Multiple binding modes of substrate to the catalytic RNA subunit of RNase P from *Escherichia coli*

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ABSTRACT

M1 RNA that contained 4'-thiouridine was photochemically cross-linked to different substrates and to a product of the reaction it governs. The locations of the cross-links in these photochemically induced complexes were identified. The cross-links indicated that different substrates share some contacts but have distinct binding modes to M1 RNA. The binding of some substrates also results in a substrate-dependent conformational change in the enzymatic RNA, as evidenced by the appearance of an M1 RNA intramolecular cross-link. The identification of the cross-links between M1 RNA and product indicate that they are shared with only one of the three cross-linked E-S complexes that were identified, an indication of noncompetitive inhibition by the product. We also examined whether the cross-linked complexes between M1 RNA and substrate(s) or product are altered in the presence of the enzyme's protein cofactor (C5 protein) and in the presence of different concentrations of divalent metal ions. C5 protein enhanced the yield of certain M1 RNA-substrate cross-linked complexes for both wild-type M1 RNA and a deletion mutant of M1 RNA ($\Delta[273-281]$), but not for the M1 RNA-product complex. High concentrations of Mg^{2+} increased the yield of all M1 RNA-substrate complexes but not the M1 RNA-product complex.

Keywords: E-S complexes; M1 RNA; photochemical induced cross-links; substrate-induced fit

INTRODUCTION

Ribonuclease P (RNase P) is an essential and ubiquitous ribonucleoprotein enzyme that catalyzes the 5' maturation of all precursor transfer RNAs (ptRNAs). Precursors to *Escherichia coli* 4.5S RNA, tm RNA (10Sa RNA), *Salmonella typhimurium* his operon mRNA, and several bacteriophage encoded RNAs are also substrates for *E. coli* RNase P (Bothwell et al., 1976a, 1976b; Guerrier-Takada et al., 1988; Mans et al., 1990; Alifano et al., 1994; Komine et al., 1994; Hartmann et al., 1995). *E. coli* RNase P is composed of a single catalytic RNA (M1 RNA, 377 nt) and a single protein cofactor (C5 protein, 119 amino acids) (Guerrier-Takada et al., 1983). For the *E. coli* ptRNA substrates, important features for recognition by M1 RNA in vitro include the T stem/loop, the length of the acceptor stem, and the canonical 3' CCA terminus (McClain et al., 1987; Kirsebom & Vioque, 1996). M1 RNA is believed to have the capacity to recognize its varied substrates through

multiple binding modes (Guerrier-Takada et al., 1989; Knap et al., 1990; Guerrier-Takada & Altman, 1992). Such multiple binding modes could reflect an ability of this enzyme to assume more than one conformation on the pathway to an optimally active conformation that would ultimately lead to a cleavage event. It has been proposed that the catalytic RNA component of *Bacillus subtilis* RNase P can assume two conformers to which a single substrate can bind, leading to productive enzyme-substrate complexes (Beebe & Fierke, 1994). Chemical probing experiments using Fe-EDTA and other methods have noted changes in M1 RNA structure following association with substrate, which could be indicative of an induced-fit type mechanism leading to the formation of a productive complex (Knap et al., 1990; Guerrier-Takada & Altman, 1993; Ciesiolka et al., 1994; Westhof et al., 1996).

The divalent metal ion and basic protein cofactors of RNase P may have key roles in modulating conformational changes of M1 RNA, both prior to and following substrate binding. An increase in Mg^{2+} concentration or the presence of C5 protein can alter the global architecture of M1 RNA (Westhof et al., 1996). Further, deleterious mutations in M1 RNA can be rescued by

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high, nonphysiological salt concentration or C5 protein, indicative of their ability to assist M1 RNA in structure formation (Lumelsky & Altman, 1988; Gopalan et al., 1994). C5 protein also increases the binding affinity of M1 RNA in a substrate-dependent manner (Peck-Miller & Altman, 1991; Gopalan et al., 1994, 1997).

To explore both how M1 RNA interacts with its RNA substrates and a tRNA product and how structural changes in M1 RNA are elicited by their binding, we have used a photochemical cross-linking approach mediated by the nucleoside analog, 4'-thiouridine. Using this approach, we have also analyzed how specific intramolecular and intermolecular cross-linked complexes between M1 RNA and substrates, or the product of its cleavage reaction, might be changed by its two cofactors, divalent metal ions and C5 protein.

RESULTS

4'-thiouridine was incorporated into M1 RNA (M1-s⁴U RNA) by transcription in vitro (see Materials and Methods) and the relative activity of M1 RNA as a function of the degree of 4'-thiouridine incorporation was measured. The rate of cleavage of the substrate precursor tRNA^{Tyr} pTyr(ACCAUCA), which has the natural 3' terminal sequence ACCAUCA (where the 5' A is the discriminator base), was not diminished when up to 9% of its uridine was substituted with 4'-thiouridine (data not

shown). At levels of substitution greater than 9%, the rate of pTyr(ACCAUCA) processing by M1-s⁴U RNA was reduced. The efficiency of generating intermolecular E-S cross-links as a function of 4'-thiouridine incorporated in M1 RNA increased in a linear manner with increasing 4'-thiouridine incorporated up to 9% incorporation (data not shown).

Irradiation of M1-s⁴U RNA with 6.5% 4'-thiouridine yielded approximately 1–4% of total, intact M1 RNA in individual, intermolecular cross-linked complexes (see Fig. 1). No intermolecular or intramolecular cross-links were produced when M1 RNA without 4'-thiouridine was irradiated under the conditions we used (data not shown).

M1-s⁴U RNA cross-linked to pTyr(ACCAUCA) and mTyr(ACCA)

Irradiation of M1-s⁴U RNA in the presence of pTyr(ACCAUCA) yields two major intermolecular, cross-linked complexes (designated complexes 1 and 2 in Fig. 1); a single, minor intermolecular cross-linked complex (designated complex 3; most easily visible in Figs. 1B and 3A); and three intramolecular cross-linked complexes (see Fig. 1A–C). In contrast, irradiation of M1-s⁴U RNA in the presence of the natural cleavage product, mature tRNA^{Tyr}, [mTyr (ACCA)], yields a single, major intermolecular cross-linked complex (designated complex

FIGURE 1. Cross-linking of M1-s⁴U RNA or M1-s⁴U Δ[270–283] RNA under varying conditions. Products of a cross-linking reaction between M1-s⁴U RNA or M1-s⁴U Δ[270–283] RNA and pTyr(ACCAUCA) or mTyr(ACCAUCA), electrophoresed on a denaturing polyacrylamide/7 M urea gel (5% w/v). **A:** M1-s⁴U RNA (internally labeled with [α -³²P]-GTP) was irradiated 3 min (lane 1) on ice in the presence of C5 protein (lane 2); pTyr (lane 3); mTyr (lane 4); C5 protein and pTyr (lane 5); C5 protein and mTyr (lane 6); pTyr in 100 mM Ca²⁺ (lane 7); mTyr in 100 mM Ca²⁺ (lane 8). **B:** M1-s⁴U RNA (internally labeled with [α -³²P]-GTP) and pTyr were irradiated in 10 mM Mg²⁺ (lane 1); in 100 mM Mg²⁺ (lane 2); in the presence of C5 protein in 10 mM Ca²⁺ (lane 3); in the presence of C5 protein in 10 mM Mg²⁺ (lane 4). **C:** M1-s⁴U RNA (M1 RNA WT) or M1-s⁴UΔ[270–283] RNA (M1 RNA MT) and pTyr (end labeled with [³²P]pCp) were irradiated in 10 mM Mg²⁺, M1-s⁴U RNA (lane 1); in 10 mM Mg²⁺, M1-s⁴UΔ[270–283] RNA (lane 2); in 100 mM Mg²⁺, M1-s⁴UΔ[270–283] RNA (lane 3); in 10 mM Mg²⁺ and C5 protein present, M1-s⁴U RNA (lane 4); in 10 mM Mg²⁺ and C5 protein present, M1-s⁴UΔ[270–283] RNA (lane 5).

2/mTyr), as well as the three intramolecular cross-linked complexes (Fig. 1A, lanes 4 and 6).

Under certain conditions [10 mM or 100 mM Ca^{2+} in $1\times$ binding buffer (400 mM NH_4OAc , 10 mM HEPES, pH 7.5, 10 mM MgOAc , 5% v/v glycerol); see Materials and Methods] the two major intermolecular cross-linked complexes between M1-s⁴U RNA and pTyr (ACCAUCA) are approximately equal in intensity (Fig. 1A, lanes 3 and 7). Under the same conditions, the presence of C5 protein in the cross-linking reaction increases the yield of complex 1 approximately twofold with a commensurate decrease in the yield of complex 2 (Fig. 1A, compare lanes 3 and 5). In the presence of C5 protein, the cross-linked complex between M1-s⁴U RNA and mTyr undergoes no detectable change in relative intensity with respect to uncross-linked M1 RNA in the same experiment (Fig. 1A, compare lanes 6 and 8). This result is in contrast to the effect of C5 protein on the M1-s⁴U RNA–pTyr cross-linked complexes 1 and 2. All the cross-linked intermolecular complexes between M1-s⁴U RNA and pTyr (ACCAUCA) have catalytic activity, as documented below.

A high concentration of Ca^{2+} (100 mM in $1\times$ binding buffer) does not appear to increase the yield or change the ratio of the M1-s⁴U RNA–pTyr cross-linked complexes 1 and 2 (Fig. 1A, lane 3 versus lane 7). In contrast, 10 mM or 100 mM Mg^{2+} in $1\times$ binding buffer promotes complex 1 over complex 2 (Fig. 1B, lanes 1 and 2). Further, unlike C5 protein, the presence of 100 mM Mg^{2+} enhances the yield of both complexes 1 and 2. C5 protein promotes complex 1 over complex 2 equally as well in 10 mM Ca^{2+} or 10 mM Mg^{2+} (Fig. 1A, lane 5; Fig. 1B, lanes 3 and 4). In regard to the cross-linked complex between M1-s⁴U RNA and mTyr, a high concentration of Ca^{2+} (100 mM) does not appear to alter its amount nor does C5 protein, as stated above (Fig. 1A).

Complexes formed with a deletion mutant of M1 RNA, $\Delta[273-281]$

Cross-linking of deletion mutant, M1 RNA $\Delta[273-281]$, to pTyr (ACCAUCA) using UV (260 nm) irradiation revealed an altered pattern in the cross-links with respect to that obtained with wild-type M1 RNA and pTyr (ACCAUCA) (C. Guerrier-Takada, pers. comm.). This mutant has a defect in both K_M and k_{cat} in the processing of pTyr (ACCAUCA). We asked if the altered binding pattern could be observed in our assay as well. Cross-linking of the deletion mutant (M1-s⁴U $\Delta[273-281]$ RNA) and pTyr (ACCAUCA) results in five cross-linked complexes (Fig. 1C, lane 3). These complexes are produced only in the presence of 100 mM Mg^{2+} (Fig. 1C, compare lanes 2 and 3). C5 protein enhances the formation of two of the five cross-linked complexes observed (Fig. 1C, compare lanes 3 and 5). Thus, a high concentration of Mg^{2+} (100 mM in $1\times$ binding buffer)

acts to increase the M1-s⁴U $\Delta[273-281]$ RNA–pTyr interaction whereas C5 protein acts not only to increase M1 RNA–pTyr binding but also appears to select for the association of pTyr with specific conformers of M1 RNA.

Identification of locations of cross-linked nucleotides

The occurrence of a cross-link or photoadduct between two nucleotides of an RNA results in the pausing or termination of complementary strand synthesis by reverse transcriptase at the nucleotide preceding the one that is cross-linked, and is commonly referred to as a “stop” in reverse transcription.

Intermolecular cross-linked complex 1, [M1-s⁴U RNA–pTyr (ACCAUCA)], appears to have a single cross-link in M1-s⁴U RNA at nucleotide U257, as identified by primer extension (see Materials and Methods) with four primers complementary to different regions of M1 RNA (Fig. 2A, lane 2; Table 1). Complex 2, [M1-s⁴U RNA–pTyr (ACCAUCA)], also appears to have a single cross-link in M1-s⁴U RNA at nucleotide U294 (Fig. 2A, lane 1). Nucleotides U257 and U294 are located on opposing strands of an internal loop termed J15/16 of M1 RNA (see Fig. 6A,B). The presence of C5 protein in the cross-linking reaction does not alter the location of the M1-s⁴U RNA cross-links to pTyr in either complexes 1 or 2, only their relative ratio (Fig. 2A, lanes 4 and 5). The [M1-s⁴U RNA–mTyr] complex 2 has a single cross-link at U257 in M1-s⁴U RNA (Fig. 2A, lane 6).

Using primers complementary to the 3' end of tRNA^{Tyr} (primers tRNA#1 and #2; see Table 1), no apparent stops are observed in pTyr in complex 1 (Fig. 2B, lane 3). Likewise, there are no stops in complex 2/mTyr using these same primers (data not shown). With complex 2, reverse transcriptase stops at –5 and –8, an indication of cross-links at –6 and –9 (Fig. 2B, lane 2). Similarly, primer extension on complex 2 with primer tRNA#1 resulted in no stops for complex 1 and complex 2/mTyr, but stops for complex 2 at –5 and –8, an indication of cross-links at –6 and –9 (data not shown). We note that AMV reverse

TABLE 1. Oligonucleotides complementary to M1 RNA and tRNA^{Tyr} used in primer extension reactions.

Oligonucleotide	Spanning nucleotides	Sequence (5' to 3')
In M1 RNA		
M1#1	369–377	AGGTGAAAC
M1#2	356–377	AGGTGAAACTGACCGATAAGCC
M1#3	204–220	AGTTTACCGTGCCACGG
M1#4	119–138	GCTCTCTGTTGCACTGGTCG
In tRNA ^{Tyr}		
tRNA#1	81–88	TGATGGTG
tRNA#2	65–81	GGTGGGGGAAGGATTCG

FIGURE 2. Identification of site(s) of cross-links by primer extension and partial alkaline hydrolysis. **A:** Primer extension with primer M1#2 (5' end labeled, see Table 1) of cross-links between M1-s⁴U RNA and pTyr(ACCAUCA) (complexes 1 and 2) or mTyr(ACCAUCA) (complex 2/mTyr), electrophoresed on a denaturing polyacrylamide/7 M urea gel (8% w/v) beside a dideoxy sequencing ladder generated using primer M1#2, to identify site(s) of cross-link. Primer extension of samples of M1-s⁴U RNA cross-linked in the presence of pTyr, complex 2 (lane 1) and complex 1 (lane 2); mTyr, complex 2 (lane 3); pTyr and C5 protein, complex 2 (lane 4) and complex 1 (lane 5); mTyr and C5 protein, complex 2 (lane 6). **B:** Primer extension with primer tRNA#2 (5' end labeled, see Table 1) of pTyr(ACCAUCA) cross-linked in complexes 1 and 2 (cross-links between M1-s⁴U RNA and pTyr), electrophoresed on a denaturing polyacrylamide/7 M urea gel (8% w/v). The dideoxy sequencing ladder was generated using primer tRNA#2. Primer extension of pTyr not irradiated or control (lane 1); in complex 2 (lane 2); in complex 1 (lane 3). **C:** Partial alkaline hydrolysis of cross-linked complexes between M1-s⁴U RNA and pTyr(ACCAUCA) that is pCp end labeled (complexes 1 and 2), electrophoresed on denaturing polyacrylamide/7 M urea gel (20% w/v). Partial T1 digest of pTyr (ACCAUCApCp; 3' end labeled), used as marker (lane 1), which cleaves 3' of the G residue at position 66 in the T-loop. Partial alkaline hydrolysis of pTyr(ACCAUCA) as control (lane 2) and cross-linked complexes 2 (lane 3) and 1 (lane 4).

transcriptase extends to full length in the control and samples lanes, not stopping at +1 in pTyr(ACCAUCA) (Fig. 2B), indicating that cross-linked complexes 1 and 2 are between M1-s⁴U RNA and the substrate and not with a cleavage product of the substrate that may be in the complex. (When pTyr(ACC) was used as substrate, the same results were obtained as for pTyr(ACCAUCA); data not shown).

To identify where U257 in M1-s⁴U RNA cross-links with pTyr and mTyr, a partial alkaline hydrolysis assay of the complex was carried out because the shortest primer (tRNA#1) spans the extreme 3' end of the tRNA sequence, thereby precluding examination of the 3' terminal nucleotides for the presence of a cross-link (Fig. 2C; Table 1). Further, as there are no stops observed with use of the primers complementary to the tRNA sequence, this suggests that the cross-link is, indeed, at the 3' terminus.

Partial alkaline hydrolysis of an RNA species generates, on a denaturing gel, a ladder of fragments derived from breaks at every phosphodiester bond. Anomalous migration of a band can be used to assess, for example, if a nucleotide in the fragment has formed a cross-

link. We note that the conditions necessary for producing a partial alkaline hydrolysis ladder of pTyr results in complete hydrolysis of M1 RNA: any altered migration of fragments derived from pTyr in the cross-linked complex is due to cross-linking to a nucleotide in M1 RNA. The partial alkaline hydrolysis ladder of pTyr in complex 2 (Fig. 2C, lane 3) indicates that there are no apparent changes in migration of bands relative to the control, uncross-linked pTyr. Therefore, there are no cross-links at the 3' terminus of pTyr in complex 2. However, partial alkaline hydrolysis of complex 1 reveals an altered migration pattern of the hydrolyzed pTyr in this complex relative to the control ladder and to partially hydrolyzed pTyr in complex 2 (Fig. 2C, compare lane 4 to lanes 2 and 3). Based on the patterns of the partial T1 digest, which cleaves 3' of accessible guanosine residues in the T-loop of the tRNA and elsewhere, and partial alkaline hydrolysis ladders, (Fig. 2C, lanes 1 and 2), we concluded that nt U257 forms cross-links with nt 83–86 of pTyr in complex 1. Partial alkaline hydrolysis indicates that U257 cross-links with the 3' terminus of mTyr in complex 2/mTyr at nt 83–86 as well (data not shown). Interestingly, the cross-links be-

tween M1-s⁴U RNA and mTyr are identical to those between M1-s⁴U RNA and pTyr in complex 1.

The third intermolecular cross-linked complex between M1-s⁴U RNA and pTyr (complex 3), is approximately three- to fourfold lower in yield than either of the two major intermolecular cross-linked complexes described above (complexes 1 and 2; Fig. 3, lanes 2–4). The nucleotide in M1-s⁴U RNA involved in this cross-link is U69, a single nucleotide bulge present in the P4 pseudoknot (see Fig. 6). U69 cross-links to pTyr in the 5' leader segment at nt -7, -8, and -10 (data not shown).

Irradiation of M1-s⁴U RNA alone or in the presence of pTyr or mTyr produces the same three intramolecular cross-linked complexes that are observed irrespective of metal ion concentration or the presence of C5 protein (unpubl. results). The intramolecular cross-linked complexes are not observed in cross-linking reactions in which either pTyr or mTyr are the only labeled species, thus verifying that the cross-links in these complexes are indeed intramolecular (Fig. 1C, lanes 1–5).

There is no detectable change in the relative intensity of the intramolecular cross-linked complexes when M1-s⁴U RNA is cross-linked in the presence of either pTyr or mTyr versus M1-s⁴U RNA cross-linked in their absence (Fig. 1A,B).

Cross-links between M1-s⁴U RNA and the pTyr 3' end variant pTyr(AC)

A previous study using light of 260 nm to induce formation of photoadducts between nucleotides close to each other in space indicated that removal of the CCA 3' terminus in pTyr resulted in a change in the pattern of cross-links (Guerrier-Takada et al., 1989). However, the exact sequence change in the 3' end of pTyr that led to the altered pattern was not delineated. Kinetic studies of the importance of the 3' terminus in the cleavage reaction governed by M1 RNA have indicated that the 3' cytosine of the 3' terminal CCA sequence is critical: the K_M changes dramatically with removal of this base (Guerrier-Takada et al., 1984; Hardt et al.,

FIGURE 3. Cross-linking of M1-s⁴U RNA with pTyr having deletions at the 3' terminus in the presence of C5 protein. Products of cross-linking reaction between M1-s⁴U RNA (internally labeled with [α -³²P]-GTP) and pTyr having 3' end deletions, electrophoresed on a denaturing polyacrylamide/7 M urea gel (5% w/v). **A:** M1-s⁴U RNA irradiated 3 min in the presence of C5 protein alone (lane 1) and with pTyr having at the 3' terminal end the sequence, 5' to 3' (where the 5' A is the discriminator base) pTyr(ACCAUCA) (lane 2); pTyr(ACCA) (lane 3); pTyr(ACC) (lane 4); pTyr(AC) (lane 5); pTyr(A) (lane 6). **B:** M1-s⁴U RNA irradiated 3 min in the presence of C5 protein and pTyr having at the 3' end deletions in its sequence, electrophoresed on denaturing polyacrylamide/7 M urea gel (5% w/v). M1-s⁴U RNA irradiated in the presence of C5 protein and pTyr having at the 3' end the sequence, 5' to 3' (where the 5' A is the discriminator base) pTyr(ACCAUCA) (lane 1) or lacking that sequence, pTyr (lane 2). There is a nonspecific stop in the reverse transcription reactions at approximately the position of nt 286 in M1 RNA.

1995; Oh et al., 1998; see Table 2 in Altman & Kirsebom, 1999). In our experiments, M1-s⁴U RNA was irradiated in the presence of pTyr that sustained various deletions in its 3' terminal sequence, ACCAUCA. The sequential deletion of nucleotides from the 3' terminus of pTyr does not change the number of cross-linked complexes, but does change their migration pattern in gels when the 3' sequence changes from ACC to AC or A (Fig. 3A, lanes 5 and 6). Further, the effect of C5 protein in enhancing the yield of one intermolecular cross-linked complex over that of others is diminished with the deletion of the critical 3' cytosine residue [pTyr(ACC) to pTyr(AC)]: all three cross-linked bands now appear in approximately equal intensity (Fig. 3A,B). Nucleotide U257 in M1-s⁴U RNA forms a cross-link with pTyr(AC) in complex 1' but not to nt 83–86 in pTyr(ACCAUCA) as is the case with complex 1. Nucleotide U257 of complex 1' cross-links to the 5' leader between –14 and –16 in contrast to the U257-3' trailer cross-link in complex 1 (Fig. 4C, lane 2). Nucleotide U294 in M1-s⁴U RNA forms a cross-link with pTyr(AC) in complex 2', similar to that of complex 2 (M1-s⁴U RNA cross-linked to pTyr(ACC); Fig. 4A, see lanes 2, 4, 6, and 8). Nucleotide U294 cross-links to the 5' leader between –7 and –9 in complex 2' as is the case for complex 2 (Fig. 4C, lane 3).

In the third cross-linked complex (complex 3') between M1-s⁴U RNA and pTyr(AC), some of the sites of cross-linking between M1-s⁴U RNA U69 and the sub-

strate are the same or adjacent to those in complex 3 (nucleotides –7, –8, and –9; Fig. 4C, lane 4) and others are not (nt 1, –1, and –2); we note that cross-linked complexes 1', 2', and 3' are present on removal of the discriminator base, A (Fig. 3B, lane 2); however, the location of the cross-links in this complex were not determined.

A new intramolecular cross-link

In each of the three cross-linked complexes 1, 2, and 3 between M1-s⁴U RNA and pTyr (ACCAUCA), there appears to be only single intermolecular cross-links. In contrast, in all three cross-linked complexes 1', 2', and 3', which are between M1-s⁴U RNA and pTyr(AC), there is a single intramolecular cross-link identified by reverse transcription as well as a single intermolecular cross-link. The intramolecular cross-link is between M1-s⁴U RNA nt U104 and G110 (nucleotides in hairpins P8 and P9, respectively; Fig. 4B, lanes 2–4; see also Fig. 6). This intramolecular cross-link may account for the altered migration of the cross-linked complexes 1', 2', and 3' relative to that of M1-s⁴U RNA cross-linked with pTyr (ACC to ACCAUCA; Fig. 2). The new intramolecular cross-link appears to represent a specific conformational change induced only by the binding of a pTyr lacking the 3' end residues CA of ACCA. We note that this intramolecular cross-link is not observed by primer extension of the three intramolecular cross-

FIGURE 4. Primer extension of cross-linked samples between M1-s⁴U RNA and pTyr. **A:** Primer extension with primer M1#2 of cross-linked complexes between M1-s⁴U RNA and pTyr having 3' terminal deletions, electrophoresed on denaturing polyacrylamide/7 M urea gel (8% w/v). Primer extension of M1-s⁴U RNA cross-linked with pTyr(ACCAUCA) (lane 1), complexes 2 (lane 2) and 1 (lane 3); pTyr(ACCA), complexes 2 (lane 4) and 1 (lane 5); pTyr(ACC), complexes 2 (lane 6) and 1 (lane 7); pTyr(A), complexes 2' (lane 8) and 1' (lane 9). **B:** Primer extension with primer M1#1 of cross-linked complexes between M1-s⁴U RNA and pTyr having the 3' terminal sequence AC (where 5' A is the discriminator base), electrophoresed on denaturing polyacrylamide/7 M urea gel (8% w/v). Primer extensions of M1-s⁴U RNA irradiated 3 min (lane 1); complex 1' (lane 2); complex 2' (lane 3); complex 3' (lane 4). **C:** Primer extension with tRNA#2 of pTyr(AC) cross-linked to M1-s⁴U RNA, electrophoresed on denaturing polyacrylamide/7 M urea gel (8% w/v). Primer extension of pTyr(AC) irradiated 3 min (lane 1); complex 1' (lane 2); complex 2' (lane 3); complex 3' (lane 4).

linked complexes present in all cross-linking experiments, in the presence and absence of pTyr(ACCUCA) or pTyr(AC) (data not shown).

Activity of cross-linked complexes

We showed that the cross-linked complexes we studied were not artifactual, unproductive E-S complexes by demonstrating that each was capable of carrying out the cleavage reaction governed by M1 RNA. The cross-linked complexes we studied were isolated from denaturing gels in which the structure of the RNA species might be denatured, and with the exception of cross-linked nucleotides, contacts between M1-s⁴U RNA and pTyr disrupted. Therefore, renaturation of the cross-linked complex was carried out prior to assaying for enzymatic activity to address this concern.

Renaturation of complex 1 in the presence or absence of 10 mM Mg²⁺ (see Material and Methods) did not increase the rate of processing within the complex (data not shown). However, processing of the substrate in the complex does occur if renaturation is carried out in 10 mM Mg²⁺ but does not in water. We also note that the complex renatured in water and then assayed in 10 mM Mg²⁺ appears to be equally as active as the complex renatured in 10 mM Mg²⁺ and then assayed in 10 mM Mg²⁺. Therefore, 10 mM Mg²⁺ is not essential for the complex to reform an active structure, nor is renaturation. Further, complex 1 is equally as active in 10 mM Mg²⁺ or 100 mM Mg²⁺ (Fig. 5), in contrast to native M1 RNA, which requires >50 mM Mg²⁺ for optimal activity (Guerrier-Takada et al., 1986). Because enzyme and substrate are already tethered together in these complexes, a high concentration of divalent metal ions may not be necessary to act as a charge shield to promote efficient substrate binding.

Activity assays were also used to verify the general location of the cross-link(s) in pTyr, that is, in the 5' leader sequence or in the mature domain of tRNA. With pTyr 3' end labeled, the cleaved complex 1 generates the expected bands if there was a cross-link only in the mature domain of tRNA (Fig. 5A, see top left panel). For complexes 2 and 3, the mature tRNA cleavage product is observed as expected if the substrate were 3' end labeled and the cross-link were present in the 5' leader (Fig. 5A, see top right panel). Similarly, we cross-linked M1-s⁴U RNA and pTyr 5' end labeled and observed the bands expected if the only cross-link were located in the 3' terminal sequence (complex 1) and in the 5' leader (complex 2) of pTyr (Fig. 5B, top panel).

Relative rates of cleavage were estimated from initial rates in the linear portion of the cleavage curves of cross-linked complexes not renatured (see Table 2). Complexes 1 and 2 are equally active whereas complex 3 is tenfold less active (Fig. 5A; Table 2). C5 protein has no effect on the activity of complex 1 (Fig. 5A,B). Similarly, the rate of cleavage of pTyr in

complex 2 does not change with the addition of C5 protein (data not shown). Complex 3 was not assayed in the presence of C5 protein.

The cross-links between M1-s⁴U RNA and pTyr(AC) were also examined for activity (Fig. 5D). Unlike the activity of complex 3 relative to complexes 1 and 2 as mentioned above, complex 3' is as active as complexes 1' and 2' (Table 2). The activity of the cross-linked complexes with pTyr(AC) is approximately tenfold slower relative to the rate of cleavage with cross-linked complexes (1 and 2) with pTyr(ACCA).

DISCUSSION

We observed that M1 RNA can form productive, cross-linked complexes with the same and different substrates in different ways. Such a phenomenon may explain the diversity of substrates that can be processed by M1 RNA. For each of the substrate variants we studied, three cross-linked complexes with M1 RNA were isolated. Each of the three catalytic complexes had a single cross-linked nucleotide in M1 RNA: U69, U257, or U294. This was verified by cross-linking the ptRNA substrates to M1 RNA having the mutations Δ U69, C257/C258, or Δ [294–295] and observing the loss of the "canonical" cross-links (unpubl. results).

Nucleotides U257 and U294 are on opposing strands of the conserved internal loop J15/16 of bacterial RNase P RNAs (see Fig. 6), a putative binding site for divalent metal ions and the binding site for the canonical CCA of the ptRNA 3' terminus (Kazakov & Altman, 1991; Kirsebom & Svard, 1993, 1994; Ciesiolka et al. 1994; Oh & Pace, 1994). Specifically, U294, G293, and G292 of M1 RNA have been demonstrated to base pair with NCC of the ptRNA 3' terminus (Kirsebom & Svard, 1994). U257 cross-linked to the 3' terminus of a ptRNA ending in the 3' terminal sequence ACC (where the 5' A is the discriminator base). Strikingly, however, U257 cross-linked to the 5' leader (nt –14 to –16) of pTyr ending in the 3' terminal sequence AC. This difference in the location of U257 cross-links in these substrates, which differed by a single 3' terminal nucleotide, represents a significant change in the orientation of the pTyr(AC) on M1 RNA when compared to pTyr(ACCAUCA). The differences in cross-linking results with these substrates is also compatible with chemical probing experiments of E-S complexes with pTyr(A) versus pTyr(ACCAUCA) (Knap et al., 1990). U294 cross-linked to nucleotides between –6 and –9 of the 5' leader, regardless of the sequence at the 3' terminus of the ptRNA. Similarly, in all substrate variants, U69 cross-linked to 5' leader nucleotides. The pTyr(AC) variant formed cross-links to nucleotides on either side of the site of cleavage as well. Therefore, while the different substrate variants can bind in distinctly different orientations, they can also participate in some similar binding modes.

FIGURE 5. Activity assays of cross-linked complexes between M1-s⁴U RNA and pTyr(ACCA) or pTyr(AC). **A:** Activity assay of cross-linked complexes 1, 2, and 3, between M1-s⁴U RNA and pTyr(ACCA), 3' end labeled, at 37 °C in 10 mM Mg²⁺. Five samples taken at time points 0 min (control), 5 min, 15 min, 25 min, and 40 min. Top left: schematic of the expected bands due to cleavage of cross-link complexes if the cross-link, as indicated by an X, were in the mature domain of the tRNA (left) or in the 5' leader sequence (right). Activity of complexes to generate the tRNA product (mTyr) and the 5' leader. The first lane is pTyr cleavage pattern by M1 RNA (control). **B:** Activity assay of cross-linked complexes 1 and 2, between M1-s⁴U RNA and pTyr(ACCA), 5' end labeled. Top right: schematic of the expected bands due to cleavage of cross-link complexes if the cross-link were in the mature domain of the tRNA (left) or in the 5' leader sequence (right). **C:** Activity assay of cross-linked complex 1, between M1-s⁴U RNA and pTyr(ACCA), 3' end labeled. Complex 1 assayed at 10 mM Mg²⁺ in the presence of C5 protein (a); 10 mM Mg²⁺ (b); 100 mM Mg²⁺ (c) at 5 min intervals. **D:** Activity assay of cross-linked complexes between M1-s⁴U RNA and pTyr(ApCp), 3' end labeled. The appearance of a band migrating with the same mobility as pTyr in some of the cleavage assays is due to the occasional trapping of uncross-linked substrate in the cross-linked E-S complexes.

TABLE 2. Location of cross-links between M1 RNA and pTyr(AC CAUCA) or pTyr(AC) and cleavage activity of cross-linked complexes.

Cross-linked complex ^a	M1 RNA	pTyr(ACCAUCA)	Activity ^b
1	U257	+83 to +86	0.8
2	U294	-6, -9	0.7
3	U69	-8, -10	0.08
pTyr(AC)			
1'	U257	-14, -15, -16	0.09
2'	U294	-7, -9	0.09
3'	U69	+1, -1, -2, -7, -8, -9	0.08

^aSee Figures 1 and 3.^bActivity of cross-linked complexes in 10 mM magnesium, 1× assay buffer (see Materials and Methods), expressed as percent substrate cleaved per minute measured during the linear phase of the reaction.

To account for a series of RNase P RNA cross-links with both ptRNA substrate or product on a computer generated three-dimensional model of M1 RNA, Westhof et al. (1996) conjectured that the ptRNA (with an undenatured aminoacyl stem) would have to assume two different orientations on M1 RNA that would differ by a rotation of 180° about the axis of the anticodon stem of the tRNA. We also find that the cross-linked complexes between U294 and the pTyr 3' trailer and between U257 and the 5' leader sequence can be understood if the ptRNA were to be fixed in one binding mode on a recent model of M1 RNA (Massire et al., 1998), and then rotated 180° about the axis of its anticodon to accommodate the other. Of course, if the aminoacyl stem is denatured when bound to M1 RNA, it is easily fitted to M1 RNA in a manner compatible with all our data. The various cross-linked complexes we describe may represent different conformers that the M1 RNA–substrate complex assumes on the pathway to the final, active conformer.

The cross-links formed by U257 to the different substrates suggests that the 3' terminal CA nucleotides (of the ACCA sequence) act both to guide the substrate to a particular mode of binding on M1 RNA and as an allosteric effector. The importance of the 3' terminal CCA as a factor in the binding and cleavage of substrates for M1 RNA has been highlighted in biochemical (Guerrier-Takada et al., 1984, 1989; Perreault & Altman, 1993) and genetic studies (Kirsebom & Svard, 1993).

A substrate-induced conformational change in the enzyme?

The cross-linked complexes between M1 RNA and ptRNA ending in the sequence AC at its 3' terminus migrated differently from the cross-linked complexes between M1 RNA and ptRNA ending in ACC at its 3' terminus. This phenomenon reflects the intramolecular

cross-link present in only the cross-linked complexes between M1 RNA and ptRNA ending in AC at its 3' terminus. The intramolecular cross-link occurs between nt 104 and 110 (helices P8 and P9) in M1 RNA (see Fig. 6B). These nucleotides must be within 4.5 Å of one another to form a cross-link in the presence of 4'-thiouridine (Favre, 1990; R. Zimmermann, pers. comm.). Protection studies of UV-induced cross-linked complexes between M1 RNA and pTyr lacking CCA at its 3' terminus indicated that helices P8 and P9 (spanning nt 92–119) are highly protected (Knap et al., 1990; see Fig. 6B). On the other hand, helices P8 and P9 in cross-linked complexes between M1 RNA and pTyr with CCA at the 3' terminus were not protected from chemical probes (Knap et al., 1990; see Fig. 6A). The distance between nt 104 and 110 is approximately 19 and 27 Å, depending on which of two recent three-dimensional computer generated models of M1 RNA one consults (Chen et al., 1998; Massire et al., 1998). Accordingly, a local change in M1 RNA of at least 15 Å would have to occur to account for the formation of the intramolecular cross-link. This series of events would, presumably, ensure that the particular substrate could determine specificity by eliciting the necessary structural changes in M1 RNA.

The nature of product inhibition

Both product and excess substrate in the cleavage reaction are inhibitors of the activity of M1 RNA (Guerrier-Takada et al., 1989). Only a single major cross-linked complex between M1 RNA and a tRNA product was isolated. This cross-linked complex shares cross-links with only one of the three M1 RNA–substrate cross-linked complexes. This suggests that product competes for one among several binding sites on M1 RNA for substrate. Kinetic studies have differed on the type of inhibition exhibited by product, both competitive and noncompetitive types have been observed (Smith et al., 1992; Guerrier-Takada & Altman, 1993). Based on the results we report here, noncompetitive inhibition by the tRNA^{Tyr} product of the enzymatic activity of M1 RNA would be expected.

The M1 RNA–pTyr cross-linked complex that has the shared cross-links with the M1 RNA–product cross-linked complex is the one that is enhanced in yield by C5 protein. However, the M1 RNA–product cross-linked complex is not enhanced by the presence of C5 protein. Perhaps C5 protein selects formation of only one specific, active M1 RNA conformer with substrate over that with product, thus minimizing product inhibition.

Roles of cofactors: C5 protein and divalent metal ions

What allows the C5 protein to enhance association between M1 RNA and substrate and to discriminate against

FIGURE 6. Proposed secondary structure of *Escherichia coli* M1 RNA (Hass et al., 1994) and secondary structure of pTyr ending in the 3' terminal sequence ACCAUCA or A. Interactions between M1-s⁴U RNA and (A) pTyr(ACCAUCA), or (B) pTyr(A), obtained in this study and in two previous studies (Guerrier-Takada et al., 1989; Knap et al., 1990). Nucleotides in M1 RNA and pTyr at less than 4.5 Å distance from each other, obtained in this study (brown, purple, and red in A and B). Nucleotides in M1 RNA interacting with substrate based on UV (254 nm) induced cross-linking (blue in A; gold and blue in B) (Guerrier-Takada et al., 1989). Nucleotides highly protected by chemical probing of E-S cross-linked complexes (indicated in green in A and B) (Knap et al., 1990). Intramolecular cross-link is indicated between P8 and P9 helices (see B).

or not enhance association with product? One possibility is that the C5 protein recognizes both a feature or conformation in M1 RNA and a feature of the substrate that differs from any found in the product. There is evidence in support of such a possibility. The effect of the *E. coli* and *B. subtilis* RNase P protein components in enhancing the processing of tRNA precursors is correlated with the length of the 5' leader sequence in the substrate (Peck-Miller & Altman, 1991; Crary et al., 1998). It was recently reported that the protein component of *B. subtilis* RNase P, modified at single cysteine residues with a photochemical cross-linking reagent, can cross-link to nucleotides in the 5' leader of a precursor tRNA, albeit the reagent used can form cross-links from a distance of at least 11 Å (Niranjanakumari et al., 1998). We note that the rate of cleavage of those M1 RNA-ptRNA cross-linked complexes that were tested are not altered by the presence of the C5 protein. Our and other results support the contention that the protein component of RNase P enhances affinity of substrate to M1 RNA. Further, our results indicate that C5 protein also selects a specific conformer of M1 RNA for binding to substrate. However, it is important to note that biochemical and genetic studies indicate that the effect of C5 protein is substrate dependent, and therefore results regarding a particular tRNA substrate might not be applicable to another substrate (Kirsebom et al., 1988; Peck & Miller, 1990; Peck-Miller & Altman, 1991; Gopalan et al., 1997). We observed that the effect of C5 protein in enhancing the yield of one intermolecular cross-linked complex over that of others is substrate dependent, as this phenomenon is not apparent when the ptRNA substrate that ends in the 3' sequence AC is used.

An increase in the concentration of Mg^{2+} , presumably acting as an electrostatic shield against repulsion of the negatively charged phosphates of the RNAs, increases the affinity of substrate for M1 RNA (Reich et al., 1988). Consistent with this notion we find that at 10 mM Mg^{2+} , M1 RNA mutants $\Delta[273-281]$, C257/C258, and $\Delta[294-295]$ are incapable of forming cross-linked complexes with substrate, whereas at 100 mM Mg^{2+} , complex formation is achieved. For wild-type M1 RNA, we find that the cross-linked complexes are formed at 10 mM Mg^{2+} , whereas at 100 mM Mg^{2+} the amount of all cross-linked complexes between M1 RNA and substrate are increased. We observe that processing within the M1 RNA-substrate cross-linked complexes show no difference at 10 mM versus 100 mM Mg^{2+} . A previous study of single turnover kinetics of M1 RNA revealed, consistent with our observation, no change in the catalytic rate constant for Mg^{2+} concentration greater than 20 mM Mg^{2+} (Tallsjo & Kirsebom, 1993).

M1 RNA is presented with a significant task in vivo, to catalyze the hydrolysis of all precursor tRNAs and the precursors to 4.5S RNA and other RNAs. The results of this study suggest that M1 RNA binds substrates

in different ways, thereby broadening its specificity. Substrate-induced conformational changes in the enzyme may also aid efficient catalysis. Furthermore, C5 protein, which appears to have multiple roles in the RNase P reaction that vary in a substrate-dependent manner (Liu & Altman, 1994; Gopalan et al., 1997), increases the binding of substrates to specific conformers of M1 RNA. Indeed, a recent report provides evidence for a conformational change in *E. coli* C5 protein upon binding to M1 RNA (Gopalan et al., 1999). The RNA and protein subunits of RNase P evolved together to enable a single ribonucleoprotein enzyme to select the conformer that best facilitated efficient and precise cleavage of each of over sixty different substrates.

MATERIALS AND METHODS

Chemicals and enzymes

The following enzymes were purchased from New England Biolabs (Beverly, Massachusetts): DNA restriction enzymes, T4 polynucleotide kinase, and calf intestinal alkaline phosphatase. T4 RNA ligase was purchased from Epicentre (Madison, Wisconsin) and T7 RNA polymerase from Promega (Madison, Wisconsin). Nucleoside triphosphates and RNase T1 were purchased from Pharmacia Biotech (Piscataway, New Jersey). The nucleoside analog 4'-thiouridine-5'-triphosphate (sodium salt) was purchased from USB (Cleveland, Ohio). Quick-spin Sephadex (G-25 and G-50) columns were purchased from Boehringer Mannheim Corp. (Indianapolis, Indiana). $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ (400 Ci/mmol), $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (3,000 Ci/mmol), and $[\text{}^{32}\text{P}]\text{pCp}$ (3,000 Ci/mmol) were purchased from Amersham Life Science (Arlington Heights, Illinois). Primer extension experiments utilized AMV reverse transcriptase (Boehringer Mannheim Corp.), and DNA primers were synthesized (solid phase) at the W.M. Keck Biotechnology Resource Laboratory (Yale University).

Preparation of RNA and protein

A plasmid (pJA2') encoding *rnpB*, the *E. coli* gene encoding M1 RNA under control of the T7 polymerase promoter, was linearized with *FokI* for run-off transcription of M1 RNA in vitro as described previously (Vioque et al., 1988). In addition, plasmids encoding the precursors to tRNA^{Tyr} (pTyr), 3' end variants of pTyr, and mature tRNA^{Tyr} (mTyr), under the control of the T7 polymerase promoter, were linearized with the appropriate restriction enzymes for transcription in vitro. RNA transcripts from the pTyr constructs have a natural 5' leader sequence (43 nt; see Fig. 6). The mTyr construct was cloned to allow initiation of transcription to begin at the +1 position: the transcript has a 5'-triphosphate rather than the 5'-monophosphate as generated by RNase P catalyzed phosphodiester bond cleavage.

4'-thiouridine triphosphate can be incorporated into an RNA transcript during T7 run-off transcription in vitro in a random manner with an efficiency four- to fivefold less than uridine triphosphate (Favre, 1990; Dubreuil et al., 1991). M1 RNA and a deletion mutant of M1 RNA ($\Delta[273-281]$) were synthesized in the presence of standard rNTPs and the nucleoside analog 4'-thiouridine triphosphate (4'-thioUTP) such that

approximately 1–9% of all uridine bases were replaced with 4'-thiouridine (4'-thioU) in individual transcripts. Following transcription in vitro, DNA templates were degraded with DNase I, protein was extracted by phenol:chloroform treatment, and RNA was then purified by size exclusion column chromatography on Sephadex (G25 or G50) columns, as described previously (Vioque et al., 1988). M1 RNA, pTyr, and mTyr were also transcribed in the presence of radiolabel for internal labeling. Radiolabeled RNA (internal or end labeled) was purified from denaturing polyacrylamide/7 M urea gels (5% w/v). The concentration of the RNA species was determined by measuring their optical density at 260 nm or for labeled RNA species by comparison to a series of RNA standards or samples of known concentration on an agarose gel (2% w/v). C5 protein was prepared and its concentration determined as described previously (Vioque et al., 1988).

Prior to the cross-linking reaction, M1 RNA containing 4'-thioU (M1-s⁴U RNA) was resuspended in 1× binding buffer and heated at 65 °C for 5 min and renatured by allowing the species to cool slowly to room temperature, for approximately 2 h. M1-s⁴U RNA was also renatured, as described above, to measure activity as a function of 4'-thioU.

Cross-linking reactions

M1-s⁴U RNA renatured in the presence of 10 mM Mg²⁺ was prepared for cross-linking in 1× binding buffer that contained either 10 mM Mg²⁺ or Ca²⁺, as indicated in the figure legends. M1-s⁴U RNA (0.01–0.1 μM), in a total volume of 30–40 μL, was incubated at 37 °C for 5 min in either the presence or absence of C5 protein (five- to tenfold molar excess in concentration over M1-s⁴U RNA or M1Δ[273–281] RNA). The sample was placed on ice for 3 min and then mixed on ice with or without substrate or product. The substrate or product molar concentration was in excess of M1-s⁴U RNA at a concentration twofold above the K_M for the particular substrate (where for pTyr(ACCAUCA) K_M = 30 nM, for pTyr(AC) K_M = 150 nM, for pTyr(A) K_M = 350 nM; see Altman & Kirsebom, 1999). Total reaction mixture was kept on ice 3.8 cm from UV bulbs and samples were immediately irradiated for 3 min. Samples were irradiated using a Stratalink UV Crosslinker with five 8 W bulbs having λ_{max} of 312 nm (Stratagene, La Jolla, California). After 3 min, the sample was mixed vigorously by vortexing (5 s) with twice its volume of 9 M urea/dye (0.05% w/v bromophenol blue, 0.05% w/v xylene cyanol ff) and then loaded on denaturing polyacrylamide/7 M urea gels (5–8%), and electrophoresed at 30–35 mA for approximately 6–7 h until the bromophenol blue dye was detected at the bottom of the gel (40 cm total length). Cross-linked reaction products were visualized by autoradiography and an image was generated by a PhosphorImager program (MacBAS, version 2.0, Fuji) for quantifying results.

Identification of cross-linked nucleotides

Slices of the gel corresponding to cross-linked species were excised. The RNA was eluted from the gel slices by incubation in 1× elution buffer (10 mM Tris, pH 7.5, 1 mM ethylene diamine tetraacetic acid (EDTA), 100 mM NaCl, 0.01% sodium dodecyl sulfate (SDS) w/v) at 37 °C for 6–8 h. The cross-linked species were then precipitated by addition of

two volumes of 95% ethanol. Cross-linked nucleotides were identified by primer extension or partial alkaline hydrolysis. Primer extension was carried out using DNA primers, 5' end labeled with [γ -³²P]ATP, as previously described (Guerrier-Takada et al., 1989). Nucleotide(s) involved in cross-links were identified on the basis of a dideoxy sequencing ladder, using the same primers complementary to regions of M1 RNA or tRNA (see Table 1) on denaturing polyacrylamide/7 M urea gels (8–10% w/v). Partial alkaline hydrolysis was performed by heating 5' or 3' end-labeled samples at 95 °C for 8–12 min in 50 μL 1× alkali buffer (50 mM NaHCO₃, pH 9.2, 1 mM EDTA). Sample was then precipitated with 0.3 M sodium acetate/95% ethanol and electrophoresed on denaturing polyacrylamide/7 M urea gels (10–20% w/v).

Activity of cross-linked complexes

The area of the gel corresponding to a cross-linked species was excised and the RNA eluted from the gel slices, as described above. Certain cross-linked species were then renatured by heating at 65 °C for 5 min and then cooling slowly to room temperature (~1.5 h).

Cross-linked complexes, with 5' or 3' end-labeled substrate, were monitored for RNase P activity in 1× binding buffer that contained either 10 mM or 100 mM Mg²⁺. The activities of the cross-linked complexes were measured in both the presence and absence of C5 protein and with or without renaturation of the complex in the presence or absence of 10 mM Mg²⁺. Activity was measured at 37 °C by taking aliquots at specified times, mixing with two volumes of 9 M urea/dye, vortexing (5 s), and subsequent electrophoresis on denaturing polyacrylamide/7 M urea gels (5% w/v).

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