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Role of the 1-72 base pair in tRNAs for the activity of *Escherichia coli* peptidyl-tRNA hydrolase

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ABSTRACT

Previous work by Schulman and Pelka (1975) J. Biol. Chem. 250, 542–547, indicated that the absence of a pairing between the bases 1 and 72 in initiator $tRNA^{Met}_f$ explained the relatively small activity of peptidyl-tRNA hydrolase towards *N*-acetyl-methionyl-$tRNA^{Met}_f$. In the present study, the structural requirements for the sensitivity of an *N*-acetyl-aminoacyl-tRNA to *Escherichia coli* peptidyl-tRNA hydrolase activity have been further investigated. Ten derivatives of $tRNA^{Met}_f$ with various combinations of bases at positions 1 and 72 in the acceptor stem have been produced, aminoacylated and chemically acetylated. The release of the aminoacyl moiety from these tRNA derivatives was assayed in the presence of peptidyl-tRNA hydrolase purified from an overproducing strain. $tRNA^{Met}_f$ derivatives with either C_1A_{72}, C_1C_{72}, U_1G_{72}, U_1C_{72} or A_1C_{72} behaved as poor substrates of the enzyme, as compared to those with C_1G_{72}, U_1A_{72}, G_1C_{72}, A_1U_{72} or G_1U_{72}. With the exception of U_1G_{72}, it could be therefore concluded that the relative resistance of $tRNA^{Met}_f$ to peptidyl-tRNA hydrolase did not depend on a particular combination of nucleotides at positions 1 and 72, but rather reflected the absence of a base pairing at these positions. In a second series of experiments, the unpairing of the 1 and 72 bases, created with C-A or A-C bases, instead of G-C in methionyl-$tRNA^{Met}_m$ or in valyl-$tRNA^{Val}_1$, was shown to markedly decrease the rate of hydrolysis catalysed by peptidyl-tRNA hydrolase. Altogether, the data indicate that the stability of the 1-72 pair governs the degree of sensitivity of a peptidyl-tRNA to peptidyl-tRNA hydrolase.

INTRODUCTION

Peptidyl-tRNA hydrolase (PTH; EC 3.1.1.29), an enzyme activity found in all cellular extracts where it had been searched for, sustains an *in vitro* hydrolytic activity converting peptidyl-tRNAs or *N*-acetyl-aminoacyl-tRNAs into free tRNAs plus peptides or *N*-acetyl-aminoacids (1, 2). Consequently, PTH is believed to play a role in the translational apparatus through the recycling of free tRNAs from the immature peptidyl-tRNAs created by abortive protein synthesis. Indeed, the study of *E.coli* temperature sensitive mutants of the *pth* gene encoding PTH has established that PTH activity was essential to a bacterial cell by preventing the accumulation of uncleaved peptidyl-tRNA molecules (3, 4).

N-formyl-methionyl-$tRNA^{Met}_f$ or *N*-acetyl-methionyl-$tRNA^{Met}_f$ were shown to be more resistant to the hydrolysis by purified PTH than any other *N*-acetyl-aminoacyl-tRNAs (1, 5). The pool of *N*-formyl-methionyl-$tRNA^{Met}_f$ in a bacterial cell is therefore likely to be protected from the hydrolysis by PTH. This behaviour would be of first importance, since the growth rate of *E.coli* cells strongly depends on the intracellular concentration of *N*-formyl-methionyl-$tRNA^{Met}_f$, as shown recently through the study of the effect of a disruption of the gene encoding methionyl-$tRNA^{Met}_f$ formyltransferase (6).

The relative insensitivity of *N*-acetyl-methionyl-$tRNA^{Met}_f$ to PTH action clearly lies on the nucleotide sequence of $tRNA^{Met}_f$ (initiator $tRNA^{Met}$) and not on the nature of the esterified aminoacid, since *N*-acetyl-methionyl-$tRNA^{Met}_m$ (elongator) is a good substrate of PTH (5). Actually, Schulman and Pelka (7) showed that chemical modification of $tRNA^{Met}_f$ with sodium bisulfite converted *N*-acetyl-methionyl-$tRNA^{Met}_f$ into a substrate of PTH as efficient as *N*-acetyl-phenylalanyl-$tRNA^{Phe}$. These authors could demonstrate that among all the bisulfite modifications, the change of C_1 into U_1, creating thereby a base pairing with A_{72}, was responsible for the increased sensitivity to PTH. Considering that $tRNA^{Met}_f$ was the only tRNA in *E.coli* with unpaired 1-72 nucleotides, the same authors proposed therefore that the absence of a base pairing between nucleotides 1 and 72 was enough to account for the resistance of a tRNA to PTH. In agreement with this attractive hypothesis, it was recently observed that the introduction of U_1A_{72} in $tRNA^{Met}_f$ led to the appearance of a significant fraction of non-aminoacylated tRNA *in vivo*, this effect being not observed in a strain carrying a thermosensitive mutation in the *pth* gene (8).

In the present work, advantage was taken of the recent characterization of the gene encoding *E.coli* PTH (9) to purify the enzyme from an overproducing strain. This allowed us to investigate whether the presence or absence of the 1-72 base

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pairing in a tRNA was actually the only element governing the sensitivity or resistance to PTH. For this purpose, series of tRNA mutants were constructed and the corresponding *N*-acetyl-aminoacyl derivatives were studied in the presence of homogeneous PTH.

MATERIALS AND METHODS

Construction of a bacterial strain overexpressing the peptidyl-tRNA hydrolase activity

Two oligonucleotides (5′-TACGTTATCTGAATTCGATAC-GCAGTTT and 5′-GAATGGAAATAAGCTTGCCTATTATAC) were used to amplify by Polymerase Chain Reaction the recently characterized *pth* gene encoding PTH (9) from *E.coli* chromosomal DNA. Mismatches (boldfaced) were introduced in the sequence of the oligonucleotides to create either an *Eco*RI or a *Hin*dIII site (underlined). A 735 base pair fragment, containing the whole *pth* gene as well as its own promoter region, was obtained after amplification; it was restricted in the presence of the *Eco*RI and *Hin*dIII restriction enzymes and ligated between the *Eco*RI and *Hin*dIII restriction sites of pUC18, yielding the pUCpth plasmid. The nucleotide sequence of the cloned fragment was fully verified by automatic double-stranded DNA sequencing (ALF system, Pharmacia). In the context of the pUCpth plasmid, the *pth* gene was under the control of the Lac promoter. JM101Tr cells carrying the pUCpth plasmid were grown in the presence of 0.3 mM IPTG. The corresponding crude bacterial extract exhibited a 360-fold increase of the PTH activity when compared to control cells (JM101Tr pUC18) grown under the same conditions.

Synthesis of tRNA genes

Oligonucleotides (18 to 33-mers) were synthesized on a Pharmacia Gene Assembler and purified by anion-exchange chromatography (Mono Q, Pharmacia). Initiator $tRNA^{Met}$, elongator $tRNA^{Met}$, $tRNA^{Val}$ genes and their derivatives were constructed by the assembly of six overlapping oligonucleotides, as described (10). tRNA genes were ligated between the *Eco*RI and *Pst*I sites of the pBSTNAV2 expression vector, a derivative of pBSTNAV (10, 11). The tRNA gene sequences were systematically verified by dideoxy-sequencing of single-stranded DNA obtained by using the R-408 helper phage (Stratagene, La Jolla, CA, USA). The genes encoding derivatives of $tRNA^{Met}{}_f$ have been described elsewhere (12, 13).

tRNA purification

Overproducing cells were grown in 1 litre of 2×TY medium containing 50μg of ampicillin per ml. Crude tRNA extracts (410 to 1,100 pmole $tRNA^{Met}/A_{260}$ Unit or 350 to 490 pmole $tRNA^{Val}/A_{260}$ Unit depending on the expressed tRNA species) were prepared as previously described (10) and further purified by an anion-exchange step on a Q-Hiload column (16 by 100 mm; Pharmacia) as follows: the extract in 10 ml of buffer A containing 20 mM Tris−HCl (pH=7.6); 8 mM $MgCl_2$; 0.1 mM EDTA; 0.2 M NaCl was loaded onto the column equilibrated in the same buffer; thereafter, a 0.36 to 0.48 M NaCl gradient in buffer A (2.5 ml/min; 0.10 M/h) was used to separate $tRNA^{Met}$ variants. 5−15 mg pure $tRNA^{Met}$ or $tRNA^{Val}$ were routinely recovered from a 1 litre culture. Acceptances by methionine or valine of the tRNA preparations ranged between 1,350 and 1,650 pmoles per A_{260} Unit.

Preparation of acetylated aminoacyl-tRNAs

$tRNAs^{Met}$ were fully methionylated within 10 minutes at 25°C in a mixture (2.5 ml) containing 20 mM Tris−HCl (pH=7.6); 7 mM $MgCl_2$; 10 mM 2-mercaptoethanol; 0.1 mM EDTA; 150 mM KCl; 114.8 μM [^{14}C]-methionine (56 Ci/mole; Dositek-France); 2 mM ATP; 40 μM tRNA; 1.5 μM methionyl-tRNA synthetase. The *E.coli* methionyl-tRNA synthetase used in the assays was the truncated monomeric M547 variant, purified as previously described (14). Valylation of $tRNAs^{Val}$ in the presence of 1.5 μM valyl-tRNA synthetase was obtained in a similar fashion (see above) except that 120 μM [^{14}C]-valine (50 Ci/mole; Dositek-France) was used instead of methionine. Homogeneous *E.coli* valyl-tRNA synthetase was from the laboratory stock. The extents of the aminoacylation reactions were followed by TCA precipitation of an aliquot, filtration through GF/C disks and subsequent scintillation counting.

After completion of the reaction, aminoacyl-tRNAs were ethanol precipitated. The aminoacyl-tRNA pellet (0.1 μmole) was then dissolved in 0.5 ml of 5 mM sodium acetate (pH = 7), and acetylation was achieved at 0°C for 15 minutes by adding 0.5 ml Dimethylsulfoxide, 0.1 ml glacial acetic acid and 0.1 ml acetic anhydride. The solution was ethanol precipitated thereafter and centrifuged. The pellet was dissoved in 0.5 ml of a solution containing 10 mM copper sulfate and 0.2 M sodium acetate (pH = 5) in order to deaminoacylate non-acetylated aminoacyl-tRNAs (15). The solution was left for 30 minutes at 37°C and the reaction was stopped by the addition of 5 μl of 1 M EDTA. tRNAs were then ethanol precipitated and centrifuged. The pellet was first washed with 0.5 ml of a solution containing 75% ethanol, 1 mM $MgSO_4$ and 1 mM EDTA and then with 0.5 ml of a solution containing 75% ethanol, 1 mM $MgSO_4$ and 2 mM sodium acetate. *N*-acetylaminoacyl-tRNAs were finally dissolved in buffer B: 20 mM K_2HPO_4 (pH=7.0), 0.1 mM EDTA, 10 mM 2-mercaptoethanol. Extent of the acetylation reaction could therefore be determined by measuring the ^{14}C radioactivity and systematically found of the order of 90%.

Purification of peptidyl-tRNA hydrolase to homogeneity

JM101Tr cells carrying the pUCpth plasmid were used to inoculate a flask of 2×TY medium containing 50μg/ml ampicillin and 0.3 mM IPTG (1 litre). Cultures were grown overnight at 37°C, harvested by centrifugation and resuspended in 40 ml of buffer B (see above). The sample was sonicated, and cell debris removed by centrifugation. Streptomycin sulphate (3% w/v) was added to the supernatant. After centrifugation, the supernatant was submitted to a $(NH_4)_2SO_4$ precipitation (70% saturation) and centrifuged. The pellet was redissolved in 10 ml of buffer B plus 50 mM KCl, dialysed against the same buffer to remove ammonium sulphate, and finally applied onto a gel filtration columm (Superose 6; 1.6cm by 50cm; Pharmacia) equilibrated in the same buffer. The column was eluted at 0.2 ml/min. Fractions containing the PTH activity were pooled, dialysed extensively against buffer B and diluted to a final volume of 20 ml in the elution buffer. The resulting sample was applied onto an anion exchanger (Q-Hiload; 1.6cm by 10 cm; Pharmacia) equilibrated in buffer B. Because of its very basic isoelectric point (pI=10), the enzyme was not retained on the column. The run-through, containing PTH activity, was pooled and stored at −30°C after extensive dialysis against buffer B containing 55% glycerol. These purification steps were enough to purify 4 mg

of peptidyl-tRNA hydrolase to more than 95% homogeneity, as judged by polyacrylamide gel electrophoresis analysis under denaturing conditions.

Peptidyl-tRNA hydrolase activity assay

Peptidyl-tRNA hydrolase activity was measured at 37°C in 100 μl assays. *N*-acetyl-aminoacyl-tRNAs (0.3 to 75 μM) were incubated in 75 μl of buffer B plus 10 mM $MgCl_2$. The assay was started by the addition of 25 μl of a solution of PTH diluted in buffer B plus 200 μg/ml bovine serum albumin (Boehringer-Mannheim). Final concentration of PTH was comprised between 2 and 250 nM. The reaction was quenched by the addition of 300 μl of 5% trichloroacetic acid and of 20 μl of carrier RNA from yeast (4 mg/ml). The mixture was then centrifuged for 5 minutes in an Eppendorf microcentrifuge (15,000 g). Under these conditions, total tRNA was recovered in the pellet, whereas released *N*-acetyl-[^{14}C]aminoacids were in the supernatant. The supernatant (100 μl) was withdrawn and added to 6 ml of Picofluor (Packard) in order to measure radioactivity by scintillation counting.

Kinetics of release of the *N*-acetyl-aminoacyl moiety from the tRNA substrate by a constant PTH concentration were systematically followed up to at least 30% of the concentration of the initially added tRNA substrate. Initial rates (v) of *N*-acetyl-aminoacyl-tRNA hydrolysis were determined from at least four values in the linear portion of the kinetics. K_m and k_{cat} values for tRNA and their associated standard errors were derived from iterative non-linear least squares fits of the Michaelis–Menten equation to the experimental v values measured in the presence of *N*-acetyl-aminoacyl-tRNA concentrations ranging between 0.5 and 75 μM.

RESULTS

Purification and characterization of *E.coli* peptidyl-tRNA hydrolase

The *E.coli* gene encoding PTH (9) was amplified by means of the Polymerase Chain Reaction from chromosomal DNA and inserted under the control of the Lac promoter within the pUC18 multicopy plasmid. The PTH activity in extracts of JM101Tr cells grown in the presence of IPTG was enhanced 360-fold by the presence the pUCpth plasmid. Polyacrylamide gel electrophoresis of the crude bacterial extract of JM101Tr pUCpth cells revealed that a 22,000 kDa polypeptide became the major soluble protein (not shown).

Overexpressed PTH could be made homogeneous through two successive chromatographic steps (Table 1). Sequencing of the 17 N-terminal residues of the purified protein confirmed its identity with the product of the *pth* gene (9). Several biochemical properties of the purified PTH could be determined. A molar absorption coefficient at 280 nm of 20,000 $M^{-1}.cm^{-1}$ was deduced from the measurements of the protein concentration by the Bradford technique (16), using bovine serum albumine as standard. Isoelectric focusing of PTH, with an isoelectric calibration kit (range pH 3–10; Pharmacia), indicated an isoelectric point of the order of 10, in agreement with the value (pI=9.3) calculated from the amino acid sequence.

The K_m value of *N*-acetyl-methionyl-tRNA$^{Met}{}_m$ in the reaction of hydrolysis catalysed by PTH was measured. Its value (K_m = 2.2±0.4 μM) is of the same order of magnitude as that associated with other enzymes catalysing reactions involving a tRNA as substrate (*e.g.* the reactions of aminoacylation or formylation). Using the same assay, a k_{cat} value of 0.7±0.1 s^{-1} could be found, taking into account a monomeric enzyme. However, this value might have been underestimated, since, in native polyacrylamide gel electrophoresis and molecular sieving experiments (not shown), the protein was observed to distribute amongst several discrete states of oligomerization.

The creation of a pairing between the 1-72 bases makes *N*-acetyl-methionyl-tRNA$^{Met}{}_f$ a substrate of PTH as efficient as *N*-acetyl-methionyl-tRNA$^{Met}{}_m$

It had been early indicated (7) that the increased resistance of *N*-formyl-methionyl-tRNA$^{Met}{}_f$ to PTH could be accounted for by the conversion of the base pair at the end of the acceptor stem into a U-A. To enlarge this observation, ten variants of tRNA$^{Met}{}_f$ with mutations at the level of the 1-72 base pair were produced. These tRNAs were aminoacylated with methionine and chemically acetylated. The enzymatic parameters of PTH in the release of *N*-acetyl-methionine from the *N*-acetyl-methionyl-tRNA$^{Met}{}_f$ variants were compared. The results, compiled in table 2, clearly showed that PTH exhibited two types of behaviour, which could not be correlated with particular nucleotide compositions but rather depended on the presence or the absence of a Watson–Crick base pairing between positions 1 and 72. *N*-acetyl-methionyl-tRNAs$^{Met}{}_f$ derivatives harbouring

Table 1. Purification of *E.coli* peptidyl-tRNA hydrolase

Purification step	Protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Relative purification factor
Crude extract	258	265	1.0	100	1.0
Ammonium sulfate	117	225	1.9	85	1.9
Superose-6	39	92	2.4	41[a]	2.3
Q-Hiload	4.3	89	20.7	34	20.1

The purification procedure and the standard assay for PTH activity measurement were as described in the Materials and Methods section. The protein content was determined by the method of Bradford (16) using the Bio-Rad protein assay kit. The Enzyme Unit (U) is defined as the amount of protein capable of releasing one nmole of *N*-acetyl-[^{14}C]methionyl from *N*-acetyl-[^{14}C]methionyl-tRNA$^{Met}{}_m$ per second in one ml of the standard assay. The relative purification factor was calculated by dividing the specific activity at each step of the preparation by that measured in the crude extract.

[a] The low yield of the molecular sieving step is due to unrecovered enzyme activity, which reflects high molecular weight aggregation of PTH.

Table 2. Effects on the deacylation reaction catalyzed by peptidyl-tRNA hydrolase of mutations altering the 1-72 pair of *E.coli* $tRNA^{Met}{}_f$

Base at positions 1 and 72		K_m (μM)	$k_{cat}(s^{-1})$	k_{cat}/K_m $(M^{-1}s^{-1})$ $\times 10^{-4}$	Relative k_{cat}/K_m
C	A [(a)]	4.5±0.3	0.08±0.01	1.7±0.2	1.0
C	G	4.0±0.9	1.4±0.2	35±4	20.9
C	C	12.9±2.1	0.34±0.04	2.6±0.2	1.6
U	A	24.5±6.9	5.1±1.1	21±1	12.3
G	C	8.0±1.0	3.7±0.3	47±2	27.4
A	U	3.7±0.8	1.1±0.2	29±3	17.2
G	U	4.9±1.5	2.7±0.5	54±4	31.8
U	G	5.3±0.8	0.10±0.01	1.9±0.1	1.1
U	C	16.3±4.3	0.21±0.05	1.3±0.1	0.8
A	C	15.3±1.9	0.34±0.03	2.2±0.1	1.3

Michaelis–Menten parameters in the reaction catalysed by PTH were measured in the presence of *N*-acetyl-[^{14}C]methionylated derivatives of $tRNA^{Met}{}_f$ having the indicated nucleotides at positions 1 and 72. Relative k_{cat}/K_m values were calculated giving an arbitrary value of 1.0 to the result obtained with wild-type $tRNA^{Met}{}_f$.
[(a)]: corresponds to wild-type $tRNA^{Met}{}_f$.

Table 3. Effect on the activity of peptidyl-tRNA hydrolase of mutations of the 1-72 base pair in several tRNAs

Starting tRNA species	Bses at position 1	72	K_m (μM)	$k_{cat}(s^{-1})$	k_{cat}/K_m $(M^{-1}s^{-1})$ $\times 10^{-4}$	Relative k_{cat}/K_m
f met	C	A[(a)]	4.5±0.3	0.08±0.01	1.7±0.2	1.0
m met	G	C[(b)]	2.2±0.4	0.7±0.1	31±20	18.2
m met	C	A	2.3±0.8	0.05±0.01	2.1±0.4	1.2
m met	A	C	nd	nd	3.4±0.5	2.0
fasm	C	A	4.5±0.6	0.035±0.004	0.8±0.1	0.5
val 1	G	C[(c)]	6.1±1.1	0.65±0.07	11±1	6.5
val 1	C	A	59±16	0.07±0.02	0.13±0.01	0.08

Michaelis–Menten parameters in the reaction catalysed by PTH of derivatives of $tRNA^{Met}$ (f met= $tRNA^{Met}{}_f$; m met= $tRNA^{Met}{}_m$; fasm= $tRNA^{Met}{}_m$ with the acceptor stem of $tRNA^{Met}{}_f$, see reference 13) or $tRNA^{Val}{}_1$ (val 1) having each the nucleotides at position 1 and 72 indicated in the table. The tRNA molecules were fully esterified with methionine ($tRNA^{Met}$ derivatives) or valine ($tRNA^{Val}{}_1$ derivatives) in the presence of the corresponding aminoacyl-tRNA synthetase and chemically acetylated as described in the Materials and Methods section. Relative k_{cat}/K_m values were calculated giving an arbitrary value of 1.0 to the result obtained with wild-type $tRNA^{Met}{}_f$.
[(a)]: corresponds to wild-type $tRNA^{Met}{}_f$.
[(b)]: corresponds to wild-type $tRNA^{Met}{}_m$.
[(c)]: corresponds to wild-type $tRNA^{Val}{}_1$.
n.d. means that the value has not been determined.

a base pair at positions 1-72 (GC, CG, AU or UA pairs) were good substrates of PTH, with k_{cat}/K_m values identical or close to those measured with *N*-acetyl-methionyl-$tRNA^{Met}{}_m$ (Table 3), while initiator tRNAs lacking the base pairing (CC, UC and AC pairs) were as resistant to the hydrolysis by PTH as unmodified *N*-acetyl-methionyl-$tRNA^{Met}{}_f$ (CA pair). An intriguing situation was encountered with the G_1U_{72} and U_1G_{72} variants. Whereas the G_1U_{72} variant was an efficient substrate of PTH, the U_1G_{72} variant was as resistant to PTH-catalysed hydrolysis as wild-type $tRNA^{Met}{}_f$ (Table 2).

The unpairing of the 1-72 positions makes *N*-acetyl-aminoacyl-tRNAs as poor substrates of PTH as iniator *N*-acetyl-methionyl-$RNA^{Met}{}_f$

To search for other determinants in the tRNA structure possibly used by PTH, the sequence of the acceptor stem of $tRNA^{Met}{}_m$ was substituted by that of $tRNA^{Met}{}_f$. The construction of this chimaeric tRNA, which is featured by a C_1A_{72} pair, has been described previously (tRNA $^{Met}{}_{fasm}$, reference 13). Measurement of the catalytic performances of hydrolysis by PTH revealed that *N*-acetyl-methionyl-$tRNA^{Met}{}_{fasm}$ had become less sensitive to PTH than *N*-acetyl-methionyl-$tRNA^{Met}{}_m$. Moreover, the k_{cat}/K_m value associated to *N*-acetyl-methionyl-$tRNA^{Met}{}_{fasm}$ was very similar to that measured in the presence of *N*-acetyl-methionyl-$tRNA^{Met}{}_f$ (Table 3). This indicated that the acceptor stem of $tRNA^{Met}{}_f$ brought the whole information required to specify the resistance of a tRNA to PTH.

The resistance to PTH of a derivative of $tRNA^{Met}{}_m$, having the C_1A_{72} unpairing of $tRNA^{Met}{}_f$ instead of the normal G_1C_{72} pair, was measured also. Again, this tRNA was found as resistant as $tRNA^{Met}{}_f$ (Table 3). This suggested that the only 1-72 pair positions was responsible for the resistance or the sensitivity of a tRNA to PTH. Another variant of $tRNA^{Met}{}_m$ with an A_1C_{72} pair was constructed and found to exhibit an increased resistance to PTH action, with a k_{cat}/K_m value close to the one found in the presence of initiator $tRNA^{Met}{}_f$ (Table 3). This suggested that the resistance of a tRNA to PTH action only depended on the unpairing of the 1-72 nucleotides and not on the nature of the

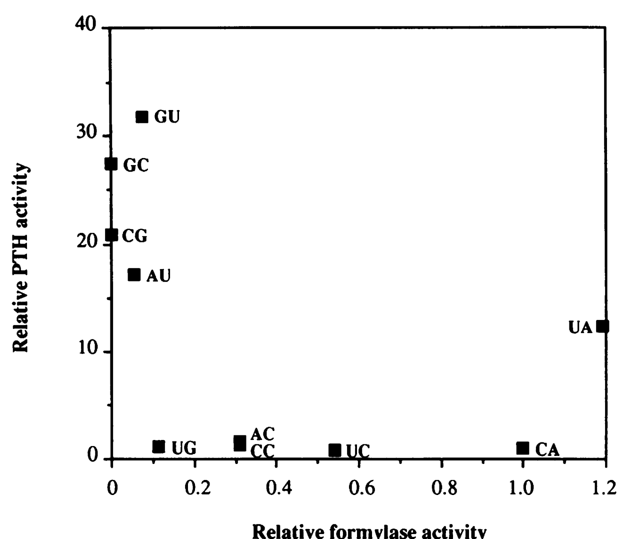

Figure 1. Comparison between (a) the susceptibility of several variants of $tRNA^{Met}_f$ to be substrates of peptidyl-tRNA hydrolase and (b) their formability by methionyl-$tRNA^{Met}_f$ formyltransferase. The nature of the 1-72 pair in the $tRNA^{Met}_f$ variant is indicated beyond each corresponding dot. The x-axis corresponds to the relative efficiency of formylation (k_{cat}/K_m) of the derivatives of $tRNA^{Met}_f$, giving a value of 1.0 to that obtained with unmodified $tRNA^{Met}_f$ (the results are taken from reference 13). Relative sensitivities to PTH (k_{cat}/K_m) of the same derivatives of $tRNA^{Met}_f$, giving a value of 1 to k_{cat}/K_m of wild-type $tRNA^{Met}_f$ (results taken from table 2, this work), are on the y-axis. The values are clustered either on the x-axis or on the y-axis, showing therefore that those tRNAs which are the most resistant to PTH are, in turn, the most sensitive to methionyl-$tRNA^{Met}_f$ formyltransferase, and *vice versa*.

bases at those positions. Finally, to verify that this conclusion was not biased by the nature of the tRNA itself or by that of the esterified aminoacid, the G_1C_{72} base pair of $tRNA^{Val}_1$ was changed into a C_1A_{72} one. In agreement with the above results, the k_{cat}/K_m value associated to *N*-acetyl-valyl-$tRNA^{Val}_1C_1A_{72}$ became 90-fold smaller than that associated to wild-type *N*-acetyl-valyl-$tRNA^{Val}_1$ (Table 3).

DISCUSSION

In the present work, the presence or the absence of a hydrogen-bonded base pair at the end of the acceptor stem is shown to markedly influence the catalytic efficiency of hydrolysis of *N*-acetyl-aminoacyl-tRNAs by *E.coli* PTH. In *E.coli*, only the $tRNA^{Met}_f$ species has unpaired nucleotides at positions 1 and 72. Consequently, its relative resistance to the hydrolysis by PTH can be simply explained. In turn, most elongator tRNAs in *E.coli* have G_1C_{72}, C_1G_{72}, A_1U_{72} or U_1A_{72} base pairs (see reference 17). This feature explains why these tRNA molecules carrying oligopeptides can be hydrolyzed by PTH more efficiently than initiator *N*-formylmethionyl-$tRNA^{Met}_f$. Two $tRNA^{Leu}$ isoacceptors ($tRNA^{Leu}_2$ and $tRNA^{Leu}_4$) have a G_1U_{72} base pair, whereas tRNAs with a U_1G_{72} base pair never occur in *E.coli*. Interestingly, the present study shows that the introduction of a G_1U_{72} base pair in $tRNA^{Met}_f$ renders this tRNA more sensitive to PTH, while, unexpectedly, that of a U_1G_{72} has no consequence on the rate of hydrolysis of *N*-acetyl-methionyl-$tRNA^{Met}_f$ catalysed by PTH.

One possible explanation for the opposite effects associated to a UG base pair *versus* a GU one might be that these pairs differently contribute to the stability of the acceptor stem through their stacking with the adjacent G_2C_{71} base pair of $tRNA^{Met}_f$. However, the two tRNAs displaying a G_1U_{72} base pair in *E.coli*, $tRNA^{Leu}_2$ and $tRNA^{Leu}_4$, have a C_2G_{71} base pair. Consequently, the stacking contribution of G_1U_{72} with C_2G_{71} can be expected to be similar to that in $tRNA^{Met}_f$ of G_2C_{71} with U_1G_{72}. Since *N*-acetyl-leucyl-$tRNAs^{Leu}_{2\ or\ 4}$ are likely to be efficient substrates of PTH, while the variant of *N*-acetyl-methionyl-$tRNA^{Met}_f$ with a U_1G_{72} pair is not, other explanations must be found. Additional contributions to the stability of a tRNA acceptor stem sequence have probably to be taken into account also. In this context, it should be noted that recent data (18) have shown that the discriminator base may influence the stability of the adjacent 1-72 pair, probably through stacking interactions.

The reaction catalysed by methionyl-$tRNA^{Met}_f$ formyltransferase already emphasized the importance of the stability of the acceptor stem of $tRNA^{Met}_f$. It was indeed demonstrated that the creation of a strong base pairing (GC or CG) at positions 1 and 72 was enough to prevent the action of this enzyme (13, 19). The data in the present study further establish that there is a marked correlation between the catalytic efficiency (k_{cat}/K_m) of the deacylation reaction catalysed by PTH in the presence of a given *N*-acetyl-methionyl-$tRNA^{Met}_f$ and that of the reaction catalysed by methionyl-$tRNA^{Met}_f$ formyltransferase (Figure 1). The more efficient a $tRNA^{Met}_f$ variant is a substrate of one of the two enzymes, the less efficient it is a substrate of the other. The AU pair is the only exception. In making these comparisons, it should be however underlined that, depending on the nature of the 1-72 pair, the efficiencies of formylation vary in the range of six orders of magnitude, whereas the range of variation of catalytic efficiencies between PTH-resistant and PTH-sensitive $tRNAs^{Met}_f$ variants is limited to 40-fold (Table 2). Such a relatively low discrimination capacity of PTH was already noticed (7) and renders difficult to fully explain the protection of *N*-formyl-methionyl-$tRNA^{Met}_f$ *in vivo*.

In this context, it is worth to evaluate the physiological relevance of the PTH action as defined *in vitro*. *In vivo*, the natural substrates of PTH, *i.e.* peptidyl-tRNAs, are unlikely to be sequestered by elongation factor EFTu-GTP (20), whereas PTH has to compete with initiation factor IF_2 for the binding of free formyl-methionyl-$tRNA^{Met}_f$ molecules. The dissociation constant values of the complexes of each of the two proteins with *N*-formyl- or *N*-acetyl-methionyl-$tRNA^{Met}_f$ are similar: 1 μM in the case of IF_2 (see (21)) and 4.5 μM in the case of PTH, assuming that the K_m parameters reflect equilibrium constants. The present work enabled us to calculate that as few as 25 PTH molecules occur in a bacterial cell, whereas 10^4 molecules were estimated in the case of initiation factor IF_2 proteins (22). Consequently, there is a great probability that a free *N*-formylmethionyl-$tRNA^{Met}_f$ molecule binds an IF_2 protein rather than PTH. Ii is likely that this binding unbalance in favour of initiation factor IF_2 also contributes to allow *N*-formylmethionyl-$tRNA^{Met}_f$ to escape the hydrolysis catalysed by PTH.

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REFERENCES

1. Kossel, H. and RajBhandary, U. L. (1968) *J. Mol. Biol.*, **35**, 539–560.
2. Gross, M., Starn, T. K., Rundquist, C., Crow, P., Olin, A. and Wagner, T. (1992) *J. Biol. Chem.*, **267**, 2073–2079.
3. Atherly, A. and Menninger, J. R. (1972) *Nature New Biol.*, **240**, 245–246.
4. Menninger, J. R. (1979) *J. Bacteriol.*, **137**, 694–696.
5. Kossel, H. (1970) *Biochim. Byophys. Acta*, **204**, 191–202.
6. Guillon, J. M., Mechulam, Y., Schmitter, J.-M., Blanquet, S. and Fayat, G. (1992) *J. Bacteriol.*, **174**, 4294–4301.
7. Schulman, L. H. and Pelka, H. (1975) *J. Biol. Chem.*, **250**, 542–547.
8. Lee, C.P., Dyson, M.R., Mandal, N., Varshney, U., Bahramian, B. and RajBhandary, U.K. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 9262–9266.
9. Garcia-Villegas, M. R., De La Vega, F. M., Galindo, J. M., Segura, M., Buckingham, R. H. and Guarneros, G. (1991) *EMBO J.*, **10**, 3549–3555.
10. Meinnel, T., Mechulam, Y. and Fayat, G. (1988) *Nucl. Acids Res.*, **16**, 8095–8096.
11. Meinnel, T., Mechulam, Y., Fayat, G. and Blanquet, S. (1992) *Nucl. Acids Res.*, **20**, 4741–4746.
12. Meinnel, T., Mechulam, Y., Lazennec, C., Blanquet, S. and Fayat, G. (1993) *J. Mol. Biol.*, **229**, 26–36.
13. Guillon, J. M., Meinnel, T., Mechulam, Y., Lazennec, C., Blanquet, S. and Fayat, G. (1992) *J. Mol. Biol.*, **224**, 359–367.
14. Mellot, P., Mechulam, Y., LeCorre, D., Blanquet, S. and Fayat, G. (1989) *J. Mol. Biol*, **208**, 429–443.
15. Schofield, P. and Zamecnik, P.C. (1968) *Biochim. Biophys. Acta*, **155**, 410–416.
16. Bradford, M. M. (1976) *Anal. Biochem*, **72**, 248–254.
17. Komine, Y., Adachi, T., Inokuchi, H. and Ozeki, H. (1990) *J. Mol. Biol.*, **212**, 579–598.
18. Lee, C.P., Mandal, N., Dyson, M.R. and RajBhandary, U. L. (1993) *Proc. Natl. Acad. Sci. USA*, **90**, in press.
19. Lee, C. P., Seong, B. L. and RajBhandary, U. L. (1991) *J. Biol. Chem.*, **266**, 18012–18017.
20. Janiak, F., Dell, V. A., Abrahamson, J., Watson, B., Miller, D. and Johnson, A. (1990) *Biochemistry*, **29**, 4268–4277.
21. Petersen, H. U., Røll, T., Grunberg-Manago, M. and Clark, B. F. C. (1979) *Biochem. Byophys. Res. Commun.*, **91**, 1068–1074.
22. Bremer, H. and Dennis, P. P. (1987) In F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, & H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology, American Society for Microbiology, Washington, D.C., pp 1527–1542.