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Specific Interaction between Anticodon Nuclease and the tRNA^{Lys} Wobble Base

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The bacterial tRNA^{Lys}-specific PrrC-anticodon nuclease cleaves its natural substrate 5′ to the wobble base, yielding 2′,3′-cyclic phosphate termini. Previous work has implicated the anticodon of tRNA^{Lys} as a specificity element and a cluster of amino acid residues at the carboxy-proximal half of PrrC in its recognition. We further examined these assumptions by assaying unmodified and hypomodified derivatives of tRNA^{Lys} as substrates of wild-type and mutant alleles of PrrC. The data show, first, that the anticodon sequence and wobble base modifications of tRNA^{Lys} play major roles in the interaction with anticodon nuclease. Secondly, a specific contact between the substrate recognition site of PrrC and the tRNA^{Lys} wobble base is revealed by PrrC missense mutations that suppress the inhibitory effects of wobble base modification mutations. Thirdly, the data distinguish between the anticodon recognition mechanisms of PrrC and lysyl-tRNA synthetase.

Keywords: modified nucleosides; lysyl-tRNA synthetase; RNA-protein interaction; RNA processing; trimethylamine N-oxide (TMAO)

Introduction

A tRNA^{Lys}-specific anticodon nuclease (ACNase) in latent form exists in *Escherichia coli* strains containing the optional locus *prr* (Kaufmann, 2000). Latent ACNase comprises the core polypeptide PrrC and type I DNA restriction-modification enzyme EcoprrI that stabilizes PrrC and masks its activity (Levitz *et al.*, 1990; Linder *et al.*, 1990; Amitsur *et al.*, 1992; Tyndall *et al.*, 1994). A phage T4-coded peptide termed Stp inhibits EcoprrI DNA restriction activity and activates ACNase (Penner *et al.*, 1995). The consequent cleavage of tRNA^{Lys} 5′ to the wobble base leaves 2′,3′-cyclic phosphate and 5′-OH termini. However, the phage-encoded RNA repair enzymes polynucleotide kinase and RNA ligase normally offset the damage. The first enzyme removes the cyclic phosphate group from one terminus and phosphorylates the other; the second enzyme joins the processed ends, restoring tRNA^{Lys} (Amitsur *et al.*, 1987).

Specific cleavage of tRNA^{Lys} in mammalian cells expressing recombinant PrrC suggests that PrrC harbors the ACNase site (Shterman *et al.*, 1995; and unpublished results). In agreement, a tRNA^{Lys} recognition site has been located in PrrC by adjacent mutations that alter ACNase cleavage site specificity and substrate preference (Meidler *et al.*, 1999). The cognate site in tRNA^{Lys} comprises most likely the anticodon sequence. This is inferred from features shared by the eukaryotic and *E. coli* tRNA^{Lys} substrates of ACNase (Shterman *et al.*, 1995) and from the presence of anticodon sequences resembling that of tRNA^{Lys} in most quasi-substrate tRNAs cleaved when PrrC is highly over-expressed in *E. coli* (Meidler *et al.*, 1999).

The specificity of ACNase was investigated here more closely by assaying unmodified and hypomodified derivatives of tRNA^{Lys} as substrates of wild-type and mutant alleles of PrrC. The data indicate, first, that the anticodon sequence and wobble base modifications play major roles in the interaction of tRNA^{Lys} with ACNase. Secondly, PrrC missense mutations that suppress the negative effects of wobble base modification mutations reveal a specific interaction between the protein and the RNA ligand. Finally, the results distinguish between the anticodon recognition mechanisms of ACNase and lysyl-tRNA synthetase (LysRS).

Abbreviations used: ACNase, anticodon nuclease; LysRS, lysyl-tRNA synthetase; aaRS, aminoacyl-tRNA synthetase; TMAO, trimethylamine N-oxide.

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Results

Unmodified tRNA^{Lys} as an ACNase substrate

Unmodified tRNA sequences help pinpoint tRNA identity elements of aminoacyl-tRNA synthetases (aaRS) (Sampson & Uhlenbeck, 1988). The absence of the modifications has impaired the aminoacylation of certain species, including *E. coli* tRNA^{Lys}. However, the residual reactivity of unmodified tRNA^{Lys} has sufficed for comparing it with mutant derivatives and allowed conclusions to be drawn relevant to the interaction of tRNA^{Lys} with the cognate aaRS (Tamura *et al.*, 1992). To find out if unmodified tRNA^{Lys} can be likewise used as a reference substrate of ACNase, the unmodified and modified molecules were compared in a standard *in vitro* core ACNase assay (see Materials and Methods). In this assay, one-third of the radioactivity of the natural substrate was converted into the labeled product, fragment 1-33 (Figure 1(a), lane 3; Table 1). Unmodified tRNA^{Lys} yielded trace amounts of a similar fragment (Figure 1(a), lane 6; Table 1). However, the extent of cleavage of the unmodified molecule could be increased considerably by adding trimethylamine N-oxide (TMAO) to the reaction mixture (Figure 1(a), compare lanes 6 and 12; Table 1). TMAO is a general protein-folding agent (Baskakov & Bolen, 1998) and stabilizes PrrC-core ACNase in its active, oligomeric form (our unpublished results). The labeled cleavage product of unmodified tRNA^{Lys} was identified then as fragment 1-33 by RNA sequencing (not shown). Its electrophoretic mobility, between analogues carrying terminal 3′-OH or 3′-P groups (Figure 1(b), compare lane 1 with lanes 3 and 4), suggested the presence of a 3′-terminal cyclic phosphate group. In agreement, treatment of the product with phage T4 polynucleotide kinase that harbors also 3′-phosphodiesterase and 3′-phosphatase activities (Cameron & Uhlenbeck, 1977; Amitsur *et al.*, 1987), reduced the mobility of the treated product, shifting it to the position of the 3′-OH-containing marker (Figure 1(b), compare lanes 2 and 4). Hence, ACNase cleaved natural and unmodified tRNA^{Lys} at the same position, producing similar cleavage termini.

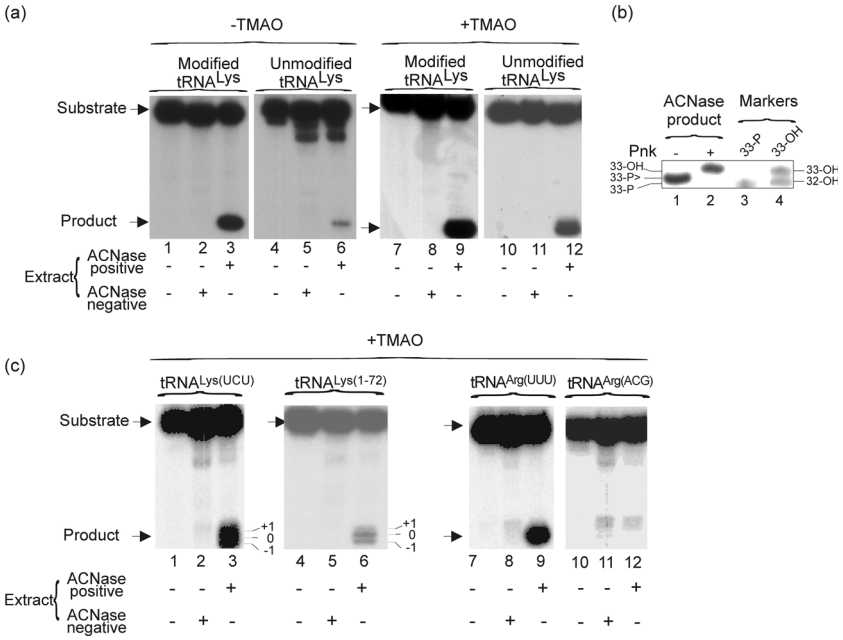

Figure 1. Unmodified tRNA^{Lys} and mutant derivatives as substrates of ACNase. (a) Comparison of modified and unmodified tRNA^{Lys}. *E. coli* tRNA^{Lys} labeled with ^{32}P in the cleavage junction (lanes 1-3, 7-9) or (5′-^{32}P)-labeled, *in vitro* transcribed tRNA^{Lys} (lanes 4-6, 10-12) were incubated under core ACNase assay conditions, as such (lanes 1, 4, 7, 10) with a control ACNase-negative extract (lanes 2, 5, 8, 11) or with ACNase-containing extract (lanes 3, 6, 9, 12). Incubation was without (lanes 1-6) or with 1 M TMAO (lanes 7-12). The products were separated by denaturing polyacrylamide gel electrophoresis and monitored as detailed in Materials and Methods. (b) Characterization of the cleavage product of unmodified tRNA^{Lys}. The unmodified product of Figure 1(a), lane 12, was separated as such (lane 1) or after being subjected to the 3′-phosphodiesterase and phosphatase activities of T4 polynucleotide kinase (lane 2). Unmodified tRNA^{Lys} fragments 1-33 with 3′-P (lane 3) or 3′-OH (lane 4) served as markers. Pnk indicates T4 polynucleotide kinase; 3′-OH, 3′-P and 3′-P> indicate fragments 1-33 of unmodified tRNA^{Lys} carrying 3′-terminal hydroxyl, phosphate or cyclic phosphate groups, respectively. The fragment 1-33 marker carrying 3′-OH is contaminated by the faster migrating, corresponding fragment 2-33 (Materials and Methods). (c) Unmodified tRNA^{Lys} derivatives and chimerical unmodified tRNA as ACNase substrates. The ACNase reactions were performed with the indicated substrates in the presence of TMAO essentially as described above.

Table 1. Unmodified tRNA^{Lys} derivatives as ACNase substrates

tRNA "body"	Anticodon	Source	TMAO	Extent cleavage (%)
Lys	mnm^{5}s^{2}UUU	*a*	-	33 ± 4
	UUU	*b*	-	0.3 ± 0.1
	mnm^{5}s^{2}UUU	*a*	+	43 ± 6
	UUU	*b*	+	16 ± 3
	CUU	*c*	+	0.5 ± 0.1
	AUU	*c*	+	nd[a]
	GUU	*b*	+	nd
	UCU	*c*	+	20 ± 4
	UAU	*c*	+	nd
	UGU	*c*	+	0.1 ± 0.05
	UUA	*c*	+	0.3 ± 0.1
	UUC	*c*	+	0.4 ± 0.1
	UUG	*c*	+	0.1 ± 0.05
Arg	UUU	*b*	+	16 ± 4
	CCG	*b*	+	0.1 ± 0.05
	CCU	*b*	+	1.2 ± 0.4
	ACG	*b*	+	nd
Lys	ACG	*b*	+	nd
Val	UUU	*b*	+	nd
	UAC	*b*	+	nd
Lys	UAC	*b*	+	nd
tRNA species	Allele	Source	TMAO	Extent cleavage (%)
Lys	A73	*b*	+	16 ± 3
	A73→G	*b*	+	7.5 ± 2
	A73→C	*b*	+	7.4 ± 2
	A73→U	*b*	+	2.5 ± 1
	1-72 (deletion)	*c*	+	11 ± 3

Core ACNase assays were performed with the indicated substrates as described in Materials and Methods. *a*, Amitsur *et al.* (1989). *b*, Template plasmids described by Tamura *et al.* (1992). *c*, This work. nd, Not detectable, i.e. <0.1 % over background.

Effects of single base substitutions in the tRNA^{Lys} anticodon on ACNase reactivity

The improved cleavage of unmodified tRNA^{Lys} by ACNase in the presence of TMAO (Figure 1(a), lanes 6 and 12) enabled us to compare the wild-type sequence with various analogues listed in Table 1. Most single base substitutions in the anticodon sequence inhibited ACNase activity. Thus, replacing the wobble base U34 by C reduced the extent of cleavage ~30 fold. Introduction of G or A in this position abolished the activity (Table 1). U35 → C, the only mutant that was fully reactive, yielded comparable amounts of the normal and two additional products. The latter arose, most likely, by one-residue shifts of the cleavage site in either direction (Figure 1(c), lane 3). The U35 → G mutation reduced ACNase reactivity by two orders of magnitude and U35 → A abolished it. The substitutions of U36 diminished the extent of cleavage between 40 and 150-fold, in a C, A → G order of intensity (Table 1).

Chimerical tRNA constructs carrying heterologous anticodons as ACNase substrates

Comparing unmodified tRNA^{Lys} and tRNA^{Arg1} sequences with corresponding chimerical constructs containing transplanted anticodons reinforced the importance of the anticodon sequence for ACNase reactivity. Unmodified tRNA$^{Arg1(UUU)}$ (carrying the tRNA^{Lys} anticodon sequence) was as reactive as unmodified tRNA^{Lys} (compare Figure 1(a), lane 12 with Figure 1(c), lane 9; Table 1), while tRNA$^{Arg1\ (CCU)}$ and tRNA$^{Arg1\ (CCG)}$ were one and two orders less reactive, respectively (Table 1). Interestingly, the presence of C in the second anticodon position of the latter two species did not relax the cleavage site specificity (not shown), unlike tRNA$^{Lys(U35 \rightarrow C)}$ (Figure 1(c), lane 3). Unmodified tRNA$^{Arg1(ACG)}$ and chimerical tRNA$^{Lys(ACG)}$ were both unreactive. In contrast to tRNA$^{Arg1(UUU)}$, tRNA$^{Val1(UUU)}$ was inert and so were unmodified tRNA^{Val1} and chimerical tRNA$^{Lys(CAU)}$ with the Val anticodon. Since the unmodified anticodon loops of tRNA^{Lys}, tRNA$^{Arg1(UUU)}$ and tRNA$^{Val1(UUU)}$ are identical in sequence, other sequence or structural features shared by the former two and absent from the latter may also contribute to the interaction with ACNase.

Acceptor-end mutants of tRNA^{Lys} as ACNase substrates

Residue A73 of tRNA^{Lys}, called the discriminator, is a major identity element of LysRS (Crothers *et al.*, 1972; Tamura *et al.*, 1992; Giege *et al.*, 1998). Substitution of A73 by G or C reduced the extent of ACNase cleavage twofold and by U sevenfold (Table 1). Trimming the 3′-terminal ACCA overhang had little effect on the reactivity. However, this deletion relaxed the cleavage site specificity similar to the U35 → C mutation except for weaker cleavage at the upstream site (Figure 1(c), compare

lanes 3 and 6). These results suggest that the acceptor-end plays some role in the tRNA^{Lys}-PrrC interaction, albeit, not as prominent as the anticodon sequence.

Hypomodified tRNA^{Lys} lacking wobble base modifications as ACNase substrates

The low reactivity of unmodified tRNA^{Lys} (Figure 1(a), compare lanes 3 and 6) suggested that at least some of the missing modifications are important for ACNase activity. Particularly relevant seemed the modifications of the wobble base 5-methylaminomethyl-2-thiouridine (mnm^{5}s^{2}U) that abuts the cleavage junction. Expressing PrrC in *E. coli* mutants lacking the s^{2}U (*mnmA*) or mnm^{5}U (*mnmE*) modification (Hagervall *et al.*, 1998) allowed us to assess the contributions of these modifying groups.

A moderate level of PrrC expression, in which core ACNase cleaves tRNA^{Lys} mainly, was achieved with plasmid pRRC6 that encodes *prrC* under control of the strong T7 promoter but weak, indigenous translation initiation region (Morad *et al.*, 1993). A higher level that elicits cleavage of additional tRNA species was attained with plasmid pRRC11, which encodes *prrC* under control of the tightly regulated phage T7-*lac* promoter and strong translation-initiation-region of T7 gene *10* (Meidler *et al.*, 1999). The higher level of PrrC expression facilitated examination of partially active PrrC mutants. It also helped demonstrate changes in the cleavage preference of ACNase due to PrrC mutations or lack of the wobble base modifications. A vector corresponding to pRRC6 and a pRRC11 derivative encoding a PrrC null mutant provided ACNase-free backgrounds. PrrC expression was induced by infection with λ phage encoding T7 RNA polymerase (λ-CE6, Studier & Moffatt, 1986) and, in the case of pRRC11, D-β-isopropylthiogalactoside (IPTG). The 3′-proximal tRNA fragments generated *in vivo* by ACNase were 5′-end labeled *in vitro* and purified by denaturing gel electrophoresis.

As shown in Figure 2(a), among the pRRC11-transformants, the *mnmA* and *mnmE* mutants contained about tenfold less ACNase cleavage products than the isogenic wild-type host. Since the PrrC protein accumulated to a similar level in all three hosts, the reduced cleavage was attributed to poor reactivity of the hypomodified substrates. Strong inhibition of ACNase cleavage by the *mnmA* and *mnmE* mutations was evident also in the pRRC6-context but low signal-to-background ratios in the mutant hosts precluded determination of the degree of inhibition (not shown).

To monitor the major tRNA species cleaved by ACNase in the *mnmA* and *mnmE* mutants and isogenic background strain, the 5′-end labeled fragments were hybridized to dot blots of complementary oligonucleotides. These probes corresponded to residues 34-73 of tRNA^{Lys}, six different quasi-substrates (tRNA^{Glu}, tRNA$^{Gln1\&2}$, tRNA^{Arg1}, tRNA^{Asn}, tRNA^{Asp}) and the unreactive tRNA^{Metf}. It is noteworthy that the quasi-substrates tRNA^{Glu} and tRNA^{Gln1} also contain mnm^{5}s^{2}U (Krüger *et al.*, 1998). As expected, at the moderate level of PrrC expression (pRRC6 context), tRNA^{Lys} was the main substrate of ACNase in the wild-type host (Figure 3(a), Table 2). However, in both *mnmA* and *mnmE* hosts, the tRNA^{Lys} fragment constituted about a quarter of the total signal, confirming the assumption that the absence of either modification rendered tRNA^{Lys} a poor ACNase substrate. In the *mnmE* host, tRNA^{Glu} yielded more than twice the signal of tRNA^{Lys}, while fragments of other species were found in trace amounts. The most reactive species in the *mnmA* host was also tRNA^{Glu}. Two other quasi-substrates, tRNA^{Arg1} and tRNA^{Asp}, were cleaved to half and three-quarters the extent of tRNA^{Lys}, respectively. It should be pointed out that the preferred cleavage of quasi-substrates in the *mnmA* and *mnmE* hosts occurred at a tenfold lower overall extent of ACNase activity compared to the isogenic wild-type host. Therefore, the shift in substrate preference is ascribed to the low reactivities of the hypomodified forms of tRNA^{Lys} rather than their saturation by excess PrrC.

At the higher level of PrrC expression (pRRC11-context, Figure 3(b)), the tRNA^{Lys} fragment comprised less than half of the total ACNase product in the wild-type host. The remainder was distributed between tRNA^{Glu} (30 %), tRNA^{Gln1} (14 %) and smaller amounts of other quasi-substrates. In the *mnmA* cells, the tRNA^{Lys} signal dropped further to 30 %, close to that of tRNA^{Glu} (27 %); the remainder

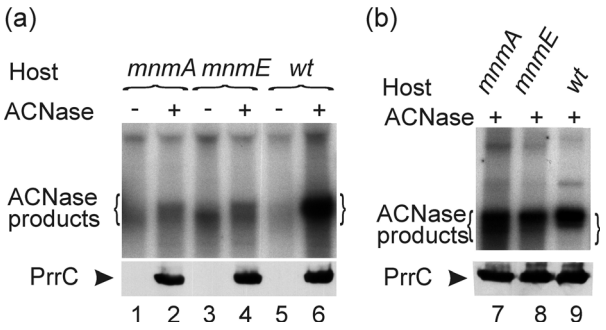

Figure 2. Effect of *mnmA* and *mnmE* mutations on the *in vivo* activity of wild-type and mutant PrrC. Pattern of ACNase products 5′-end labeled *in vitro*. Following induction of expression of (a) wild-type or (b) the D287Q alleles of PrrC from plasmid pRRC11 in the indicated host strains, low-mass RNA was extracted, 5′-end labeled and separated by denaturing polyacrylamide gel electrophoresis as described in Materials and Methods. A derivative of pRRC11 encoding a null mutant of PrrC provided the ACNase negative background. The level of PrrC expression was determined in parallel by immunoblotting. In the Western blot shown, the ACNase negative background is represented by cells devoid of PrrC rather than the null mutants of PrrC used for preparation of the RNA background in the upper panel.

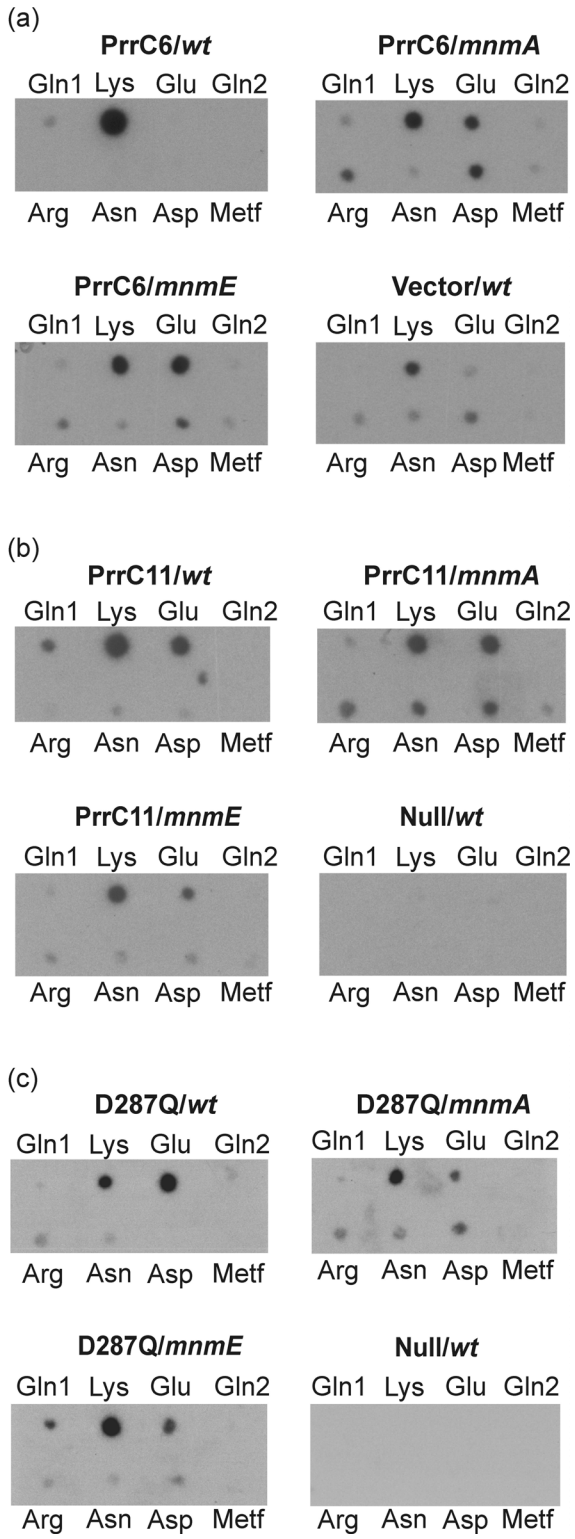

Figure 3. Identification of ACNase cleavage products generated in *E. coli* mutants deficient wobble base modifications. The labeled ACNase reaction products from the indicated PrrC expressing host cells isolated as described for Figure 2 were extracted from the gel and hybridized to dot blots containing DNA probes complementary to the 3′-portions of the indicated tRNA species. (a) pRRC6 context; (b) pRRC11-context; (c) pRRC11-D287Q.

was distributed between tRNA^{Asp}, tRNA^{Arg1} and tRNA^{Asn}, while tRNA^{Gln1} was not detectably cleaved. The *mnmE* profile resembled the wild-type, except that tRNA^{Lys} cleavage was enhanced at the expense of tRNA^{Gln1}. However, considering the tenfold lower overall extent of ACNase cleavage in the *mnmE* mutant relative to the isogenic wild-type strain (Figure 2(a)), the actual cleavage of quasi-substrates in the mutant host was taken to indicate that the mnm^{5}U-deficient tRNA^{Lys} was a poor substrate also in the pRRC11 context.

Another way to assess the reactivities of the hypomodified forms of tRNA^{Lys} was to compare the labeled 5′-terminal nucleotides of the 3′ fragments resulting from ACNase cleavage in the various hosts. These end-groups were released by nuclease P_1 digestion and separated by two-dimensional thin-layer chromatography. The amounts of the specific products were normalized with respect to the common nucleotides. The latter reflected, in part, non-specific RNA breakdown, seen also in the ACNase-negative controls and, in part, cleavage of certain quasi-substrates. The results indicated that the hypomodified end-groups were under-represented both at the low and the high expression levels of PrrC (Figure 4(a) and (b), respectively). The effect was more pronounced with the s^{2}U than mnm^{5}U end groups, suggesting that the missing 5-methylaminomethyl modification is more critical for ACNase reactivity. Interestingly, while only traces of s^{2}U were detected in the *mnmE* thin-layer pattern, the proportion of unmodified U was significantly higher than in the control and in other PrrC-expressing hosts. Presumably, the excessive U resulted from cleavage of tRNA^{Lys} or certain quasi- substrates 5′ to the second or third anticodon positions.

Some ACNase activity was expressed from pRRC11 even without induction of T7 RNA polymerase. This was evidenced by the presence of ACNase cleavage products in the respective transformants (not shown) and by the slower growth of the cells and exacerbation of this growth defect by IPTG (Figure 5). Since cells over-expressing a null allele of PrrC grew normally, the cytotoxicity was attributed to ACNase activity. Although the hypomodified substrates were poorly cleaved, the *mnmE* strain was sensitive to pRRC11, like wild-type cells, and the *mnmA* strain was even more sensitive.

Because the *mnmA* and *mnmE* mutations may exert complex effects *in vivo* (Hagervall *et al*., 1998; Cabedo *et al*., 1999), the respective hypomodified tRNA^{Lys} molecules were assayed as ACNase substrates also *in vitro*. Low-mass RNA isolated from the untransformed *mnmA*, *mnmE* and wild-type strains were 5′-end labeled. Sub-fractions enriched for tRNA^{Lys} were incubated then with core ACNase or control extracts. Comparison of the cleavage products indicated that the RNA from the *mnmE* strain yielded far less cleavage products than the *mnmA* counterpart (Figure 6(a), compare lanes 1 and 2), suggesting again that the 5-methyl-

Table 2. ACNase products in *E. coli mnmA* and *mnmE* strains expressing different PrrC alleles

PrrC allele	Host	tRNA species						
		Gln1	Lys	Glu	Gln2	Arg	Asn	Asp
D287H	*mnmA*	18 ± 4	58 ± 10	20 ± 6	-	-	2 ± 1	1 ± 0.8
	mnmE	14 ± 5	48 ± 8	22 ± 5	3 ± 2	5 ± 3	3 ± 1	5 ± 2
	Wild-type	1 ± 1	28 ± 6	50 ± 8	4 ± 2	11 ± 4	6 ± 3	-
D287 K	*mnmA*	5 ± 3	29 ± 5	29 ± 4	-	10 ± 3	10 ± 3	17 ± 6
	mnmE	5 ± 2	36 ± 6	37 ± 8	-	8 ± 3	3 ± 1	11 ± 3
	Wild-type	-	90 ± 3	7 ± 3	-	-	2 ± 1	-
D287N	*mnmA*	3 ± 2	49 ± 8	21 ± 5	6 ± 3	5 ± 2	7 ± 2	8 ± 3
	mnmE	10 ± 4	49 ± 9	21 ± 6	1 ± 0.5	5 ± 3	3 ± 1	11 ± 5
	Wild-type	3 ± 1	31 ± 5	30 ± 5	1 ± 0.5	11 ± 3	8 ± 2	16 ± 5
D287Q	*mnmA*	-	40 ± 5	16 ± 6	-	14 ± 2	10 ± 3	20 ± 5
	mnmE	11 ± 3	44 ± 6	22 ± 4	-	8 ± 3	4 ± 2	12 ± 4
	Wild-type	2 ± 1	28 ± 6	45 ± 6	7 ± 2	11 ± 3	6 ± 3	1 ± 1
Wild-type	*mnmA*	-	30 ± 5	27 ± 4	-	11 ± 2	10 ± 3	20 ± 3
	mnmE	-	57 ± 6	26 ± 5	2 ± 0.5	3 ± 1	4 ± 2	8 ± 3
	Wild-type	14 ± 3	43 ± 5	30 ± 4	2 ± 1	3 ± 1	5 ± 2	3 ± 2
Wild-type (pRRC6)	*mnmA*	1 ± 0.5	26 ± 4	37 ± 5	1 ± 0.5	13 ± 3	1 ± 0.5	20 ± 5
	MnmE	-	28 ± 4	68 ± 8	-	2 ± 1	-	1 ± 0.5
	Wild-type	3 ± 2	95 ± 3	-	-	-	-	-

PrrC mutant alleles were expressed in the pRRC11-context, wild-type also from pRRC6. The 3′ tRNA fragments generated by ACNase were 5′-end-labeled *in vitro* and hybridized to dot blots as in Figure 3. Hybridization efficiency is presented as percentage of the overall signal corrected for background hybridization. Dash indicates <1 % . All values for tRNA^{metf} were <1 %.

aminomethyl group is more critical to ACNase reactivity than the 2-thio group. On the other hand, the *in vitro* assay did not detect a difference between the extents of cleavage of the *mnmA* and wild-type RNAs (compare lanes 1 and 3). Partial RNase T_1 digestion yielded the typical tRNA^{Lys} ladder in each case, including the less-abundant product obtained with the *mnmE* RNA. Hence, tRNA^{Lys} seemed to be the major *in vitro* substrate in the three cases (Figure 6(b)).

Missense mutations in PrrC reverse the effect of the *mnmA* and *mnmE* mutations

Among PrrC mutations selected by loss of ACNase-dependent cytotoxicity, the adjacent mutations D287Y and S288P alter the cleavage-site specificity of ACNase and its order of substrate preference. These phenotypes and the presence of anticodon sequences similar to that of tRNA^{Lys} in most quasi-substrates have implicated the mutated residues with anticodon recognition (Meidler *et al.*, 1999). To examine this notion, we determined the cleavage patterns of several PrrC alleles carrying missense mutations at position 287 in the *mnmA*, *mnmE* and isogenic wild-type hosts. One of these mutants, D287Q, favored the mnm^{5}U-deficient derivatives of tRNA^{Lys} and tRNA^{Gln1} over the natural, fully modified forms (Figure 3(c) and Table 2). In the wild-type host, D287Q cleaved tRNA^{Glu} more efficiently than tRNA^{Lys} (45 *versus* 28 % of the overall signal, respectively) and weakly cleaved tRNA^{Gln1} (2 %). These preferences were opposite those of wild-type PrrC (30, 43 and 14 %, respectively, Figure 3(b), Table 2). However, in the D287Q- expressing *mnmE* cells, hypomodified tRNA^{Lys} was restored as the major substrate (44 %), while tRNA^{Glu} yielded only half this signal. Moreover, the proportion of the mnm^{5}U-deficient tRNA^{Gln1} cleaved by the D287Q mutant approached that of fully modified tRNA^{Gln1}/wild-type PrrC (11 *versus* 14 %). In the *mnmA* host, D287Q also restored tRNA^{Lys} to a predominant position (40 %); the remaining signal was distributed between comparable products of tRNA^{Glu}, tRNA^{Arg1}, tRNA^{Asn} and tRNA^{Asp}, while tRNA^{Gln1} was a trace signal. It is also noteworthy that D287Q elicited similar overall extents of tRNA cleavage in the three hosts (Figure 2(b)), contrary to wild-type PrrC that yielded a tenfold lower extent of cleavage in the mutant hosts. Hence, the compensatory effects of the D287Q mutation were absolute. Two other replacements, D287H and D287N, yielded similar phenotypes, although the latter was less pronounced. Interestingly, the charge-reversing mutation D287K did not alter the wild-type preferences significantly (Table 2).

Discussion

Role of the anticodon sequence in ACNase specificity

Substitution of single bases of the anticodon sequence of unmodified tRNA^{Lys} almost invariably inhibited ACNase activity (Table 1). Some of these mutant phenotypes deserve particular consideration. At the wobble base position, the extent of ACNase cleavage was reduced about 30-fold by the U34 → C replacement. Considering the positive contributions of the wobble base modifications to ACNase reactivity, it is expected that replacement of the natural wobble base by C would reduce the reactivity even further. Nonetheless, mammalian tRNA^{Lys3}, which contains a similar modified base at the wobble position (5-methoxycarbonylmethyl-

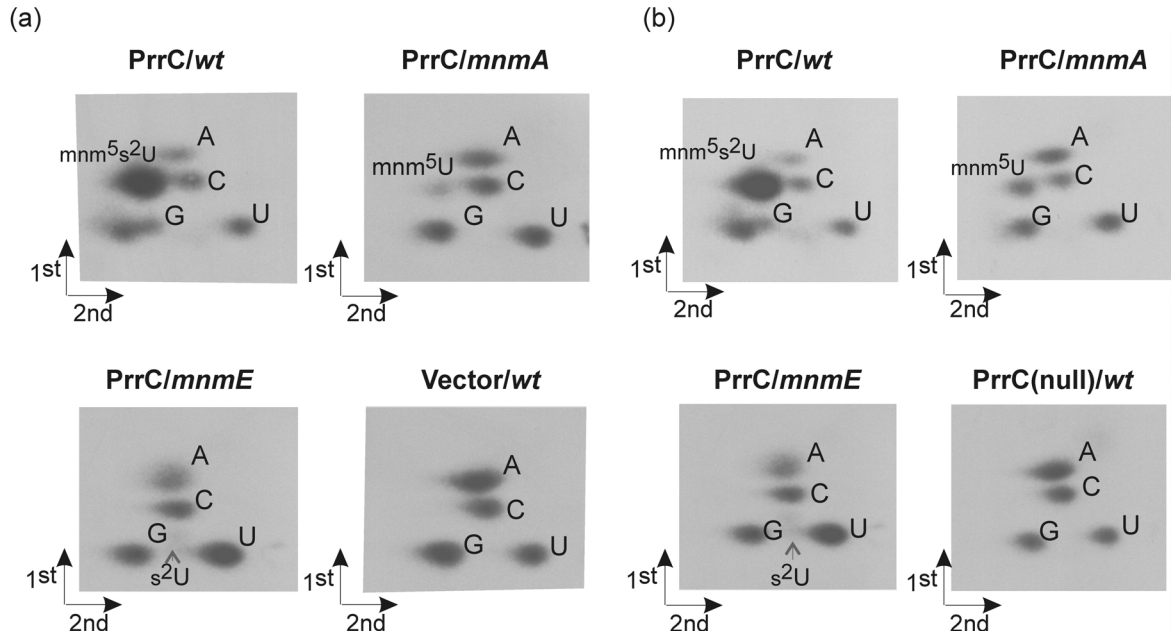

Figure 4. Determination of 5′-end groups of 3′-tRNA fragments produced by ACNase in *mnmA*, *mnmE* and isogenic wild-type *E. coli* strains. End-labeled tRNA fragments produced by ACNase in (a) pRRC6 or (b) pRRC11 transformants were extracted from the gel regions in indicated in Figure 2(a). These fragments were digested with nuclease P_1. The resultant mixtures of 5′-NMPs were separated by 2D thin-layer chromatography (Silberklang *et al.*, 1979).

2-thiouridine, mcm^{5}s^{2}U), is cleaved by ACNase less efficiently than tRNA$^{Lys1\&2}$, which contain C instead (Shterman *et al.*, 1995; Sprinzl *et al.*, 1998). If ACNase ignores the slight differences between the modified adenosine bases at position 37 of the different isoacceptors (2-methylthio-6-threonylcarbamoyladenosine [ms^{2}t^{6}A] in tRNA^{Lys3} *versus* 6-threonylcarbamoyladenosine [t^{6}A] in tRNA$^{Lys1\&2}$) and other differences outside the anticodon domain, then the distinctive mcm^{5}U modification may be largely responsible for the lower reactivity of tRNA^{Lys3}. In other words, the different wobble bases may confer ACNase reactivities in the decreasing mnm^{5}s^{2}U > U > C > mcm^{5}s^{2}U order.

The low reactivity conferred by the mcm^{5}U group may be attributed to differences in structure and/or hydrogen bond forming groups between it and the prokaryotic mnm^{5}U counterpart. Consequently, direct interaction between PrrC and the wobble base may be disrupted or weakened. An indirect effect of the mnm^{5}U → mcm^{5}U change through alteration of the overall anticodon loop conformation seems less likely, since both groups are thought to secure an anticodon conformation that is ready to bind the complementary mRNA codon on the ribosome (Sundaram *et al.*, 2000).

The U35 → C mutation did not reduce overall ACNase reactivity but relaxed the cleavage site specificity of ACNase (Figure 1(c)). Hence, the 4-keto group of U35 may be important for maintaining this specificity. That the 4-keto group interacts somehow with PrrC is suggested by retention of residual ACNase reactivity by the U35 → G mutant, as opposed to the inert U35 → A (Table 1). That is, the 6-keto group of G could provide a weak substitute for the 4-keto group of U35. However, the unmodified tRNA$^{Arg1(CCU)}$ and tRNA$^{Arg1(CCG)}$ derivatives that contain C at position 35 did not exhibit relaxed cleavage site specificity, indicating that cleavage site determination is more complex. Furthermore, relaxation of the cleavage site specificity was also caused by trimming the 3′ overhang of tRNA^{Lys} (Figure 1(c)) and by certain PrrC mutations (Meidler *et al.*, 1999). It remains to be established whether a common

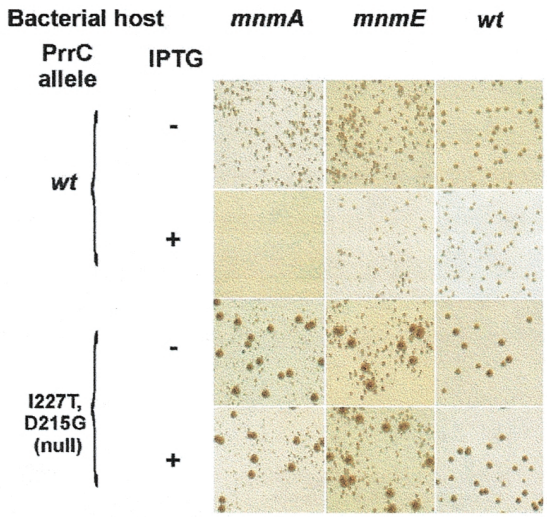

Figure 5. Effect of PrrC expression on cell growth of the *mnmA* and *mnmE* mutants. *E. coli* strains TH168 (*mnmA*), TH169 (*mnmE*) and TH170 (isogenic background) transformed with plasmid pRRC11 or derivative encoding a PrrC null mutant were plated on LB medium with or without 1 mM IPTG.

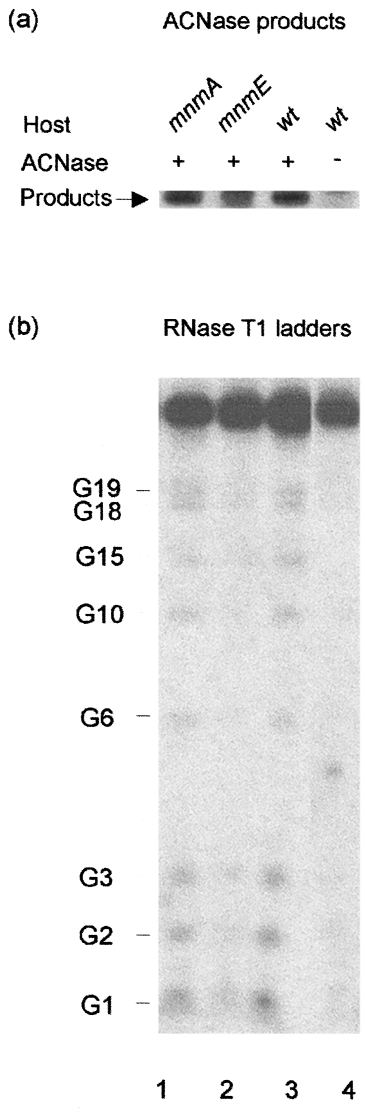

Figure 6. *In vitro* ACNase cleavage of hypomodified tRNA^{Lys}. (a) Low-mass RNA fractions isolated from the indicated *E. coli* strains were 5′-end labeled *in vitro* and incubated with ACNase-containing or control extracts and the products were separated by denaturing gel electrophoresis. The strip shown corresponds to the 30-35 nt size range containing the labeled ACNase products. (b) RNA from gel portions in (a) corresponding in position to the ACNase product band were partially digested with RNase T_1 and separated by denaturing gel electrophoresis.

mechanism underlies the relaxation of the cleavage site-specificity by these different mutations.

The comparable reactivities of unmodified tRNA^{Lys} and tRNA$^{Arg1(UUU)}$ (Figure 1(a) and (c); Table 1) reinforce the role of the anticodon sequence as a cue for ACNase, against a backdrop of an apparently inert remaining part. Nonetheless, weak interaction of ACNase with sequences or conformational features outside the anticodon loop must be considered because tRNA$^{Val1(UUU)}$ was not a substrate although it shares the anticodon loop sequence with tRNA^{Lys} and tRNA$^{Arg1(UUU)}$. Furthermore, mild inhibition of ACNase activity by the substitutions of the discriminator base A73 and relaxation of the cleavage site-specificity by deletion of the 3′-terminal ACCA overhang suggest that the 3′ end of the molecule also influences the interaction with the enzyme. However, ACNase cleaves the fully modified anticodon stem-loop of tRNA^{Lys} (Y.J., D. R. Davis and G.K., unpublished results), reinforcing the predominant contribution of this domain to ACNase specificity.

Importance of the wobble base modifications for ACNase substrate specificity

Unmodified tRNA^{Lys} was a less reactive ACNase substrate than fully modified tRNA^{Lys} (Figure 1(a), Table 1), indicating that at least some of the base modifications play a role in substrate recognition and/or reactivity. Such contributions may be attributed to the s^2U and mnm^5U modifications of the wobble base, since mutant forms of tRNA^{Lys} missing either modification were poorly cleaved *in vivo* and, in the case of the mnm^5U-deficient molecule, also *in vitro* (Figures 2-4, and 6). Impairment of ACNase activity by the absence of s^2U or mnm^5U modification may be attributed to the looser conformation of the anticodon (Sundaram *et al.*, 2000). Alternatively, or in addition, the inhibition may be due to loss of direct contacts between the wobble base and PrrC. The existence of a direct contact between the mnm^5U-modification and PrrC was proposed above.

The cleavage patterns of certain PrrC mutants in the *mnmA* and *mnmE* host backgrounds (Figure 3, Table 2) provide an additional vantage point from which to assess the above possibilities. Both hypomodified tRNA^{Lys} species were preferred to the natural, fully modified tRNA^{Lys} by the PrrC mutant alleles D287Q, D287H and D287N, contrary to the bias shown by wild-type PrrC (Figure 3 and Table 2). It is doubtful that the suppressor phenotypes of the three PrrC mutants reflect charge compensation, because the charge-reversing D287K mutation did not alter the wild-type bias (Table 2). However, the suppressing mutations could elicit local protein conformation better suited for interaction with a looser anticodon and/or provide alternative opportunities for direct contact between the protein and the hypomodified substrate. Such explanations may also apply to the suppression of the cleavage phenotype of the s^2U-deficient substrate by D287Q, N and H. The compensatory effects of these mutations also raise the prospect of creating novel ACNase derivatives endowed with desired cleavage specificities.

The absence of the s^2U modification reduced the *in vivo* extent of tRNA^{Lys} cleavage by an order of magnitude (Figures 2 and 3), yet made the cells more vulnerable to PrrC expression (Figure 5). This discrepancy may be reconciled by proposing that the hypomodified tRNA^{Lys} is less reactive than the wild-type but retains the affinity for PrrC. As a

result, the hypomodified tRNA could be captured in a harmful complex. Alternatively, enhanced cleavage of other tRNA species or synthetically lethal effects account for the greater cytotoxicity of PrrC in the *mnmA* background.

The absence of s^2U or mnm^5U modification from $tRNA^{Glu}$ did not impair its cleavage of by ACNase, unlike $tRNA^{Lys}$. Consequently, the contribution of the two modifying groups to ACNase reactivity must be considered in a broader context of anticodon domain or even entire tRNA structure. An important feature that distinguishes $tRNA^{Lys}$ from $tRNA^{Glu}$ is the nature of the modified adenosine base at position 37, t^6A *versus* 2-methyladenosine (m^2A), respectively. It has been proposed that destabilization of the $tRNA^{Lys}$ anticodon stem-loop by t^6A is balanced, in part, by the wobble base modifications, an effect that may be unique to $tRNA^{Lys}$ (Sundaram *et al*., 2000). It remains to be determined whether the hypomodified forms of $tRNA^{Glu}$ are cleaved 5′ to the wobble base like $tRNA^{Lys}$ or at a downstream site. The presence of 5′-terminal unmodified U in 3′ fragments generated by PrrC in the *mnmE* host hints at such aberrant cleavage of the hypomodified $tRNA^{Glu}$ (Figure 4). It should be pointed out that cleavage of fully modified $tRNA^{Glu}$ downstream to the normal site has been observed with the PrrC alleles D287Y and S288P (Meidler *et al*., 1999).

ACNase and lysyl-tRNA synthetase recognize the anticodon differently

The anticodon of $tRNA^{Lys}$ is also a major recognition element of the cognate aaRS. Yet, LysRS and ACNase seem to interact with the anticodon quite differently. Although aminoacylation of $tRNA^{Lys}$ is severely impaired by the removal of all base modifications (Tamura *et al*. 1992), absence of only the anticodon loop modifications results in wild-type-like V_{Max} and only a fourfold higher K_m (Commans *et al*., 1998). Likewise, the *in vivo* aminoacylation of $tRNA^{Glu}$, $tRNA^{Lys}$ and $tRNA^{Gln1}$ is not reduced by mnm^5U-deficiency and the lack of the s^2U group reduced the charging of $tRNA^{Glu}$ only (Krüger *et al*., 1998). In contrast, cleavage of $tRNA^{Lys}$ by ACNase was severely inhibited *in vivo* and *in vitro* by the absence of the mnm^5U modification and, at least *in vivo*, by lack of s^2U. Moreover, the absence of the mnm^5U or s^2U group from $tRNA^{Lys}$ was partially suppressed by certain PrrC mutations. The proposed stabilizing effect of the wobble base modifications on the anticodon conformation (Sundaram *et al*., 2000) suggests that wild-type PrrC readily recognizes the rigid anticodon conformer set to interact with the mRNA codon on the ribosome. In contrast, LysRS disrupts this conformation (Cusack *et al*., 1996).

ACNase and LysRS also differ in their responses to changes in the unmodified anticodon sequence. A U34 → G wobble base substitution reduces the relative aminoacylation reactivity 20-fold (Tamura *et al*., 1992) while ACNase cleavage was diminished below detection (Table 1). U36 → G reduces the relative V_{max}/K_m of aminoacylation fivefold (Tamura *et al*., 1992), whereas the extent of ACNase cleavage declined a 150-fold. Moreover, the U36 → A mutant was more reactive than U36 → G as an ACNase substrate (Table 1) yet only the latter is a substrate for aminoacylation (Tamura *et al*., 1992). Finally, the U35 → C mutation abolished aminoacylation, whereas its main effect on ACNase was relaxation of the cleavage site specificity. Presumably, ACNase and LysRS form different contacts with individual anticodon bases, in addition to their different dependencies on the wobble base modifications.

The different modes of contact of LysRS and ACNase with the anticodon must be matched by different structures of the respective protein domains. Secondary structure predictions suggest that the C-proximal region of PrrC, implicated with anticodon recognition, is rich in α-helices (Figure 7) and, hence, may not adopt the β-strand-rich OB-fold of the anticodon-binding domain of LysRS (Cusack *et al*., 1996). Furthermore, LysRS residues that contact anticodon bases belong to different β-strands and are scattered in the linear protein sequence (Cusack *et al*., 1996). In contrast, the anticodon-recognizing residues of PrrC may be clustered. This suspicion is based on selection of juxtaposed PrrC mutations that alter the cleavage phenotype (D287Y and S288P) by a procedure that failed to yield mutations with similar phenotypes elsewhere in PrrC (Meidler *et al*., 1999; and unpublished results). ACNase and LysRS may also interact differently with portions outside the anticodon region, because LysRS aminoacylates chimerical $tRNA^{Arg1(UUU)}$ and $tRNA^{Val1(UUU)}$ with comparable reactivities (Tamura *et al*., 1992), whereas only the

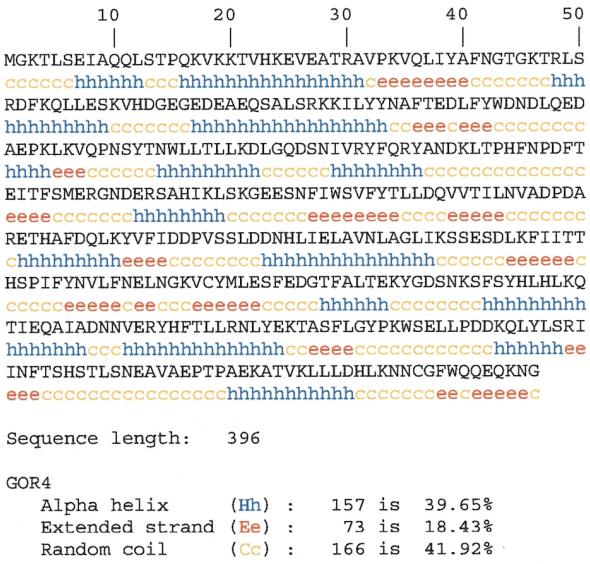

Figure 7. Predicted secondary structure of PrrC. PrrC's secondary structure was predicted by the GOR algorithm (Garnier *et al*., 1996).

former was a substrate of ACNase (Figure 1(c), Table 2).

Another RNA-protein recognition principle that may be relevant to ACNase is exercised by certain translation factors that interact with mRNA codons by adopting an anticodon stem-loop-like structure (RNA-protein mimicry, Nakamura *et al.*, 1996). Translation elongation factor EF-G interacts in this way with almost any codon, the release factors with a limited subset or only a single codon. Because tRNA anticodons interact readily with complementary anticodons (Grosjean *et al.*, 1976), the principle of RNA-protein mimicry may be extended to include protein-anticodon recognitions as well, covering, perhaps, such interactions as that of ACNase with its ligand (Kaufmann, 2000). Some of the observations discussed above lend support to this notion. Namely, certain requirements of ACNase can be fitted into the codon-anticodon rather than aaRS-anticodon interaction scheme, specifically, the dependence of ACNase on the anticodon rigidifying wobble base modifications. Another similarity between ACNase and the RNA-mimicking proteins could be the clustering of amino acid residues involved in substrate recognition. The release factors RF-1 and RF-2 contain a tripeptide ''anticodon'' that suffices for deciphering the divergent second and third letters of the stop-codon (Ito *et al.*, 2000). By analogy, the two juxtaposed amino acid residues of PrrC implicated with anticodon recognition (Meidler *et al.* 1999) may belong to a limited stretch that deciphers the tRNA^{Lys} anticodon.

Materials and Methods

Materials

Phage T7 RNA polymerase and the Klenow fragment of *E. coli* DNA polymerase I were purchased from New England Biolabs and phage T4 polynucleotide kinase was bought from USB or Amersham Inc. Calf intestinal alkaline phosphatase and Protease Inhibitor Cocktail Tablets (Complete Mini) were from Boehringer-Mannheim. Taq DNA polymerase was from MBI-Fermentas and radioactive nucleotides were from Amersham Inc. DNA oligonucleotides were from Life Technologies.

Bacterial strains, bacteriophages and plasmids

Plasmid pUC19 derivatives carrying the T7 promoter and the indicated tRNA^{Lys} and mutant tRNA^{Lys} genes were transformed into *E. coli* strain DH10B. Many of these clones have been described (Tamura *et al.*, 1992) and additional clones were derived from them here, as indicated in Table 1. Plasmids pRRC6 (Morad *et al.*, 1993), pRRC11 and pRRC11 derivatives encoding the PrrC mutants I227T-D215G (null), D287Y (Meidler *et al.* 1999) as well as D287H, Q, N and K generated here by PCR mutagenesis were employed. They were expressed in *E. coli* TH168 (*mnmA*, deficient in 2- thiolation of U34), TH169 (*mnmE*, deficient in 5-methyaminomethylation of U34) and the isogenic background strain TH170 (Hargervall *et al.*, 1998). PrrC expression was induced in the transformants by infection with λ-CE6 encoding T7 RNA polymerase (Studier & Moffatt, 1986). Directed mutagenesis of PrrC residues 287 and 288 was performed by PCR using degenerate oligonucleotides featuring all four bases at positions corresponding to the respective codons followed by identification of individual clones isolated with or without selection for loss of ACNase-dependent cytotoxicity (Meidler *et al.*, 1999).

Preparation of template DNAs and *in vitro* transcripts

Templates for *in vitro* transcription were prepared from plasmid pUC19 derivatives carrying the T7 promoter and encoding tRNA genes and mutant derivatives (Tamura *et al.*, 1992; Table 1) by cleavage with *Bst*NI nuclease. Alternatively, the templates were prepared by PCR. The PCR product was purified by electrophoresis on TBE/2% agarose gel and then cleaved with *Bst*NI nuclease followed by removal of the 3′-overhang by Klenow polymerase. *In vitro* transcription of tRNA^{Lys} fragment 1-34 was done according to the procedure of Milligan & Uhlenbeck (1989). However, this protocol yielded in addition fragment 2-34. The 5′-triphosphate of the transcripts was replaced by 5′-^{32}P by alkaline phosphatase digestion followed by phosphorylation with polynucleotide kinase. The products were purified by denaturing 15% polyacrylamide gel electrophoresis. Conversion of fragment 1-34 into fragment 1-33 carrying a 3′-terminal phosphate group entailed periodate-oxidation and β-elimination. The 3′-terminal phosphate group was removed by polynucleotide kinase.

In vitro core ACNase assays

The *in vitro* ACNase assay was performed essentially as described (Amitsur *et al.*, 1989; Morad *et al.*, 1993). Core ACNase containing extracts were prepared from *E. coli* K38:pGp1-2 containing the pRRC11-D222E or a vector plasmid (pT7-6). The cells were grown at 30 °C in LB medium containing 800 μg/ml ampicilin and 50 μg/ml kanamycin. At a density of A_{595} of 0.8, the cultures were shifted to 42 °C and 1 mM IPTG was added. After one hour, the temperature was lowered to 30 °C and incubation was continued for one hour. The cells were harvested and washed first with buffer I (10 mM Tris-HCl (pH 7.5), 15 mM $MgCl_2$, 1 M KCl, 5 mM EDTA, 10% glycerol and 10 mM 2-mercaptoethanol) followed by buffer II (buffer I with only 50 mM KCl). They were quickly frozen and thawed in two volumes of buffer III (10 mM Tris-HCl (pH 7.5), 15 mM $MgCl_2$, 60 mM NH_4Cl, 10% glycerol, 5 mM 2-mercaptoethanol and protease inhibitor cocktail). The suspension was passed twice in a 4 ml Aminco pressure cell at 16,000 p.s.i. (1 p.s.i. ~6.9 kPa). The resultant crude extract was centrifuged first in refrigerated Sorvall centrifuge (SS34 rotor) at 13,000 rpm for 20 minutes followed by centrifugation of the supernatant at 45,000 rpm for four hours at 4 °C (Beckman L5-75B ultracentrifuge, 50.2 Ti rotor). The high-speed supernatant was stored at −70 °C.

Core ACNase assays were conducted as follows. The reaction mixture (10 μl) contained 6 μl of the core ACNase-containing fraction, 36 mM NH_4Cl, 6 mM Tris-HCl (pH 7.5), 9 mM $MgCl_2$, 3 mM 2-mercaptoethanol, 1 M TMAO, 6% glycerol, protease inhibitor cocktail (Complete, Mini, Boehringer Mannheim), diluted according to the supplier's instructions; 2-3 fmol of (5′-^{32}P)-labeled tRNA substrate. The substrates employed were natural tRNA^{Lys}, labeled at the cleavage junction, (5′-^{32}P)-labeled unmodified tRNA transcripts or (5′-^{32}P)-

labeled low-mass RNA fractions isolated from *E. coli* strains TH168-170. The assays of each substrate were replicated four times. Following three hours incubation at 10 °C, aliquots were de-proteinized and separated by gel electrophoresis. The extent of cleavage was calculated from the amounts of remaining substrate and product evaluated by densitometric tracing of the gel autoradiogram using Hewlett Packard ScanJet 3p and TINA software (Raytest Isotopenmessgeräte GmbH), compatible with the TINA-PCBAS and TIFF files of the scanner.

In vivo core ACNase assays

E. coli TH168, TH169 and TH170 transformed by the indicated *prrC* plasmid were grown in LB medium containing 0.02 % maltose, 10 mM $MgSO_4$ 800 μg/ml ampicilin and 50 μg/ml kanamycin. At a cell concentration of 5×10^8/ml, λ-CE6 phage was added at m.o.i. of 5-10 and the cells were harvested 45 minutes later. Low-mass RNA was extracted by phenol and 3′ tRNA fragments generated by ACNase *in vivo* were 5′ end labeled and separated by denaturing polyacrylamide gel electrophoresis (Meidler *et al*., 1999). Aliquots of 10,000-40,000 c.p.m were hybridized to 10 pmol dots of synthetic oligonucleotide probes immobilized on nylon membranes; these probes were complementary to fragments 34-73 of the indicated tRNA species. Each RNA preparation was prepared three times from independent cultures and each preparation was analyzed by hybridization three times. RNA sequencing and end-group determination of the labeled ACNase products were performed essentially as described (Silberklang *et al*., 1979). Detection of PrrC protein in the transformants by Western blotting was as described (Meidler *et al*., 1999).

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