrophoresis on gels made with 3.6% acrylamide:bis-acrylamide (70:1), 8.3 M urea, and BTBE buffer (30 mM bis-Tris, 30 mM boric acid and 2.5 mM EDTA, pH 6.8) as previously described (*9*). For purification, the location of the bands containing un-cross-linked and cross-linked 16S rRNA were detected with a phosphorimager. Bands were cut out and eluted by ultracentrifugation through cushions containing 2 M CsCl and 0.2 M EDTA, pH 7.4, for 16 h at 40 000 rpm (*11*). RNA pellets were redissolved in 250 μL of H_2O, phenol extracted, and reprecipitated before further analysis.

Determination of Cross-Linked Sites Identity and Frequency. Cross-linking sites in separated 16S rRNA were found by primer extension analysis using 11 DNA primers complementary to regions throughout 16S rRNA (*9*, *12*). This allows reading of the 16S rRNA, except for the 3′-terminal 40 nucleotides. Frequency of cross-linking was determined from phosphorimager data (ImageQuant, Molecular Dynamics Inc.) of duplicate independent experiments. To normalize for RNA loading, cross-link band intensity was referenced to the same cross-link band (C54 × A353) in each respective lane. The C54 × A353 band showed less than 10% variance in all lanes when referenced to the un-cross-linked 16S rRNA parent band in the same lane.

RESULTS

Comparison of Cross-Links in 16S rRNA in 30S and 70S Ribosomes. The separation of 16S rRNA molecules containing UV cross-links induced in 30S and 70S ribosomes is shown in Figure 1. The identities of the cross-links made

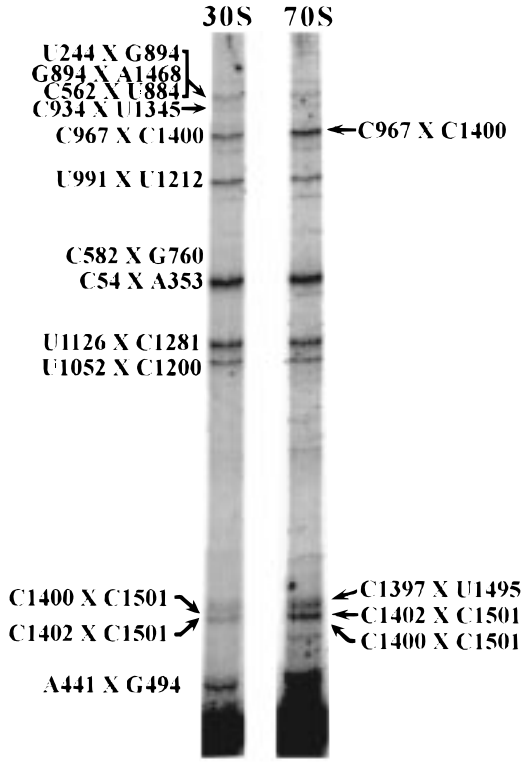

FIGURE 1: Comparison of cross-links identified in 16S rRNA in 30S subunits and 70S ribosomes in solutions containing 20 mM Mg^{2+}. 16S rRNA from irradiated 30S subunits or 70S ribosomes was isolated and purified on agarose gels, ^{32}P-labeled and separated under denaturing conditions on polyacrylamide gels. Cross-links were subsequently identified by primer extension experiments. Cross-links identified in 16S rRNA in 30S subunits are listed on the left of the gel. Cross-links indicated on the right of the gel show frequency increases upon subunit association and the cross-link C1397 × U1495 is not detected at all in the 30S subunit.

in the 30S subunit were determined previously by primer extension experiments on RNA isolated from each of the bands that appear in the gel (*9*). Assignments were based on the occurrence of pairs of primer extension stops in specific fractions that must arise from a particular type of cross-linked molecule. One additional cross-link (C1400 × C1501) has been added to the list of cross-links formed in the 30S subunit because molecules from the indicated band produced reverse transcription stops indicating cross-links at C1400 as well as C1402. RNA in this fraction also produced a stop indicating a cross-link at C1501. Primer extension experiments have been repeated for the cross-links made in 70S ribosomes and have been found to be the same as those found in the 30S experiments except for an additional cross-link, C1397 × U1495 in the 16S rRNA decoding region (see below). The frequency of 11 of the cross-links does not change significantly during subunit association; however, the cross-links C967 × C1400 and C1402 × C1501 increase in frequency. Another cross-link, A1093 × C1182, decreases upon subunit association; this is not well seen in Figure 1 but can be seen as a band above C1402 × C1501 in Figures 2 and 3. Three additional reverse transcription stops at positions A353, U534, and U793 were identified during the analysis of the 70S cross-linked samples. These should be parts of cross-links because they appear in RNA that has reduced gel electrophoretic mobility. How-

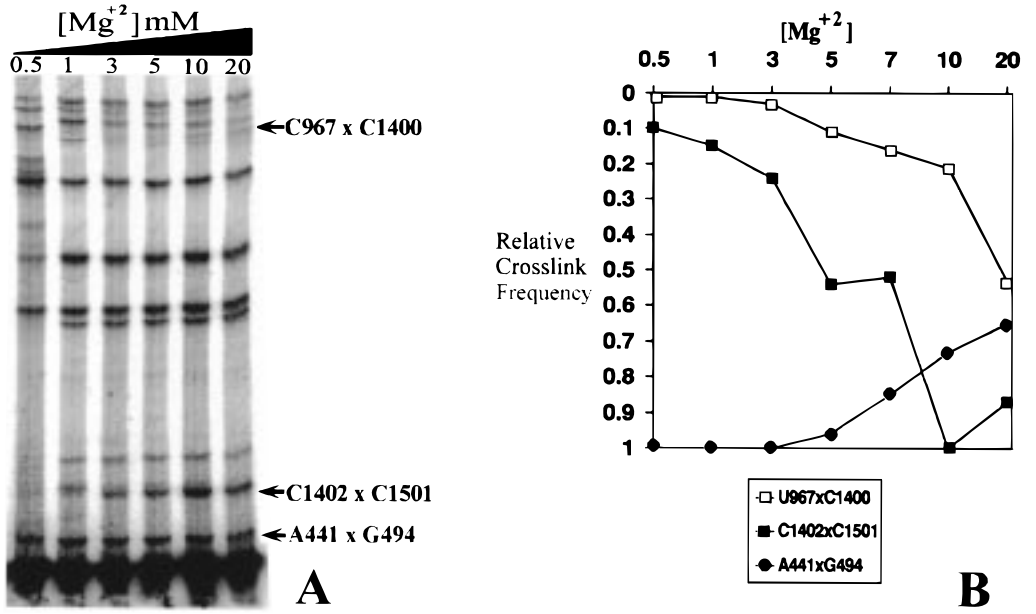

FIGURE 2: Effect of Mg^{2+} on cross-link frequency in 16S rRNA in 30S subunits detected by gel electrophoresis analysis. (A) Effects of varying Mg^{2+} concentrations from 0.5 to 20 mM on cross-link frequency. The affected cross-links are indicated with arrows. (B) Graph of relative cross-link frequency verses Mg^{2+} concentration. Values of 1 represents the highest observed frequency of the noted band; a value of 0 represents no observable cross-link. Relative frequencies were calculated as a fraction of the highest frequency observed.

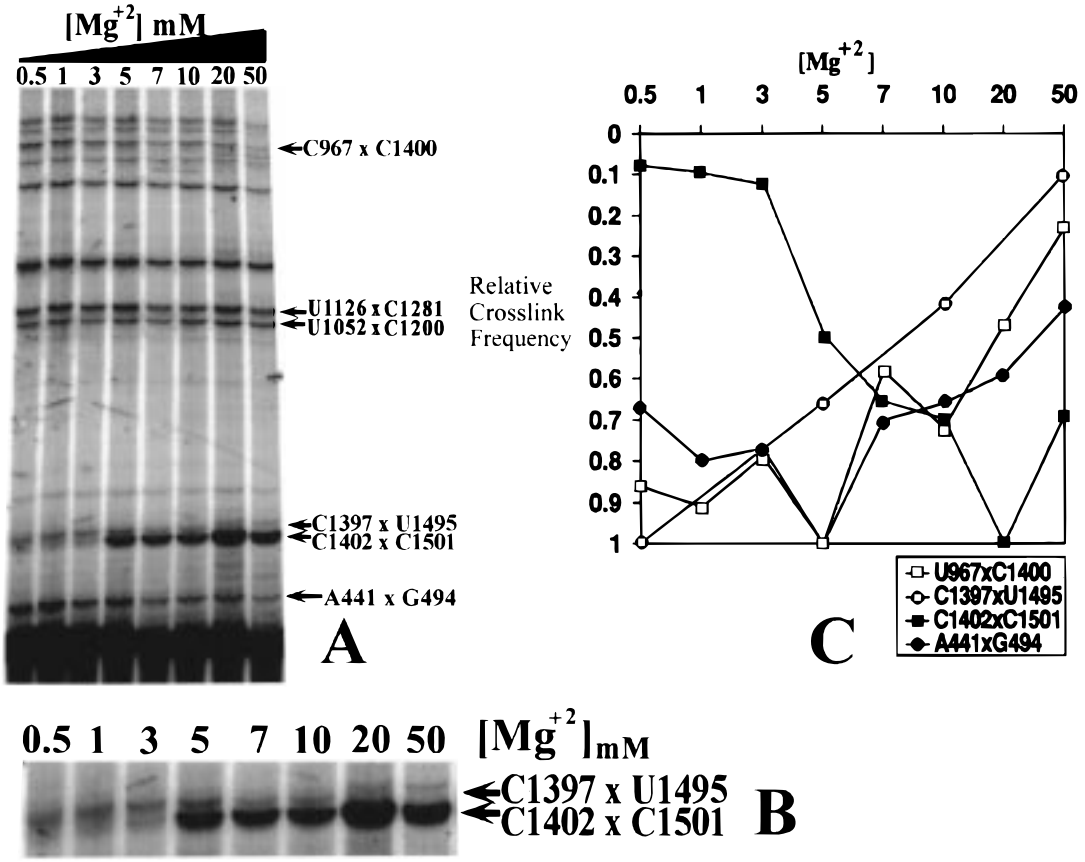

FIGURE 3: Effect of Mg^{2+} on cross-link frequency in 16S rRNA in 70S subunits. (A) Effects of varying Mg^{2+} concentrations from 0.5 to 50 mM on cross-link frequency. The affected cross-links are indicated with arrows. (B) Enlargement of the bottom part of the gel in panel A, showing the cross-links found in the decoding region. The cross-links affected by a change in Mg^{2+} are noted with arrow. The band appearing above the C1397 × U1495 cross-link in the 20 mM and 50 mM Mg^{2+} sample lanes is unidentified. (C) Graph of relative cross-link frequency verses Mg^{2+} concentration. Cross-links U1126 × C1281 and U1052 × C1200 (not shown) vary according to the text. Values of 1 represents the highest observed frequency of the noted band; a value of 0 represents no observable cross-link. Relative frequencies were calculated as fractions of the highest frequency observed.

ever, no reverse transcription stops that would indicate the other part of the cross-linking site were discovered in these molecules. Molecules containing U793, as one-half of a cross-link, had been reported previously in 30S (*9*).

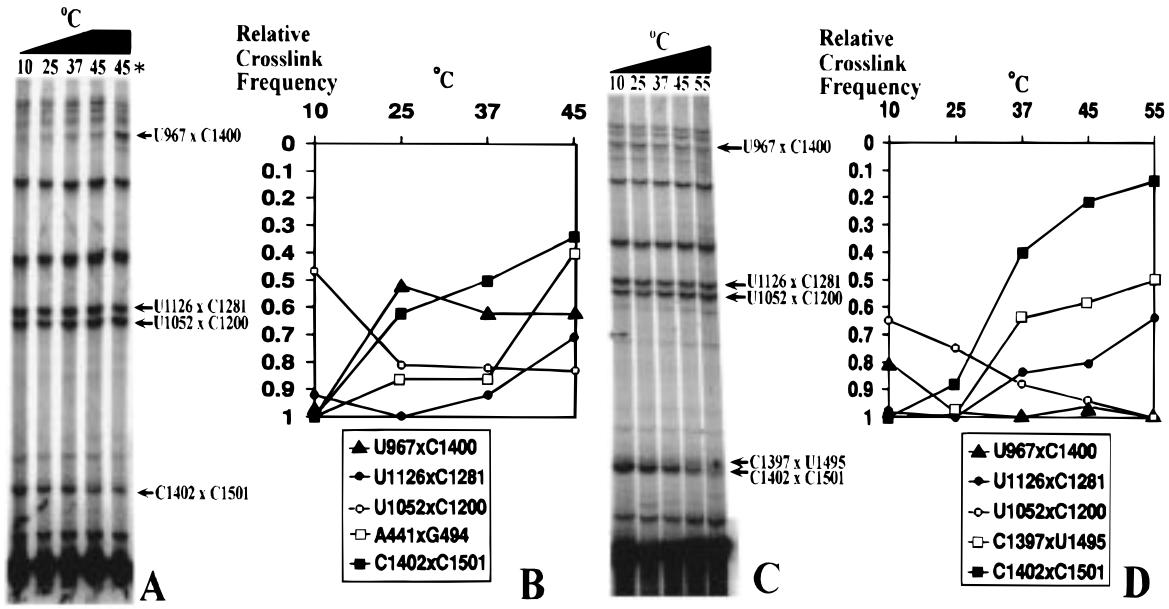

FIGURE 4: Effects of temperature on cross-linking frequency in 16S in 30S subunits and 70S ribosomes. (A) Frequency of cross-linking in 16S rRNA in 30S subunits at different temperatures detected by gel electrophoresis analysis. Lane 45* is a cross-linking performed at 45 °C, with 50 mM Mg^{2+} added to the sample prior to UV irradiation. (C) Frequency of cross-linking in 16S rRNA in 70S ribosomes at different temperatures detected by gel electrophoresis analysis. Affected cross-links are indicated by arrows. (B) and (D) are the corresponding graphs showing the relative cross-link frequency verses temperature in 30S and 70S ribosomes. Values are relative cross-linking frequency normalized so that a value of 1 represents the highest observed frequency of the noted band; a value of 0 represents no observable cross-link.

Mg^{2+}-Induced Changes in Cross-links in 16S rRNA in 30S and 70S Ribosomes. The 30S subunit was first studied by Mg^{2+} titration experiments over a concentration range of 0.5 to 20 mM Mg^{2+} (Figure 2) and 50 mM Mg^{2+} (data not shown) in the presence of 20 mM Tris, HCl, pH 7.5, 100 mM NH_4^+, and 4 mM β-mercaptoethanol to determine if there were changes in identity or frequency of the cross-links. At 0.5 mM Mg^{2+}, many cross-links were decreased in frequency and there was the appearance of several new cross-link species which have not been characterized. At Mg^{2+} concentrations of 1 mM and more, three cross-links were affected: C967 × C1400 increased continuously in frequency with increasing Mg^{2+} concentration; the frequency of C1402 × C1501 increased to a maximum at 10 mM Mg^{2+} with a slight decrease above 10 mM Mg^{2+}; the frequency of A441 × G494 decreased in frequency as Mg^{2+} concentration was increased to 20 mM. The relative frequency shifts for the affected cross-links in 30S subunits are shown in Figure 2b.

The cross-linking pattern in the 16S rRNA from samples containing 30S and 50S subunits in different Mg^{2+} concentrations is shown in Figure 3. In these experiments, 30S and 50S were mixed in solutions containing different Mg^{2+} concentrations; there should not be stable subunit association at Mg^{2+} concentrations of 1 mM or less and complete subunit association should occur in solutions of 5 mM or more (*5*). The cross-link C967 × C1400 was present even at 0.5 mM Mg^{2+} concentration, showed an increase to a maximum at 5 mM, and decreased in frequency above 5 mM. In contrast, the cross-link C1402 × C1501 behaved as it did in 30S subunits, appearing at 5 mM, increased to a maximum at 20 mM Mg^{2+}, and decreasing above 20 mM Mg^{2+} (data shown in Figure 3 only). U1052 × C1200 and U1126 × C1281 both showed gradual intensity increases with increasing Mg^{2+} concentration, and A441 × G494 showed a maximum at 5 mM Mg^{2+}.

One cross-link was identified in 70S ribosomes that is not present in 30S subunits. In samples containing 30S and 50S subunits at low Mg^{2+} concentrations, gel electrophoresis showed a second band adjacent to the band containing the cross-links C1402 × C1501 and C1400 × C1501 (Figure 3B). The second band decreased in frequency and was eclipsed by its neighboring band at higher Mg^{2+} concentrations. Isolation of the band from samples made in low Mg^{2+} buffers followed by reverse transcription analysis allowed its identification as C1397 × U1495 (results not shown). Furthermore, the frequency of cross-linking (although low in all cases) is determined by the Mg^{2+} concentration and not the subunit activity since inactive subunits diluted into a buffer containing 20 mM Mg^{2+} in the cold contained the same frequency of the C1397 × U1495 cross-link as active subunits in the same buffer (results not shown).

Temperature-Induced Changes in Cross-links in 16S rRNA in 30S and 70S Ribosomes. Samples were UV cross-linked at temperatures up to 55 °C in 10 mM Mg^{2+} to determine their thermal stability. In 30S subunits, five of the cross-links showed significant temperature dependence over the range investigated (Figure 4). Four of these gradually decreased in frequency as temperature increased, and one, C1052 × C1200, increased by doubling in frequency between 10 and 25 °C. Another 30S sample was cross-linked at 45 °C in the presence of 50 mM Mg^{2+} to determine if higher Mg^{2+} concentrations would prevent the decrease in band frequency (Figure 4A, lane 45*). In this sample, C967 × C1400 increased dramatically, resembling the 30S sample cross-linked in 50 mM Mg^{2+} at 4 °C and 70S ribosomes

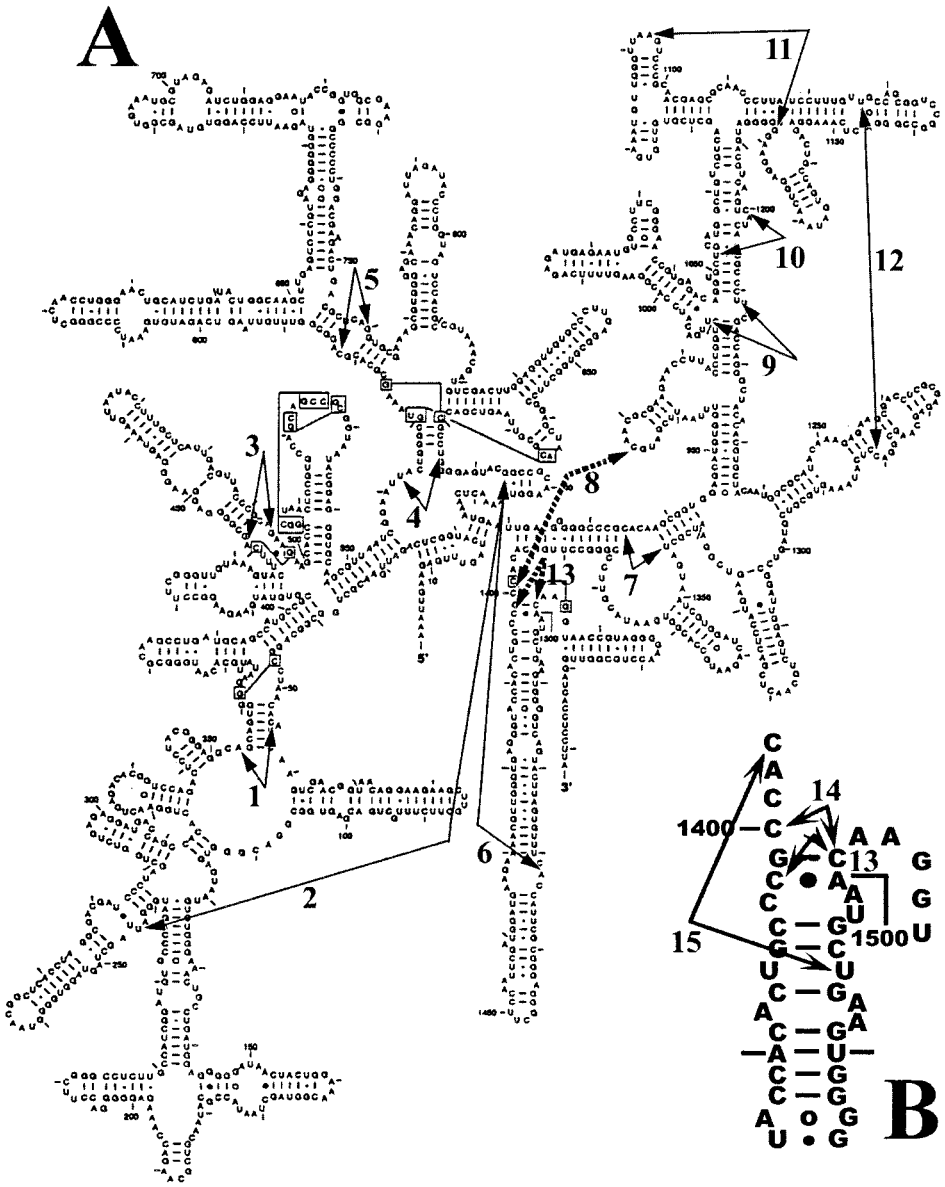

FIGURE 5: Secondary structure of 16S rRNA (Gutell et al., 1994) with cross-links identified in 30S and 70S ribosomes. (A) Thirteen cross-links previously identified (10) in 16S rRNA in 30S subunits. Numbering is as follows: 1 (C54 × A353), 2 (G894 × U244), 3 (A441 × G494), 4 (C562 × U884), 5 (C582 × G760), 6 (G894 × A1468), 7 (C934 × U1345), 8 (C967 × C1400), 9 (U991 × U1212), 10 (U1052 × C1200), 11(A1093 × C1182), 12 (U1126 × C1281), 13 (C1402 × C1501), 14 (C1400 × C1501), 15 (C1397 × U1495). Cross-link 14, determined in this study (panel B) is formed in 30S subunits and 70S ribosomes. Cross-links 8 and 13, indicated with dashed arrows, increase in frequency upon subunit association. (B) Enlargement of the decoding region in 16S rRNA. The additional cross-link seen in 70S ribosomes, C1397 × U1495, is included.

cross-linked in 10 mM Mg^{2+} at 4 °C. However, the remaining cross-links were not affected by the high Mg^{2+} concentration at 45 °C.

Changes in the frequency of cross-links in 16S rRNA in 70S ribosomes at different temperatures were also determined (Figure 4, panels C and D). The relative frequencies of only four cross-links are sensitive to temperatures up to 55 °C in 70S ribosomes. C1402 × C1501 and U1126 × C1281 decrease in frequency at higher temperatures, while U1052 × C1200 increases in intensity with temperature. These three cross-links respond similarly to temperature in 30S and 70S ribosomes. C1397 × U1495, seen only in 70S ribosomes, also decreases as the temperature increases. C967 × C1400, in contrast to its behavior in 30S subunits, is relatively insensitive to temperatures up to 55 °C.

DISCUSSION

The presence of a UV cross-link between a pair of nucleotides should be an indicator of a close distance relationship within the ribosome since cross-linking joins nucleosides by single covalent bonds or cyclobutane bridges (*13*). Because the lifetime of the monophotonic activated state is several hundred nanoseconds to a microsecond (*14*, *15*), structural fluctuations may occur so that two sites, which are normally separated by more than a covalent bond length or are in incorrect geometrical alignment, may come close together or may come in correct alignment and then can be captured as a cross-link. One of the reasons for the present experiments was to determine if the frequencies of cross-linking would be very sensitive to Mg^{2+} concentration and

temperature, which are two perturbations known to alter RNA structure and dynamics (*15, 16*). For the majority of the cross-linking sites, this is not the case, so we conclude that there are not changes in conformation or dynamics large enough to affect the cross-linking efficiency within the Mg^{2+} and temperature ranges we have been able to test. The exceptions to this are several cross-links in the 16S rRNA decoding region. There are enough cross-linking sites that follow this pattern to conclude that this is a general property of the 30S subunit—the decoding region is specifically subject to greater conformational freedom and responsiveness than the rest of the subunit.

Four cross-links in the 16S rRNA are affected by subunit association. C967 × C1400 and C1402 × C1501 increase in frequency upon subunit association, indicating alterations in the positions or dynamics of the involved nucleotides. An additional cross-link, C1397 × U1495 is induced by subunit association. The cross-link C967 × C1400 occurs between the end loop of the secondary structure element helix 31 (*17*) contained within nucleotides 960−975 and nucleotide C1400, so it must involve an interaction or very short distance between these sites. The cross-links C1402 × C1501 and C1397 × U1495 all involve nucleosides that are in the secondary structure element H44a (*17*), involving base-pairing interactions between nucleotides in the intervals 1399−1407 and 1494−1504 (see Figure 5). UV irradiation in normal RNA (*18*) or in RNA containing 4-thiouridine (*19*) does not usually produce cross-links between nucleotides that are situated opposite and adjacent to one another within double-stranded helical RNA, and no other cross-links within regular double-stranded structures were seen in this study. The presence of these cross-links in the 44a region therefore suggests the presence of unusual geometry at least under the conditions in which these experiments have been done. Chemical modification data on 16S in 30S subunits and 70S ribosomes shows only minor changes upon subunit association [protections at A790, G791, and A909 (*20*)]. Therefore, there are probably no base-pairing changes in this region during subunit association. The cross-links C967 × C1400 and C1402 × C1501 are near nucleotides G966 and G1401 that are chemically reactive in the 70S ribosome and become chemically unreactive when tRNA is in the P site (*21*). Methylation of either G966 (positions N1 and N2 with kethoxal) or G1401 (N7 with dimethyl sulfate) interferes with tRNA binding (*21*), indicating that the two regions are intimate parts of the tRNA binding site and are not in direct contact with the large subunit. Thus, the induction and change in intensity of these cross-links is due indirectly to subunit association.

One additional cross-link A1093 × C1182 is affected by subunit association, decreasing by half in frequency. We have recently estimated a distance of not more than 15 Å between C1092 and G9 (Juzumiene and Wollenzien, unpublished data), indicating that the 16S rRNA region around nt 1100 probably is located at the subunit interface. The change in frequency of this cross-link during subunit association is consistent with this location.

In 30S subunits, three cross-links were affected by changes in Mg^{2+} concentration. The cross-links C967 × C1400 and C1402 × C1501 increase in frequency and the cross-link A441 × G494 decreases in frequency with increasing Mg^{2+}. There is an alteration in the Mg^{2+} response for some of the cross-links in the 70S ribosomes. C1402 × C1501 behavior mimicked that in 30S subunits. However, for the cross-links C967 × C1400 and A441 × G494, a maximum frequency of cross-linking occurred at 5 mM Mg^{2+} rather than there being the monotonic increase in frequency with increasing Mg^{2+} seen in the 30S subunit. C967 × C1400 was present even at 1 mM Mg^{+2} concentrations in mixtures of 30S and 50S subunits, but not in 30S subunits. Thus, the presence of the 50S subunit dampens the Mg^{2+} response for these two cross-links. The responses to Mg^{2+} indicate that 0.5 mM Mg^{2+} may be sufficient to stabilize much of the global structure of the ribosome, but not the essential structural elements needed for the active conformation. A fourth cross-link that shows a response to Mg^{2+} concentration is C1397 × U1495. It is detected only when 30S subunits were incubated in the presence of 50S subunits and was most intense at the lowest Mg^{2+} concentration (0.5 mM) studied. The fact that it is seen only in the presence of 50S subunits suggests that even though there are not stable interactions between subunits at this Mg^{2+} concentration, there must be transient contacts or unstable interactions that alter the 30S structure.

Temperature studies on 30S subunits and 70S ribosomes indicate that C967 × C1400 and A441 × G494 both decreased in intensity as temperature rose in 30S subunits, but were relatively insensitive to temperature in 70S ribosomes. This indicates the additional stability induced in 16S rRNA at these sites by subunit association. The C1397 × U1495 and C1402 × C1501 cross-links showed a strong temperature dependence, decreasing in frequency with increasing temperature. U1126 × C1281 and U1052 × C1200 also showed intensity changes with rising temperature; the former increased in intensity, while the latter decreased. The increase in U1052 × C1200 cross-link may be explained because of the arrangement of domain III in 16S rRNA. The melting of the domain III tertiary structure, reflected in the decrease in the frequency of U1126 × C1281, which is a tertiary contact, requiring a specific arrangement of domain III, may allow a more favorable geometry for the region containing the interaction between U1052 and C1200 or may allow it to be more dynamic.

The interactions in the decoding region that result in the cross-links C1402 × C1501, C1400 × C1501, and C1397 × U1495 may be mutually exclusive. It is possible that only one of the cross-links C1402 × C1501 or C1397 × U1495 is conformationally permitted at a time, since these two cross-links show opposite responses to changes in Mg^{2+} concentration. This would result in a population of separate conformational states at intermediate Mg^{2+} concentrations. C1400 must also be available, either some of the time or in some ribosomes for cross-linking to C967. In addition, it is known that C1400 is photo-cross-linked to the tRNA anticodon loop of $tRNA^{Val}$ (*22*) and $tRNA^{Ser}$ (*23*) when bound to the P site in the ribosome. All of these observations suggest that this region of the 30S subunit has a potential for a number of alternate conformations. A UV cross-link between nucleotides in the intervals 1402−1403 and 1498−1501 was described by Doring et al. (*24*), which is consistent with the cross-link C1402 × C1501 reported here. However, Doring et al. reported the presence of the cross-link in 30S subunits but not in the tight couple 70S ribosomes which they studied, indicating that mRNA and tRNA binding, the process of

arresting translation during ribosome isolation, or other factors may interfere with the C1402 × C1501 cross-link.

The cross-linking frequency responses described here indicate new aspects of the 16S rRNA three-dimensional arrangement. Much of the 16S rRNA must be quite highly constrained with respect to molecular motions given the lack of responsiveness to Mg^{2+} concentration changes and temperature. The exceptional region is the decoding region itself. Furthermore, subunit association greatly alters the 16S rRNA decoding region properties. This suggests that there is some type of structural reorganization upon subunit association that may be an important step in activating the 30S subunit for its role in protein synthesis.

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