Sequence-specific recognition of colicin E5, a tRNA-targeting ribonuclease

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ABSTRACT

Colicin E5 is a novel *Escherichia coli* ribonuclease that specifically cleaves the anticodons of tRNA^{Tyr}, tRNA^{His}, tRNA^{Asn} and tRNA^{Asp}. Since this activity is confined to its 115 amino acid long C-terminal domain (CRD), the recognition mechanism of E5-CRD is of great interest. The four tRNA substrates share the unique sequence UQU within their anticodon loops, and are cleaved between Q (modified base of G) and 3′ U. Synthetic minihelix RNAs corresponding to the substrate tRNAs were completely susceptible to E5-CRD and were cleaved in the same manner as the authentic tRNAs. The specificity determinant for E5-CRD was YGUN at −1 to +3 of the 'anticodon'. The YGU is absolutely required and the extent of susceptibility of minihelices depends on N (third letter of the anticodon) in the order A > C > G > U accounting for the order of susceptibility tRNA^{Tyr} > tRNA^{Asp} > tRNA^{His}, tRNA^{Asn}. Contrastingly, we showed that GpUp is the minimal substrate strictly retaining specificity to E5-CRD. The effect of contiguous nucleotides is inconsistent between the loop and linear RNAs, suggesting that nucleotide extension on each side of GpUp introduces a structural constraint, which is reduced by a specific loop structure formation that includes a 5′ pyrimidine and 3′ A.

INTRODUCTION

Colicins are plasmid-encoded toxins that kill *Escherichia coli* cells not harbouring the same or a cognate plasmid. The modes of killing have long been divided into three classes: formation of ion channels in the inner membrane, deoxyribonuclease (DNase) activity and ribonuclease (RNase) activity (1,2). The DNase-type colicins nonspecifically cleave the genomic DNA of sensitive cells (3–5), and the RNase-type colicins inhibit protein synthesis of sensitive cells by cleaving a specific site near the 3′ end of 16S rRNA (6–8).

Colicin E5 inhibits protein synthesis by specifically cleaving tRNA^{Tyr}, tRNA^{His}, tRNA^{Asn} and tRNA^{Asp} of sensitive *E.coli* cells; this led to the introduction of the fourth type of colicin 'tRNase' (9). Colicin E5 cleaves these tRNAs between the 34th queuosine (Q) and 35th uridine (U) that correspond to the first and second letters of the anticodon triplets, yielding a 2′,3′-cyclic phosphate and a 5′-OH terminus. Q is a nucleoside with a unique base, namely, queuine. Queuine is a highly modified guanine (G) base that is widely found at the above-mentioned position in the above four tRNA species in prokaryotes and eukaryotes (10–13). This modified base is introduced by a base exchange reaction of the precursor tRNA, which is catalysed by tRNA-guanine transglycosylase (TGT) (14). Although Q is a nucleoside unique to the substrate tRNAs of both colicin E5 and TGT, colicin E5 also kills TGT-defective *E.coli* cells (15) whose tRNAs possess a G instead of the Q; in fact, colicin E5 cleaves Q-deficient tRNA^{Tyr}, tRNA^{His}, tRNA^{Asn} and tRNA^{Asp} both *in vivo* and *in vitro* (9) (data not shown). Thus, the base modification unique to colicin E5-sensitive tRNAs does not appear to be recognized by colicin E5.

Then, how does colicin E5 distinguish the target tRNAs from a large number of cellular RNAs including other tRNA molecules? The active domain of colicin E5 C-terminal ribonuclease domain (CRD) is composed of only 115 amino acids; this size is comparable with those of RNase A and RNase T1. However, the substrate specificity of E5-CRD is quite different from that of RNase A or RNase T1 that recognizes only a single pyrimidine or G (16). Moreover, the cleavage by E5-CRD is specific to anticodon loops; this suggests that its recognition mechanism is more complicated than that of RNase A and RNase T1. E5-CRD shows no sequence homology with traditional RNases and also lacks a catalytic His that is essential for these RNases; hence, the unique interaction of E5-CRD with RNA is of considerable interest to researchers. The molecular size of E5-CRD is only approximately half those of tRNAs; this suggests that E5-CRD recognizes only limited portions of tRNAs to distinguish the target tRNAs from other tRNAs. Since all E5-CRD-sensitive tRNAs contain a unique sequence UQU (or UGU in the precursors of these tRNAs) and are cleaved between the Q and the second U, we considered this UQU sequence as a candidate sequence that is recognized by E5-CRD. In this study, we determined

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the structural requirement(s) for cleavage by E5-CRD using synthetic anticodon arms and linear RNAs as substrates, and discussed the recognition mechanism of E5-CRD from the viewpoint of the target substrate structure.

MATERIALS AND METHODS

Materials

T4 polynucleotide kinase (Toyobo, Osaka) and [γ-^{32}P]ATP (NEN Life Science Products, Inc., Boston, MA) were used to label the 5′ end of the substrates. Ribonuclease T1 was purchased from Sigma (St Louis, MO). Alkaline phosphatase (*E.coli* A19; TaKaRa, Tokyo) was used to remove the 5′-monophosphate from *in vitro*-transcribed RNAs. We prepared a plasmid pTO502 to produce E5-CRD and the wild-type inhibitor protein (ImmE5) because the previously reported plasmid pTO501 (9) had a point mutation in the *immE5* gene. The methods used for overexpression and purification of E5-CRD and ImmE5 were as described previously (9). E5-CRD was stored in 20 mM sodium phosphate buffer (pH 7.0) containing 50% glycerol at −20°C.

In vitro transcription, purification and 5′ labelling of minihelices

'Minihelices' (MHs) corresponding to the tRNA anticodon arms were prepared as described previously (17) by *in vitro* transcription using His-tagged T7 RNA polymerase provided by Dr Tsutomu Suzuki (University of Tokyo). In a typical procedure, 550 pmol of a synthetic 17mer DNA for the T7 promoter sequence is annealed to a synthetic 34mer template DNA (450 pmol), half of which is complementary to a 17mer anticodon arm. The T7 RNA polymerase barely initiates transcription with C and prefers a GG sequence as the initiation sequence (17). Among the tRNAs of interest, C is the start base of the 5′ end of the anticodon arm of both tRNA^{His} and tRNA^{Asp}; hence, a set of 19mer MHs was also prepared by adding GG to the 5′ end of 17mer MHs. Each semi-duplex obtained was mixed with 1 ml of reaction buffer comprising 40 mM Tris–HCl, pH 8.0, 14 mM MgCl$_2$, 5 mM DTT, 1 mM spermidine (Sigma), 2 mM of each NTP, 20 mM 5′-GMP, 1 U/ml of inorganic pyrophosphatase from bakers yeast (Sigma) and 50 μg/ml of BSA (Roche, Basel). The His-tagged T7 RNA polymerase (20 μl) was added directly from the purification column, followed by incubation for 1 h and then supplemented with 20 μl of the T7 RNA polymerase and further incubated for 1 h. On performing this method, MHs with 5′-monophosphate were obtained. The reaction was stopped by the addition of an equal volume of 2× loading solution (9 M urea, 0.02% bromophenol blue and 0.02% xylene cyanol), followed by direct application to a 20% preparative polyacrylamide gel containing TBE buffer (90 mM Tris–borate and 1 mM EDTA) and 7 M urea. The MHs were visualized by the UV-shadowing method and eluted from the gel. They were then 5′ end-labelled with [γ-^{32}P]ATP and T4 polynucleotide kinase after treatment with alkaline phosphatase (TaKaRa).

Determination of the extent of cleavage using MHs

The cleavage reaction mixture comprised 20 mM Tris–HCl, pH 8.5, 50 mM NaCl, 100 μg/ml BSA and 4 μM of a MH.

E5-CRD was added to this reaction mixture to yield a final concentration of 0.2 nM, and the mixture was then incubated at 37°C. The MH used here contained a trace amount of 5′ end-labelled MH. At intervals, 10 μl of the solution was withdrawn and mixed with an equal volume of 2× loading solution. The cleavage of the MHs was analysed by electrophoresis of the mixtures on a 20% polyacrylamide gel containing 7 M urea and TBE buffer, and then the gel was brought in contact with an imaging plate (FUJI FILM). The imaging plate was then analysed by a FLA-3000 (FUJI FILM) for visualization of the mobility pattern. Subsequently, radioactivity of the two bands—the intact MH and 5′ end sequence of the cleavage products—was quantified. The extent of the cleavage was calculated using the following formula: extent of reaction (%) = (5′ end of the cleaved fragment) × 100/(intact MH + 5′ end of the cleaved fragment).

Oligonucleotide analysis

Oligonucleotides, dimers to tetramers, were chemically synthesized by Genset (France). GpUp was purchased from SIGMA. ApUp, GpCp and UpGp were provided by Dr Kazuya Nishikawa (Gifu University). These oligonucleotides were purified by reversed-phase high-performance liquid chromatography (HPLC) on an ODS-3 column (4.6 × 250 mm; GL Sciences) equilibrated with 100 mM triethylamine acetate buffer, pH 7.0, followed by elution with a linear gradient of acetonitrile. The purified oligonucleotides were then lyophilized and dissolved in milli-Q water. To identify the reaction products from GpUp with E5-CRD, several authentic oligonucleotides were mixed and run on the reversed-phase column.

Determination of kinetic constants of E5-CRD depending on various pH conditions with GpUp

The reaction was performed in a 1 cm path-length cuvette containing 20 mM of Tris–HCl ranging from pH 7.5 to 9.5, 50 mM NaCl and 50 μg/ml BSA. The reaction mixture was incubated with 21–78 μM of GpUp and 276–322 pM of E5-CRD incubated at 25°C. E5-CRD (20 μl) was added, and the increase in absorbance at 275 nm was monitored using the DU-65 spectrometer (Beckman). The molar extinction coefficient of GpUp used was 1150 (M^{-1} cm^{-1}) at 275 nm. Initial velocities were calculated from the plot where the extent of reaction increased linearly against time. The k_{cat} and K_m values with GpUp as the substrate were determined from the $[S]_0 - [S]_0/v$ plot at various pH.

Determination of kinetic constants of E5-CRD with oligoribonucleotides

To determine the kinetic constants with oligoribonucleotides, 3 μl of E5-CRD, ranging in concentration from 304.9 to 1524.4 pM, was added to 197 μl of the reaction buffer. The final reaction mixture comprises 20 mM Tris–HCl, pH 8.5, 50 mM NaCl, 100 μg/ml BSA and various amounts of oligonucleotides. The concentration of each oligonucleotide added was as follows: GpUp, 6.0–145.5 μM; UpGpU, 12.8–770.0 μM; UpGpUp, 25.4–254.0 μM; GpUpA, 36.0–228.0 μM; UpGpUpA, 37.1–307.4 μM and ApGpUpA, 30.6–275.4 μM. After the addition of E5-CRD, a 90 μl aliquot was immediately taken and mixed with one-third

volume of acetic acid to stop the reaction. The reaction mixture was incubated at 37°C until the extent of the reaction reached 10–15%. At this time, acetic acid was added to another 90 µl aliquot of the reaction mixture. These two samples were applied to an ODS-3 column (4.6 × 150 mm; GL Sciences), and then the peak areas of the intact substrate were measured. These values were then converted to the initial and post-reaction amounts of the intact substrate, and the initial velocity of each cleavage reaction was calculated. The kinetic constants were determined according to a previously published program (18) based on more than 16 initial velocities measured at various substrate concentrations. Since the amount of ApGpUpA available was limited, the reaction volume with ApGpUpA was set at 100 µl. The volumes in the following steps were also decreased to half; however, no difference was observed when compared with the reaction using a volume of 200 µl.

RESULTS

Cleavage of MHs by E5-CRD

We assumed that E5-CRD distinguishes the target tRNAs from other tRNAs by direct recognition of the UQU sequence in the anticodon loop. Then, we prepared a series of short 17mer RNAs referred to as MHs corresponding to the anticodon arms of an E5-CRD-sensitive tRNA^{Tyr} and an E5-CRD-resistant tRNA^{Lys} as well as their variants (Figure 1). The MHs were named, e.g. XMH is the MH mimicking the anticodon arm of the tRNA for amino acid X. The anticodon triplet corresponds to positions 8–10.

YMH, which corresponds to tRNA^{Tyr}, has a UGU sequence that is an unmodified form of UQU, at positions 7–9. When 5′ labelled YMH was incubated with E5-CRD, a band of the shorter fragment appeared as shown in Figure 2A (lanes 1 and 2). Size estimation of this new fragment revealed that the cleavage site was between G8 and U9 (Figure 2B, lanes 1–3, and Figure 2C); this indicated that YMH is cleaved by E5-CRD in the same manner as tRNA^{Tyr}. Consistently, KMH is not cleaved like the original tRNA^{Lys} (Figure 2A, lanes 9 and 10) (9). These and the following results show that YMH is actually cleaved by E5-CRD and

that the anticodon arm is sufficient to be recognized by E5-CRD. In addition, it was confirmed that the modification of G to Q is not required for colicin E5 action.

Determination of the minimal component of MHs recognized by E5-CRD

In order to evaluate the significance of the UGU sequence, a variant, YMH(G8A), in which G8 was replaced with A to obtain purine as the base, was incubated with E5-CRD. No cleavage product was detected on the polyacrylamide gel (Figure 2A, lanes 5 and 6). Similarly, YMH(U9C) was not cleaved (Figure 2A, lanes 7 and 8). On the contrary, YMH(U7C) was cleaved by E5-CRD to almost the same extent as YMH (Figure 2A, lanes 3 and 4 versus lanes 1 and 2), and as in the case of YMH, the cleavage occurred between G8 and U9 (Figure 2B, lanes 4–6). The mutations at G or the second U of the UGU sequence of YMH abolished susceptibility to E5-CRD; however, the mutation of the first U, at least to C, did not change the activity at all. These results suggest that the GU sequence corresponding to first and the second letters of the anticodon triplet is the minimum requirement for E5-CRD as a target molecule.

In this case, an E5-CRD-resistant tRNA must be converted to an E5-CRD-sensitive tRNA through a simple replacement yielding an anticodon GUN (where N is any nucleotide). To test this hypothesis, we prepared a variant of KMH, namely, KMH(U8G), in which U has been replaced with G at position 8 to create the GU sequence in the anticodon. When incubated with E5-CRD and analysed by electrophoresis, KMH(U8G) was in fact cleaved by E5-CRD (Figure 2A, lanes 11 and 12) exactly between G8 and U9 (Figure 2B, lanes 7–9). The extent of the reaction was lower than that in the case of YMH; this is discussed later. It was possible to convert an E5-CRD-resistant MH to an E5-CRD-sensitive MH by introducing a GU sequence into the anticodon, and confirmed that the GU sequence is the minimal component recognized by E5-CRD. We determined the optimal condition for E5-CRD with YMH as a substrate (data not shown), and adopted 20 mM Tris–HCl, pH 8.5, and 50 mM NaCl as the basal buffer for the reaction mixture. Under these conditions, the nonenzymatic cleavage of MHs was not
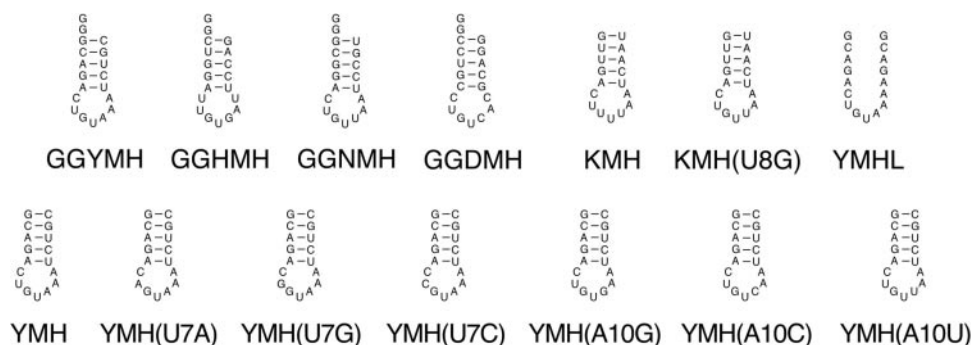

Figure 1. Minihelices used in this study. Each minihelix (MH) is referred to as XMH where X is the amino acid that is accepted by the parental tRNA. For example, YMH is the MH mimicking the anticodon arm of tRNA^{Tyr}. In the models, the top left is the 5′ end of each oligoribonucleotide, and each ribonucleotide is numbered from this position. GGXMH indicates a guanylyl-guanosine that was added at the 5′ end of XMH. If ribonucleotide X at position *n* was changed to Y, then it is indicated in parentheses as XnY. The horizontal lines indicate the putative H-bonds.
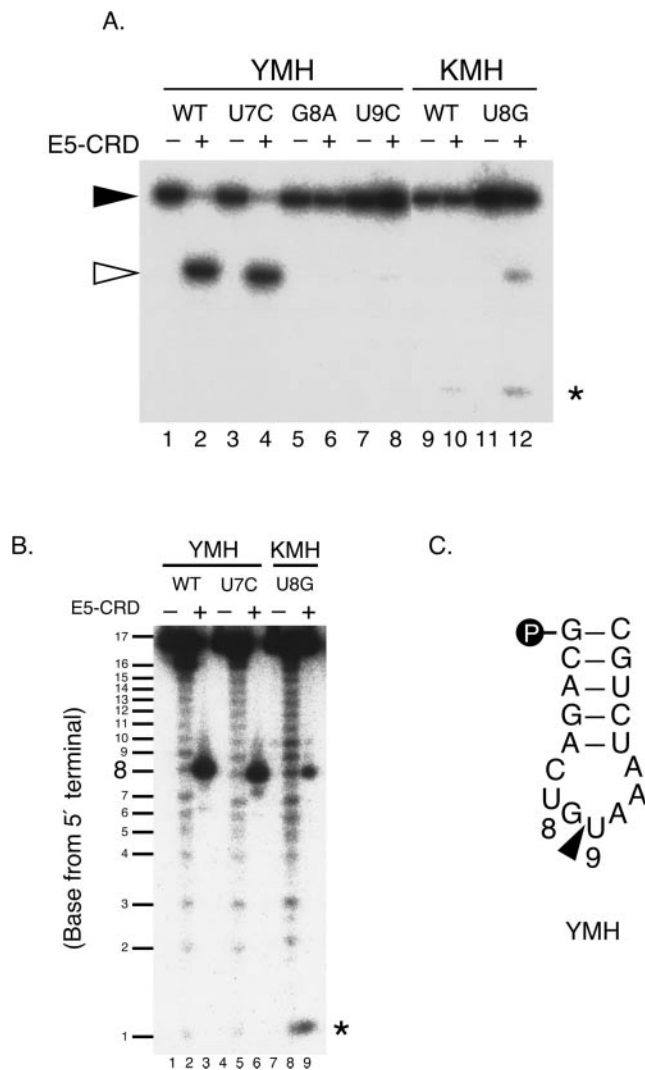
Figure 2. The potential of minihelices with wild-type or mutated sequences to be cleaved by E5-CRD. (**A**) YMH and KMH (5 μM each) were incubated with or without 10 nM of E5-CRD in 20 mM Tris–HCl (pH 7.5) and 20 mM KCl for 15 min at 37°C, and then analysed by electrophoresis on a 20% polyacrylamide gel containing 7 M urea (lanes 1, 2, 9 and 10, respectively). The visible mobility pattern was obtained with a FLA-3000 (FUJI FILM). Lane numbers are shown beneath the picture. The closed triangle indicates the band of intact MH. If cleavage of a MH occurred, a band of a shorter fragment appeared (indicated by the open triangle). Variants of YMH and KMH with a site-directed mutation were also incubated with E5-CRD and then analysed. Each mutation is indicated above the picture. The asterisk indicates the 5′-labelled G released from KMH and KMH(U8G) on cleavage between G1 and U2. (**B**) YMH, YMH(U7C) and KMH(U8G) were incubated with (lanes 3, 6 and 9, respectively) or without (lanes 1, 4 and 7, respectively) E5-CRD, and then analysed by electrophoresis. The bands were visualized with a FLA-3000 (FUJI FILM). Lane numbers are shown beneath the picture. In lanes 2, 5 and 8, fragments of partially digested YMH, YMH(U7C) and KMH(U8G) with 40 mM sodium carbonate, pH 9.0, respectively, were electrophoresed. The asterisk indicates the 5′-labelled G released from KMH(U8G) on cleavage between G1 and U2. (**C**) Graphical representation of the putative structure of 5′-labelled YMH and the cleavage site by E5-CRD. Cleavage (as indicated by a closed triangle) occurs between G8 and U9.

observed. The activity of E5-CRD does not require divalent cations, and is highly heat stable since it retained almost the same activity after boiling for 15 min at 95°C (data not shown).

Influence of the nucleotides surrounding the GU sequence on the cleavage between G and U

In the previous paper (9), we reported that tRNA[Tyr] is the most susceptible tRNA within a cell after the challenge with colicin E5, and that the order of susceptibility of cytoplasmic tRNAs is tRNA[Tyr] > tRNA[Asp] > tRNA[His], tRNA[Asn]. In order to determine whether the susceptibilities of the tRNAs reflect some local structural features around their anticodon arms, we prepared four 19-mer MHs corresponding to the four tRNAs (GGXMHs in Figure 1), and the cleavage efficiency of these MHs toward E5-CRD was compared. As shown in Figure 3A, GGYMH was the most susceptible among the four GGXMHs, and the extent was comparable with that of the 17mer YMH. The susceptibilities of GGYMH, GGDMH, GGHMH and GGNMH decreased gradually in that order; this is consistent with the properties of the parental tRNAs.

Among the sequence variations of the four GGXMHs, we presumed that the susceptibility primarily depends on the 3′ proximal ribonucleotide of the GU sequence, i.e. A, C, G or U at position 12 of the 19mer GGXMHs. Then, the susceptibilities of YMH and its variants, in which A10 was replaced with C, G or U, were compared (Figure 3B). The susceptibility decreased in the following order: YMH >> YMH(A10C) > YMH(A10G) > YMH(A10U). The order of susceptibility among the four YMH variants with only a single nucleotide change at position 10 was highly comparable with the order of susceptibility among four GGXMHs with corresponding anticodons.

Is the susceptibility affected by the ribonucleotide 5′ proximal to the GU sequence that is invariably U in all *E.coli* tRNAs (19)? We have observed already that YMH(U7C) is as susceptible to E5-CRD as YMH (Figure 2A, lanes 3 and 4 versus lanes 1 and 2). Figure 3C confirms this observation and, furthermore, shows that the susceptibilities of the other two variants YMH(U7A) and YMH(U7G) were extremely diminished. This indicates that E5-CRD exclusively prefers a pyrimidine to a purine ribonucleotide at the 5′ side of the GU sequence.

Cleavage of oligoribonucleotides by E5-CRD

The results with the MHs suggested that the substrate specificity of E5-CRD is determined only by a local sequence, including the GU sequence, within an anticodon loop. Does E5-CRD cleave linear RNAs? A YMH variant, YMHL, was prepared by changing the sequence of the 3′ strand of the stem so as to avoid helix formation. YMHL was found to be cleaved by E5-CRD; however, the rate was lower than that in the case of YMH (Figure 3D). The suggestion that a linear RNA can be a substrate of E5-CRD raised the next question: does E5-CRD cleave a guanylyl-uridine dribonucleotide as the ultimate structure of GU-containing sequences? To answer this, four dinucleotides with a 3′ phosphate, GpUp, ApUp, GpCp and UpGp, and those lacking 3′ phosphate, GpU, ApU, GpC and UpG, were incubated with E5-CRD, and then the products were analysed by HPLC on a reversed-phase column. Among these, only GpUp was cleaved by E5-CRD (Figure 4A), yielding 2′,3′-cyclic GMP and 3′-UMP (Figure 4B). The replacement of the 5′ G with an A or the 3′ U with a C as well as the exchange of
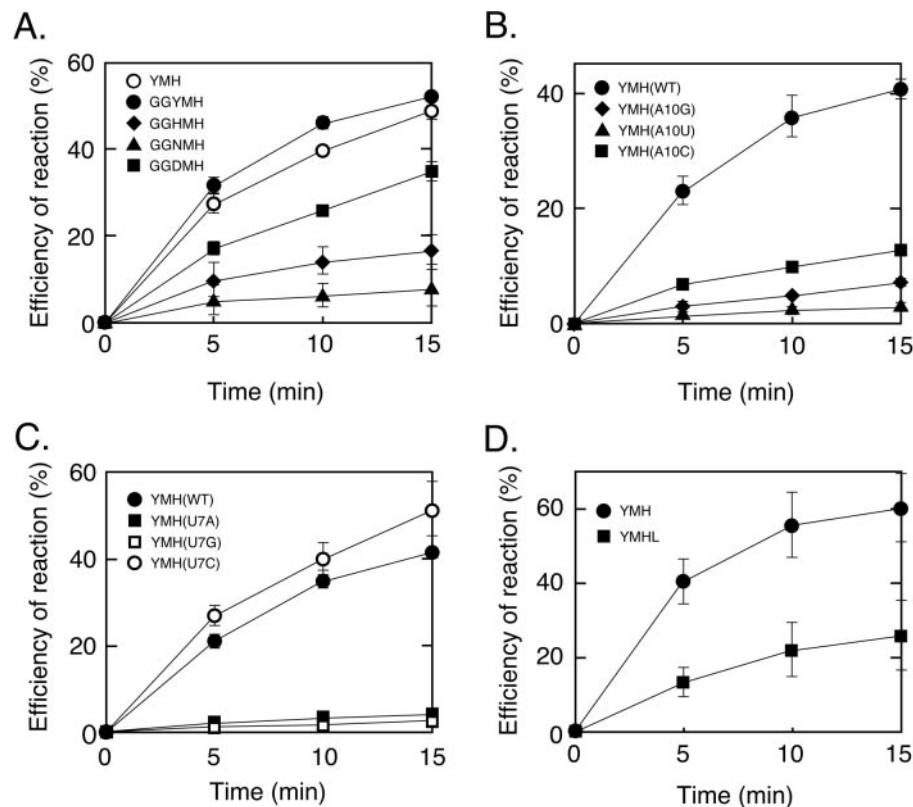
Figure 3. Time-dependent cleavage of various minihelices by E5-CRD. Various minihelices, summarized in Figure 1, were incubated and the extent of cleavage was examined at each time. (**A**) Minihelices GGYMH, GGHMH, GGNMH and GGDMH that mimicking tRNA[Tyr], tRNA[His], tRNA[Asn] and tRNA[Asp], respectively, were used. YMH was used as a control, and it was confirmed that $5'$ extended two guanine residues do not affect the susceptibility. (**B**) A mutation was introduced at A10 of YMH, and the resulting YMH, YMH(A10G), YMH(A10U) and YMH(A10C) were used as substrates. (**C**) A mutation was introduced at U7 of YMH, and the resulting YMH, YMH(U7C), YMH(U7A) and YMH(U7G) were used as substrates. (**D**) YMH and its derivative, YMHL, with the stem structure broken, were used. Error bars indicate the standard deviation of three independent assays.

G and U rendered the dinucleotide resistant to E5-CRD. These results indicate that GpUp conserves the same cleavage specificity as those in tRNAs. Using the GpUp oligo-nucleotide as the minimal substrate, we determined the pH dependence of the kinetic constants of E5-CRD, and revealed that both k_{cat} and K_m increases with pH (Figure 5A and B). However, the extent of increase in k_{cat} is much higher than that in K_m, and k_{cat}/K_m is the highest at pH 9.0 (Figure 5C).

Effect of the presence of ribonucleotide(s) adjacent to GpUp on cleavage

In the case of an MH, the ribonucleotide contiguous to the GU sequence significantly influenced the susceptibility. In order to determine the dependence of the enzymatic activity on the sequence of substrate RNAs, kinetic constants of E5-CRD were determined for several oligoribonucleotides containing the GpU sequence (Table 1). UpGpUpA was, in fact, the most susceptible among all oligonucleotides studied here. However, contrary to our expectation, the extent was almost the same as that of GpUp. UpGpUpA and GpUp were comparable in their k_{cat}/K_m values; however, both k_{cat} and K_m values of UpGpUpA nearly doubled those of GpUp; this suggested that the susceptibilities of GpUp and UpGpUpA are not the same intrinsically.

The $3'$ extension of GpUp with A alone largely decreases k_{cat}/K_m, and the $5'$ extension with Up by itself does not improve k_{cat}/K_m. In addition, the $5'$ extension with Ap, when compared to that with Up, does not decrease the k_{cat}/K_m value as expected from the decreased susceptibility in terms of the MH (Figure 3C). These results suggest that the extension of a ribonucleotide to each side of GpUp by itself do not improve or, in some cases, even diminish the ability to be cleaved by E5-CRD.

We also examined GpU. However, as seen in Figure 4A, GpU was not cleaved at all under the condition employed, as expected. When trinucleotides with the $5'$ extension of Up to GpU and GpUp were compared, the k_{cat}/K_m of UpGpU was much smaller than that of UpGpUp, mainly due to the decrease in the k_{cat} value. These findings indicate that the $3'$ phosphate of GpUp is essential for cleavage by E5-CRD.

DISCUSSION

We have shown that an MH corresponding to the anticodon arm of tRNA[Tyr] is effectively cleaved by E5-CRD at the same position where colicin E5 cleaves target tRNAs. Analysis using various MH derivatives as substrates revealed the critical role of the GU sequence at the cleavage site,
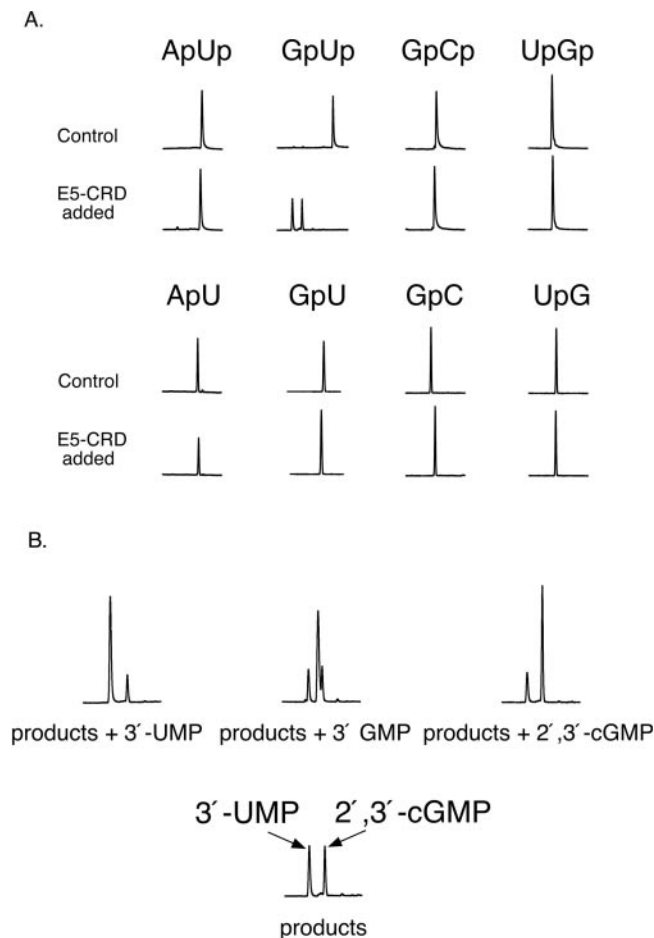
Figure 4. Cleavage of oligonucleotides by E5-CRD. (**A**) Various diribonucleotide diphosphates (125 μM) or those lacking 3′ phosphate (125 μM) were incubated with or without 50 nM of E5-CRD for 15 min at 37°C. In order to stop the reaction, 250 nM of ImmE5 was added. Then, the reaction solutions were directly applied on a reversed-phase column and the products were analysed. The upper (Control) and lower (E5-CRD added) charts of each panel were adjusted for retention times. (**B**) After the incubation of E5-CRD and GpUp, the reaction solution was mixed with authentic 3′-UMP, 3′-GMP or 2′,3′-cyclic GMP and applied on the column to compare retention times. 3′-UMP and 2′,3′-cyclic GMP were produced from GpUp by the cleavage reaction of E5-CRD.

corresponding to the first and the second letters of the anticodon (Figure 2). Furthermore, we revealed that the dinucleotide GpUp is a good substrate that conserves the recognition properties for E5-CRD. From these findings, we concluded that E5-CRD is an 'RNA restriction enzyme' that recognizes the GU sequence, preferentially in the anticodon loop, discriminating target tRNAs from other tRNAs (9,20,21). Dinucleotide GpU is barely cleaved by E5-CRD under the conditions employed in this study, and the 3′ phosphate of U was shown to be essential in the catalytic reaction affecting the k_{cat} value.

When MHs were used as substrates of E5-CRD, the 5′ pyrimidine to GU was essential (Figure 3C) and the 3′ A to GU was preferred to the 3′ C, G and U, with decreasing susceptibility in that order (Figure 3B), accounting for the differences in the susceptibility of *E.coli* tRNAs for Tyr, His, Asn and Asp. This implies that the tetranucleotide

YGUN (where Y is a pyrimidine and N is any nucleotide) at −1 to +3 of anticodons mostly determines the susceptibility of the tRNAs to E5-CRD. The E5-CRD-resistant KMH could be converted to E5-CRD-sensitive only by a single mutation, U8G. However, as described, the cleavage was not completely comparable to the authentic YMH (Figure 2A, lanes 11 and 12 versus lanes 1 and 2). This is probably because the U at the third letter of the anticodon of MH is the 'worst' nucleotide at this position for cleavage by E5-CRD. In Figure 2A, a lower molecular weight band indicated with an asterisk is shown. This proved to have been released from the 5′ end of KMH(U8G) through cleavage between G1 and U2 (Figure 2B, lane 9). The same cleavage at the 5′ end was also detected, but only faintly, for the reaction product of KMH whose anticodon loop is resistant to E5-CRD (Figure 2A, lanes 9 and 10). We interpreted that the 5′ terminal GU of KMH undergoes only limited denaturation, but that of KMH(U8G) becomes single-stranded and more sensitive to E5-CRD after the cleavage of the anticodon by E5-CRD.

Then, oligonucleotides were studied in contrast to MHs, the sequence GpUp was the minimum and almost the best substrate of E5-CRD that retained its sequence preference (Table 1). The 5′ extension of Up or the 3′ extension of A to GpUp were expected to enhance the susceptibility to E5-CRD as in the MH. However, they did not increase k_{cat}/K_m. Even UpGpUpA exhibited k_{cat}/K_m values comparable with those of GpUp; although, the k_{cat} and K_m values of UpGpUpA were slightly higher than those of GpUp. On the contrary, when the 5′ Up of UpGpUpA was replaced with the 'bad' nucleotide, Ap, the k_{cat}/K_m value decreased to only approximately half the original; this is not consistent with the drastic decrease in the susceptibility of YMH(U7A) (Figure 3C). These results suggest that nucleotides contiguous to the GU sequence are not positively involved in and, in some cases, may even be obstructive to the enzyme-substrate binding. What causes the inconsistency between the MHs and the oligonucleotides? The GU sequence within a loop structure may generally be preferred by E5-CRD to those within the linear RNA, as suggested in Figure 3D. In addition, most plausibly, the local conformation around the GU sequence in the loop, which may vary accoring to the nucleotides contiguous to GU, plays a role in the variation of susceptibility. In MHs, the loop is fixed by the stem structure, and the conformations of both the nucleotides contiguous to GU are limited. On the other hand, both the termini of an oligonucleotide are free-ended and undergo flexible conformation changes. This may also cause larger differences in the effect of changing the nucleotides contiguous to GU on MHs and oligonucleotides.

As a specific fixed structure, the U-turn is found in anticodon loops of tRNAs (22), tetra loops of GNRA (where N is any nucleotide and R is a purine nucleotide) (23) and some other RNA molecules (24–27). The U-turn is a sharp turn of a phosphate backbone at the conserved U33 of tRNAs and is reported to contribute to tRNA-ribosome binding (28,29). The U33 in tRNA^{Tyr} corresponds to U7 of YMH used in this study. The drastic loss of susceptibility to E5-CRD caused by a base change of U7 to A or G of YMH (Figure 3C) is possibly due to the destruction of the U-turn fold. However, at present, it cannot be concluded
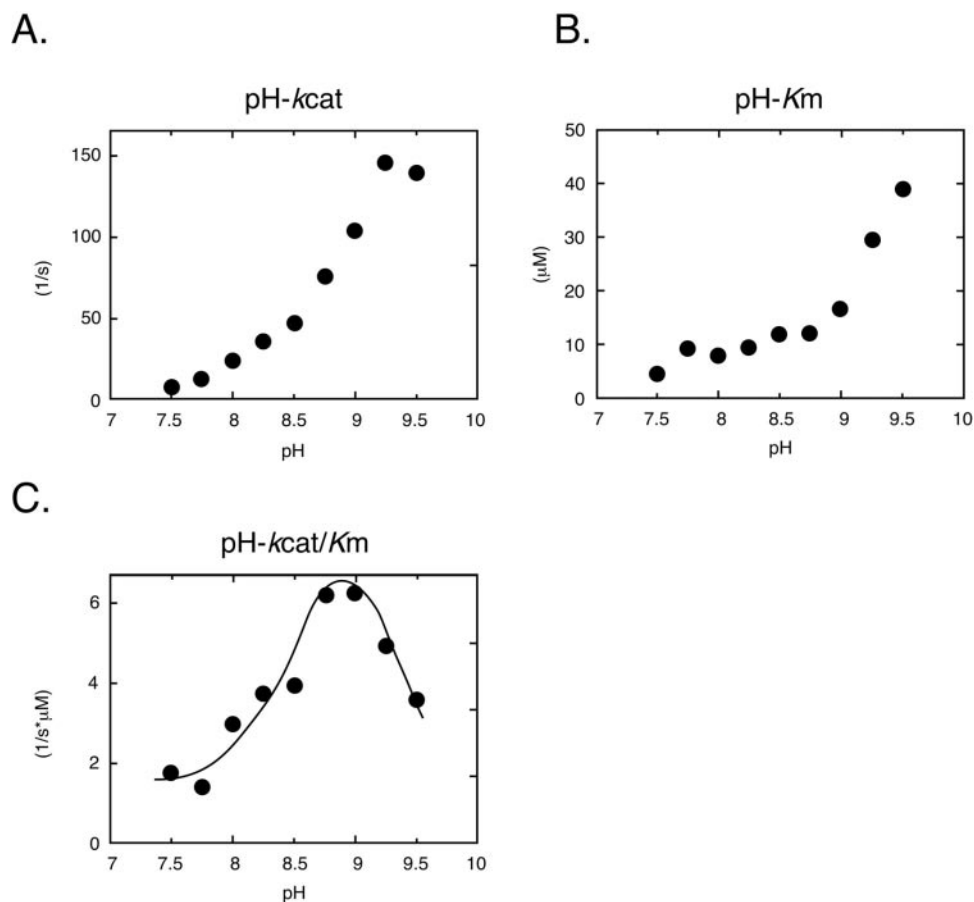
A.

pH-$k$cat

B.

pH-Km

C.

pH-$k$cat/Km

Figure 5. pH dependence of the kinetic constants of E5-CRD with GpUp as the substrate. (**A**) k_{cat} values are plotted against pH. (**B**) K_m values are plotted against pH. (**C**) k_{cat}/K_m values are plotted against pH.

Table 1. Kinetic parameters of E5-CRD towards various oligoribonucleotides

Substrate	K_m (μM)	K_{cat} (S^{-1})	K_{cat}/K_m ($\times10^6$ M^{-1} S^{-1})
GpU	ND	ND	ND
GpUp	32.9 (5.5)	42.9 (3.2)	1.30 (0.13)
UpGpU	143 (38)	3.57 (0.34)	0.0251 (0.0047)
UpGpUp	41.3 (16)	34.0 (3.5)	0.823 (0.23)
GpUpA	53.6 (16)	11.7 (1.1)	0.218 (0.046)
UpGpUpA	59.5 (18)	84.2 (7.1)	1.41 (0.32)
ApGpUpA	90.6 (23)	65.8 (6.4)	0.73 (0.12)

Numbers in parentheses indicate the standard errors obtained from $n > 16$ experiments. ND, not detected.

that the U-turn is necessary to serve as a good substrate of E5-CRD, since the change of U7 to C also retained the susceptibility. Recently, we found that the E5-CRD structural preference for small RNA loops containing GU was demonstrated by an *in vitro* selection method (Y. Harada, T. Ogawa, H. Masaki, S. Yokoyama and I. Hirao, manuscript in preparation).

When growing *E.coli* cells were challenged by colicin E5, QU in the anticodon loop of the target tRNAs was specifically cleaved, but GU sequences in other parts of the tRNA molecules were not (9). However, E5-CRD can cleave a GU in a single-stranded linear RNA (Figure 3D), although with less efficiency, and it also cleaves a GpUp dinucleotide (Figure 4A). Then, why are the GU sequences in mature

tRNA molecules, other than the QU in anticodons, not cleaved? Since tRNA molecules are tightly packed, the anticodon loop is the only naked part and other single-stranded parts such as the TΨC-loop and D-loop are folded into the L-shape form. Therefore, we assume that the bases in the TΨC-loop and D-loop are not easily accessible to E5-CRD from outside the tRNA molecule and only the anticodon loop is cleaved. We determined recently the tertiary structure of E5-CRD bound to a substrate analogue dGpdUp by crystal structure analysis (S. Yajima, S. Inoue, T. Ogawa, T. Nonaka, K. Ohsawa and H. Masaki, accompanying paper). The active site forms a narrow cleft on the surface of E5-CRD, to which small protruding loops such as the anticodon loop are suggested to fit. In the resolved structure, the nucleotide conformations are *syn* and *anti* for dG and dU, respectively. The guanine has three hydrogen bonds with Val103, and stacks with the indole ring of Trp102; while the uracil has hydrogen bonds with Ser52, Phe53 and Lys55, and stacks with the pseudo-ring formed by Asp105 and Arg107 (Figure 6). The Q base of tRNAs was implicated to extend its modified group at N7 of G without interacting with E5-CRD. This is consistent with our observation that colicin E5 does not distinguish G and Q bases.

The idea that the tight conformation of tRNA molecules contributes to the anticodon-specific activity of E5-CRD is also supported by other findings. When E5-CRD was incubated with *in vitro*-transcribed tRNAs devoid of modified
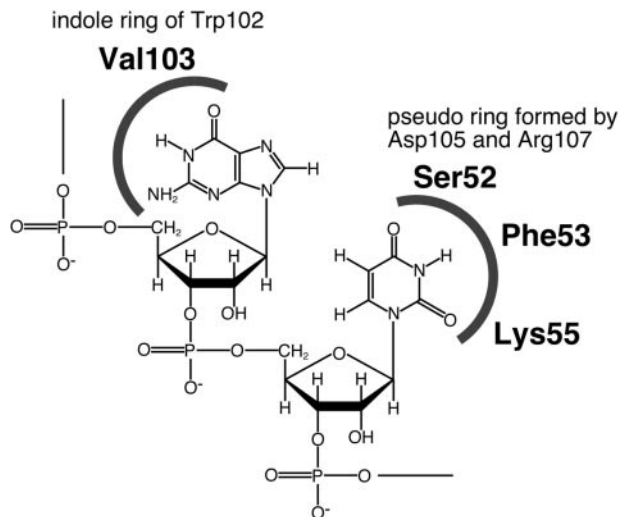
Figure 6. Schematic representation of the substrate recognition of E5-CRD. Amino acids forming hydrogen bonds are indicated in boldface. In the crystal structure, guanine (*syn* form) and uracil (*anti* form) bases are found to stack with the indole ring of Trp102 and the pseudo ring formed by Asp105 and Arg107, respectively.

bases, weaker cleavage at several positions, including GU, was observed; however, the anticodon loop was the most sensitive (data not shown). The conformation of the tRNA molecule is stabilized by the tertiary interactions maintained by modified bases (30,31). Therefore, the extra cleavages by E5-CRD are possibly caused by the less tight conformations of *in vitro*-transcribed tRNAs. The finding that an *in vitro*-transcribed tRNA exposes additional recognition sites is also reported for the recognition of UGU in the anticodon loop by tRNA-guanine transglycosylase (TGT: 32). Kung *et al.* (33) demonstrated that *E.coli* TGT could recognize UGU located in the TΨC arm of *in vitro*-transcribed yeast tRNA[Phe], but not when the wild-type tRNA[Phe] with modifications was used.

Recently, Lin *et al.* (34) reported that E5-CRD, which lacks the N-terminal seven amino acids as compared with that of our construct, recognizes the GU residues of a substrate MH. Our present results are consistent with those of the authors; however, we have shown that the dinucleotide GpUp is the ultimate specific substrate of E5-CRD. We also suggested that the nucleotides that extended to both sides of the GU are not positively involved in binding to E5-CRD; however, at least in the anticodon stem–loop context, there appears to be a wide range of preference of nucleotides at both sides of the GU sequence, which explains the extent of preference of the four actual tRNA species. The above-mentioned authors concluded that replacement of the first U of UGU with C decreases the cleavage efficiency, but this is not in agreement with our result (Figure 3C). They carried out an alanine scan of all acidic and basic residues around the putative RNA binding cleft of E5-CRD. Furthermore, they solved the crystal structure of E5-CRD, and the structure of the anticodon-arm of tRNA[Phe] was manually docked into the cleft of E5-CRD. From these results, they suggested that D97 and R99, corresponding to D105 and R107 of E5-CRD constructed by us, respectively, were involved in the recognition of G34. But, in our model, D105 and R107

contribute to the recognition of U35 by stacking of the pseudo-ring formed by two residues.

The specific activity of E5-CRD increases in a basic pH range, though higher affinity to the substrate GU sequences is observed under the physiological pH condition as seen in lower K_m values (Figure 5). We are proposing a novel enzymatic mechanism accounting for the unique pH dependence of the reaction (S. Inoue, S. Yajima, T. Ogawa, M. Hidaka and H. Masaki, manuscript in preparation). In a way, it is interesting to know that such suboptimal level of the tRNase activity is sufficiently strong for colicin E5 to cleave four target tRNAs and to cause sensitive cells to die. Why did colicin E5 acquire such specificity? In order to possess stronger ability to kill other cells, it would be favourable if E5-CRD had a nonspecific RNase activity instead of such a specific one. This is also the case with other RNase-type colicins such as E3, E4 and E6 that cleave 16S-rRNA at the 49th phosphodiester bond from the 3′ end. The other tRNase-type colicin, namely, colicin D also exhibits a narrow substrate specificity that only cleaves tRNA[Arg] isoacceptors (35). Anticodon nuclease (ACNase), found in a naturally isolated *E.coli prr*⁺ strain and activated by T4 phage infection also exhibits tRNA[Lys]-specific RNase activity (36–38). ACNase, which is known as a suicidal enzyme, plays a specific role in killing the host cells. Recently, Lu *et al.* (39) revealed that zymocin—a type of killer toxin produced by the yeast *Kluyveromyces lactis*—targets tRNA[Glu]mcm⁵s²UUC, tRNA[Lys]mcm⁵s²UUU and tRNA[Gln]mcm⁵s²UUG. Why are these enzymes specific? It is intriguing to postulate the existence of some ancestral RNases, the roles of which were specialized to regulate the expression of certain genes by cleaving their transcripts. Later, these RNases might have integrated as ribonuclease domains into colicins, ACNase or zymocin, and have evolved the current features. This hypothesis allows us the supposition of the 'tRNase family' of which these enzymes are members (20). At present, we may be aware of only some of these enzymes; however, in the future, we may be able to determine all tRNases.

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