Point and deletion mutations eliminate one or both methyl group transfers catalysed by the yeast TRM1 encoded tRNA \((m^{2}G_{26})\)dimethyltransferase

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ABSTRACT

Guanosine at position 26 in eukaryotic tRNAs is usually modified to \(N^{2}, N^{2}\)-dimethylguanosine \((m^{2}G_{26})\). In \textit{Saccharomyces cerevisiae}, this reaction is catalysed by the TRM1 encoded tRNA \((m^{2}G_{26})\)dimethyltransferase. As a prerequisite for future studies, the yeast TRM1 gene was expressed in \textit{Escherichia coli} and the His-tagged Trm1 protein (rTrm1p) was extensively purified. rTrm1p catalysed both the mono- and dimethylation of \(G_{26}\) in vivo in \textit{Escherichia coli} tRNA and in vitro in yeast trm1 mutant tRNA. The TRM1 gene from two independent wild-type yeast strains differed at 14 base positions causing two amino acid exchanges. Exchange of the original Ser467 for Leu caused a complete loss of enzyme activity in vitro against trm1 yeast tRNA. Comparatively short N- or C-terminal deletions from the 570 amino acid long Trm1 polypeptide decreased or eliminated the enzyme activity, as did some point mutations within these regions. This indicated that the protein is not a two domain peptide with the enzyme activity localised to one of the domains, but rather that both ends of the polypeptide seem to interact to influence the conformation of those parts that make up the RNA-binding site and/or the active site of the enzyme.

INTRODUCTION

Transfer RNA (tRNA) genes from all organisms are transcribed into precursor tRNAs, which are then processed into mature tRNAs in a series of steps involving trimming by exo- and endonucleases, splicing out of any existing introns and modifications of some of the bases in the polynucleotide chain. At this time 80 modified nucleosides, many of them methylated, have been isolated from eukaryotic and prokaryotic tRNAs. Most of them are formed post-transcriptionally. The methylating enzymes are highly specific both with respect to the methylated base and to the site of methylation in the tRNA molecule.

The formation of a modified base in a pre-formed polynucleotide depends on the interactions between this polynucleotide, the enzyme protein polymer and the donor of the modifying group. Predetermined point mutations introduced stepwise into the genes for the respective polymers might unravel how the substrate tRNA and the modifying enzyme recognize each other. We have explored such techniques on tRNA genes to elucidate which elements in the tRNA are required for dimethylation of position \(G_{26}\) in the junction between the D stem and the anticodon stem in yeast tRNAs. This was done by use of modifying enzymes either in vitro from yeast cells, as a crude enzyme extract, or in vivo in \textit{Xenopus laevis} oocytes, into which tRNA was microinjected. Besides the improving effects of certain secondary structures in tRNAs, yeast homologous dimethylation of \(G_{26}\) was shown to require two G-C base pairs preceding \(G_{26}\) and at least five bases in the extra loop of the yeast tRNAs. The \(m^{2}G_{26}\) modification is found in tRNAs from all Eukarya and Archaea, but not in eubacterial tRNAs. In \textit{Saccharomyces cerevisiae} the dimethylation of \(G_{26}\) to \(m^{2}G_{26}\) is catalysed by an enzyme encoded by the nuclear \textit{TRM1} gene, which corresponds to a 63 kDa protein of 570 amino acids. Which parameters in Trm1p and other tRNA methylating enzymes make them so specific are not yet known. The only eukaryotic tRNA \((m^{2}G_{26})\)dimethyltransferase that has been purified to homogeneity was the 200–240 kDa protein isolated from the protozoan \textit{Tetrahymena pyriformis}. No yeast methylating enzyme has been isolated in a pure form. We have now extensively purified the yeast tRNA \((m^{2}G_{26})\)methyltransferase as a recombinant protein in \textit{Escherichia coli} and shown that the enzyme activity is very sensitive to even very short terminal deletions and to certain point mutations in specific internal regions of the polypeptide.

MATERIALS AND METHODS

Reagents and chemicals

Restriction enzymes and DNA modification enzymes were purchased from New England Biolabs and Boehringer-Mannheim. Isopropyl-\(\beta\)-D-thiogalactoside (IPTG) came from Sigma and S-adenosyl\((^{14}\text{C}-\text{methyl})\)-L-methionine (53 mCi/mmol) from Amersham.

Plasmids, bacteria and yeast strains

The \textit{E.coli} expression plasmid pET15b (Novagen) was derived from pBR322. The \textit{E.coli} strains used were: DH5\(\alpha\) [supE44, supE44]...
Expression of wild-type and truncated recombinant Trm1 proteins in E.coli

The TRM1 gene in plasmid pGT554 (18) was amplified by the PCR method (PCR kit N808-0913; Perkin Elmer), starting immediately after the second start codon at bp position +49 by using the complementing primers oligo-1 (forward, 5′-GCCGCTCGAGTGAAGCTGCTA TTA TCC-3′) and oligo-2 (reverse, 1697–1713, 5′-GCCGCTCGAGTCTATGATGAGGTTGTTGGGAC-3′) carrying the XhoI site at the outside borders. The XhoI-cut PCR product, cloned into the E.coli expression vector pET15b, gave pJL104, which coded for an in-frame fusion of six N-terminal histidines and Trm1p (reverse, 15). We denote the shorter translation product, corresponding to the amino acid sequence N-terminal histidines and Trm1p (Fig. 1). We denote the shorter translation product, corresponding to the amino acid sequence N-terminal histidines and Trm1p (reverse, 15).

Oligonucleotide-directed mutagenesis

All oligonucleotides used were synthesized at CMB (Umeå University, Sweden). The Transformer® Site-Directed Mutagenesis Kit (Clontech) was used to make specific mutations and N-terminal deletions in the TRM1 gene in pJL104. The trans oligo selection primer oligo-6 (1221–1244, 5′-CCAGAGATGATCGTGATGAA-CACG-3′) eliminated the unique NdeI site by a 1 bp change at bp 1232 of TRM1 (cf. Fig. 1). Each of the oligos oligo-3 (91–104, 5′-GGTCGGCCGCCCAGCCGCTT-TTCTATAATCCC-3′), oligo-8 (139–157, 5′-GGTCGGCCGCCCAGCCGCTT-TTCTATAATCCC-3′) and oligo-9 (145–164, 5′-GGTCGGCCGCCCAGCCGCTT-TTCTATAATCCC-3′) all complementary to the thrombin cleavage site in pJL104 were used together with oligo-6 (1221–1244) to loop out different N-terminal-portions of TRM1 in pJL104, giving plasmids pJL109–pJL113 (Table 1). In plasmid pJL105 the BglII TRM1 fragment from plasmid Ycp (TRM1 7.4) was cloned into the BamHI site in plasmid pET15b. Oligo-7 (627–651, 5′-CATCGAGTTGGACCCTACGGTACC-3′) and oligo-14 (5′-CCAGGATCTGATGAGGTTGTTGGGAC-3′), causing loss of a PstI site at position 4958 in the vector) annealed to pJL104 gave pJL115, in which the BamHI site within the TRM1 gene was disrupted without changing the amino acid sequence.

The C-terminal TRM1 deletions were constructed by PCR using the forward primer oligo-6 (1221–1244) and oligo-12 (1695–1677, 5′-GGTCGGCCGCCCAGCCGCTT-TTCTATAATCCC-3′), oligo-10 (1665–1642, 5′-GGTCGGCCGCCCAGCCGCTT-TTCTATAATCCC-3′), oligo-16 (5′-CCAGGATCTGATGAGGTTGTTGGGAC-3′) and oligo-11 (1620–1603, 5′-CCAGGATCTGATGAGGTTGTTGGGAC-3′), causing loss of a PstI site at position 4958 in the vector) annealed to pJL104 gave pJL115, in which the BamHI site within the TRM1 gene was disrupted without changing the amino acid sequence.

Point mutation plasmids pJL104E47A, pJL104G48A, pJL104S467L and pJL104S570A were constructed by PCR-based site-directed mutagenesis (Perkin Elmer). The synthetic antisense oligonucleotides used to generate exchanges of single amino acids were oligo-15 (5′-GATTTCATATGTCAGGCGAGAAAAGCAGAAATTC-3′), oligo-16 (5′-CAATAGTCAGGCGAGAAAAGCAGAAATTC-3′), oligo-17 (5′-CCATGAGTTGGACCCTACGGTACC-3′) and oligo-18 (5′-CCAGGATCTGATGAGGTTGTTGGGAC-3′). Mutated positions (underlined) were screened for in the transformants by DNA sequencing (ABI Prism™ Dye Terminator Cycle Sequencing Ready Reaction Kit, P/N 402078; Perkin Elmer).
Table 1. Constructions of wild-type and truncated TRM1

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Oligo used for construction</th>
<th>Deletion or point mutation</th>
<th>Length of Trm1 (amino acids)</th>
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The two ATG start codons (+1 and +49) in the TRM1 gene give two polypeptides that differ in 16 N-terminal amino acids (13). In this study, wt refers to the translation product rTrm1p from the first amino acid (Leu18) after the second ATG in the TRM1 gene. AN and ΔC indicate the number of amino acids deleted from the N(Leu18)- and the C(Ser570)-termini of rTrm1p, respectively.

Generation of antibodies and immunoblot analysis

The purified rTrm1p, concentrated to 1 mg/ml (Centriplus®; Amicon), was used to immunise two New Zealand white male rabbits (AgriSera AB). Aliquots of 100–200 μg of rTrm1p were injected at days 0, 14, 26 and 90. Before use, the collected sera were incubated with rabbit (AgriSera AB). Aliquots of 100–200 μg of rTrm1p were injected at days 0, 14, 26 and 90. Before use, the collected sera were incubated with rabbit (AgriSera AB).

For immunoblotting tests, crude cell extracts from the same amount of cells were separated by 10 or 12% SDS–PAGE, transferred to PVDF membranes (BioRad) and blotted at 25 °C. The same amount of cells were separated by 10 or 12% SDS–PAGE, transferred to PVDF membranes (BioRad) and blotted at 25 °C.

Formation of m^2G_{26} and m^2_{2}G_{26} in tRNA in vitro and in vivo

For assay of methylation in vitro (17), crude enzyme extract was incubated at 30 °C for 30 min with an excess of yeast D4 tRNA substrate and S-adenosyl[14C]-methyl-L-methionine as methyl donor (initial rate determinations). Methyl label in re-isolated tRNA was measured by scintillation counting.

Isolated, in vivo modified bulk tRNA from plasmid-carrying E.coli (21) was re-purified on a Nucleobond cartridge Ax500 column (Macherey-Nagel GmbH). About 120 μg of tRNA was hydrolised with nuclease P1 and bacterial alkaline phosphatase to nucleosides and analysed on a Supelcosil LC-18S column using a Waters HPLC system (22). The tRNA samples were free of contaminating RNA as shown by the complete lack of markers unique for tRNA.

RESULTS

Purification of yeast tRNA (m^2_{2}G_{26})methyltransferase (rTrm1p)

The S.cerevisiae TRM1 encoded tRNA (m^2_{2}G_{26})dimethyltransferase catalyses the transfer of both methyl groups from the methyl donor S-adenosyl-L-methionine uniquely to position G_{26} in pre-tRNA (7,16,23). The enzyme is of fairly low abundance in yeast cells. Even when the cloned TRM1 gene (18) was over-expressed in yeast cells, the amount of enzyme per cell was too low to allow isolation of the amounts of pure enzyme needed for future structural and mechanistic studies. We therefore over-expressed the wild-type and mutant forms of the yeast TRM1 gene in E.coli. The affinity column technique was then used to purify the tRNA (m^2_{2}G_{26})dimethyltransferase as a histidine-tagged protein. Escherichia coli completely lacks tRNA G_{26}methylating enzymes (24) and therefore G_{26} in E.coli tRNA is a site for modification in vivo when yeast TRM1 is expressed in E.coli cells (12), as well as in vitro by the yeast enzyme (25). The homologous methylation was tested with yeast D4 tRNA specifically lacking G_{26} modifications (7,16).

The S.cerevisiae TRM1 gene has two ATG start codons at bp positions +1 and +49, respectively, both of which can initiate translation into functional proteins (13). The TRM1 sequence in plasmid pJL104 starts with the position (Leu18) immediately after the second start codon downstream of a T7 Lac promoter and a His6-tag (Fig. 1). IPTG-induced cells carrying pJL104 could after the second start codon downstream of a T7 Lac promoter and a His6-tag (Fig. 1). IPTG-induced cells carrying pJL104 could produce up to 3 mg of rTrm1p protein/l culture. Pooled peak fractions, eluted from the separation column, were purified to at least 96% (Fig. 2). The rTrm1p protein had the expected size of 63 kDa (Fig. 2) and was enzymatically active. The enzyme has a theoretical pl of 9.2, does not enter non-denaturing gels with a pH range of 8.3–9.5, has a tendency to aggregate, precipitates if stored at 4 °C for several days, but is stabilised by sodium and potassium salts. When kept frozen in small portions the activity was maintained for at least several months.

The first 27 N-terminal amino acids in rTrm1p are not needed for G_{26} modification activity in vitro or in vivo

Some bacterial tRNA modifying enzymes simultaneously harbour both a modification activity and a second function in the polypeptide, presumably in different domains/regions of the protein (26). Whether the Trm1 polypeptide might be a dual domain protein (27) with the modification activity located in either the N- or the C-terminal portion of the protein was investigated by successively deleting N- and C-terminal amino acids. The TRM1 gene in pJL104, denoted “wild-type” by us, started at position 52 (cf. Fig. 1). Thus it lacked the first 17 N-terminal amino acids compared with the original TRM1 gene. As seen in...
Figure 2. Purification and western blots of rTrm1p. Lanes 1–4, purification of rTrm1p analysed by 10% SDS–PAGE separation and stained with Coomassie brilliant blue. Crude extract from E. coli BL21(DE3) after IPTG induction of the His 6 tagged yeast gene TRM1 carried in plasmid pLL104 (lane 1). Flow-through (lane 2) and wash (lane 3) from the Ni 2+ affinity column. Eluate, pooled peak fractions (3 ml) of rTrm1p (lane 4). The position of rTrm1p is indicated. Lanes 5–7, western blots of rTrm1p. Samples, separated by 10% SDS–PAGE, were electroblotted onto a PVDF membrane and incubated with anti-rTrm1p antibodies for 1 h. Crude extract (lane 5) and different amounts of purified rTrm1p (lanes 6 and 7).

Figure 3. Expression of truncated rTrm1p in E. coli. Crude extracts from equal amounts of E. coli BL21(DE3) cells harbouring plasmids with different N- and/or C-terminal TRM1 deletions (cf. Table 1) were separated by 12% SDS–PAGE, transferred onto a PVDF membrane and blotted with anti-rTrm1p antibodies. Negative control, plasmid pET15b.

Figure 4, neither ΔN13aa nor ΔN27aa substantially influenced the modification activity, which was still 96% of the wild-type rate.

However, if another two amino acids were deleted (ΔN29aa), the activity drastically decreased to only 25% of the wild-type rate and for ΔN31aa the enzyme activity was totally lost (Fig. 4). In yeast cells also the corresponding deletion led to an inactive enzyme (28). Thus, the 27 first N-terminal amino acids from the second AUG start in Trm1p (up to Ile44; cf. Fig. 5) had no impact on those parts of this polypeptide that are important for the enzyme (28).

These in vitro data, measuring the enzyme rates, did not indicate whether the different truncated forms of the enzyme could perform both steps in the m 2 2 G, m 2 G modification reaction or only the first one. Different truncated rTrm1p variants were therefore expressed for 3 h in vivo in E. coli (cf. Fig. 3) and tRNA was isolated, degraded and analysed by HPLC. The wild-type yeast rTrm1 enzyme efficiently catalysed the transfer of both methyl groups to G 26 in the bacterial tRNAs in vivo (Table 2) to a level of 1.9 ± 0.1 mol CH 3 /mol tRNA (the theoretical optimum is 2) with 94% in the form of m 2 2 G. For ΔN13aa and ΔN27aa the levels were 2.0 and 1.7 mol CH 3 /mol tRNA, respectively, still with 94% as m 2 2 G in both cases, i.e. in essence a wild-type situation.

The ΔN29aa deletion gave an effect. Like in vitro (measuring enzyme rates), the in vivo data (measuring level of methylation) showed a decrease compared with the wild-type levels. In vivo the total amount of methylated guanosine (m 2 G + m 2 2 G) was decreased by 25% to 1.5 mol CH 3 /mol tRNA. Furthermore, the ability of the enzyme to perform the second methylation step was drastically altered, giving almost equal amounts of m 2 2 G and m 2 G compared with the normal ratio of at least 16:1. After deletion of an additional two amino acids (ΔN31aa) neither m 2 G nor m 2 2 G was detected. This truncated protein was therefore unable to perform either of the two methylation steps in vitro or in vivo. We conclude that the enzyme activities needed for the formation of the two reaction products are differently sensitive to the size of the N-terminal deletions: the dimethylation step is the more sensitive one, since data on ΔN29aa could be interpreted as
not necessarily affecting the monomethylation step, while in ΔN31aa monomethylation was also non-functional.

The C-terminal part of Trm1p was important for G26 modification in tRNA

To define if G26 modification activity was independent of a hypothetical C-terminal domain in the Trm1 polypeptide, we deleted 5, 10, 15 or 30 amino acids from the C-terminus (Table 1). All ΔCTrm1p variants were efficiently expressed in E.coli (Fig. 3). ΔC5aa caused the in vitro modification activity to drastically decrease to 27% of the wild-type rate (Fig. 4). Additional deletions up to ΔC30aa further lowered the activity to ~7% of the wild-type value. Since both C-terminal and N-terminal ΔTrm1p deletions influenced the modification activity we conclude that neither the C-terminus nor the N-terminus by themselves make up a separate domain to which the Trm1p activity was localised.

In vivo in E.coli (Table 2), the ΔC5aa total formation of m2G + m2G was measured as 1.8 mol CH3/mol tRNA, which was close to wild-type levels, but the ratio of m2G:m2G was now 3:1 compared with 16:1 in the normal case. Additional C-terminal deletions only slightly affected the total modification level, except for ΔC30aa for which the total level was lowered by 75% to 0.51 mol CH3/mol tRNA which contained practically no m2G26 (0.01 mol CH3/mol tRNA). In ΔC10aa an increase of m2G formation relative to m2G was observed, but with further C-terminal deletions the monomethylation activity was also severely lowered. Thus, C-terminal-deleted rTrm1p maintains some enzymatic activity in vivo but the truncated enzymes are especially inefficient in performing the second step in the dimethylation reaction.

Combined N- and C-terminal deletions

Drastic effects of short C-terminal deletions might indicate that the C-terminus could have a more profound influence on the overall conformation needed for rTrm1p activity than the N-terminus and that the C- and N-termini might interact with each other. We therefore combined ΔC15aa with various ΔN deletions (Table 1), expressed such ΔC15aa ΔN variants in E.coli (Fig. 3) and assayed for G26-specific tRNA methyltransferase activity in vitro. Combining ΔC15aa with ΔN13aa or ΔN27aa barely affected the low rate of ~11% caused by ΔC15aa by itself (cf. Fig. 4), while a combination with ΔN29aa (by itself causing a decrease of 25%) totally eliminated all activity. In vivo (Table 2) the level of m2G + m2G formed in ΔC15aa was 1.7 mol CH3/mol tRNA. Combining ΔC15aa with ΔN13aa had only a slight effect, giving a value of 1.6 (ΔN13aa only, 2.0). However, the combination ΔC15aa ΔN27aa gave 0.85 (ΔN27aa only, 1.7) and ΔC15aa ΔN29aa gave 0 (ΔN27aa only, 1.4) mol CH3/mol tRNA. Thus, in vivo these two combinations of N- and C-terminal deletions exceeded the added individual effects. Furthermore, in ΔC15aa ΔN27aa monomethylated G26 was strongly dominant with a ratio m2G:m2G of 0.1:1, which is remarkable since ΔN27aa by itself gave the ratio 16:1, a difference of 160 times, while the ratio of m2G:m2G in ΔC15aa and ΔC15aa ΔN13aa was ~1:1. In ΔC15aa ΔN29aa the residual activity was only an extremely inefficient monomethylating activity (0.06) in comparison with ΔN29aa by itself, where the m2G:m2G ratio was 1:1. We conclude that N-terminal deletions up to Ile44 affect the inherent modification activity in partly C-terminal deleted Trm1p in a way that exceeded additivity and that both the mono- and dimethylation steps during the G26 modification were influenced. Thus, the two ends of the Trm1 polypeptide seem to interact in creating the enzyme activity.

Internal point mutations: a mode to localise crucial regions of Trm1p

In TRM1 homologues (GenBank), Glu47, Gly48 and the C-terminal Ser570 are conserved amino acids. They are part of such N- and C-terminal deletions that affected the Trm1p activity. We therefore exchanged Ser570 for Ala, which left the enzyme fully active, Glu47Ala gave only 7% and Gly48Ala 16% of wild-type activity (Fig. 5). Thus, the identity of the amino acids at positions 47 and 48 are pivotal for the enzyme activity.
The yeast mutant D4 is a m^2G-deficient mutant that was isolated after UV treatment of a wild-type strain, YF^*, isolated from a brewery in Belgium (16). Its relatedness to the standard yeast strain is not known. We have cloned and sequenced the YF^* TRM1 gene (GenBank accession no. AF086825) and the D4 trm1 gene (GenBank accession no. AF086826). The YF^* and DB745 TRM1 sequences differed in 14 positions, but only two of them lead to amino acid shifts (Fig. 5), namely Thr203Ser and Gly517Arg. These shifts have no effect on the enzyme activity. Besides these 14 positions, the D4 trm1 sequence had five additional base exchanges, one of them causing serine at position 467 to be exchanged for leucine. To prove that this exchange was the cause of the lack of activity in D4 Trm1p, leucine was introduced into Trm1p from the wild-type gene from DB745 and was shown to cause a complete loss of tRNA (m^2G26)methyltransferase activity. We conclude that position 467 is of crucial importance for the activity and that serine but not leucine at this position is instrumental in the enzyme activity.

**DISCUSSION**

Structural, functional and mechanistic studies on how a modifying enzyme exerts its activity and which parts of the enzyme interact with its specific set of tRNAs ultimately require a pure enzyme. The now available techniques finally allowed us, after three decades of attempts (25), to purify *S.cerevisiae trm1* encoded tRNA (m^2G26)methyltransferase to at least 96% purity. In crude yeast cell extracts the enzyme is invariably quite stable, but an increasing degree of purity, as well as longer storage, is mostly accompanied by loss of activity. When the yeast rTrm1p was overproduced in bacteria over a short time period and then isolated in an efficient and fast two-step procedure, the enzyme remained active and the protein could withstand storage for some time. With the quantity and quality of the protein now available, attempts to crystallise the enzyme have been initiated.

The TRM1 sequence implies a 63 kDa protein, which is the size of the pure rTrm1p. We and others (24) have observed that the enzymatic activity under non-denaturing conditions was usually associated with much higher molecular weights, thus the native form of the enzyme might be a multimeric form of or a complex with the 63 kDa polypeptide. The tendency of the native, quite basic protein to form aggregates and the stabilising effects of salts bear clear similarities to another tRNA modifying enzyme, the tRNA-acceptor transglycosylase (Tgt) from *Zymomonas mobilis* (29). It is suggestive that an enzyme that exerts its activity in the yeast nucleus (30), where it shares its substrate with many other modifying and maturating enzymes, might optimise its access to the substrate by being present as a member of a multicomponent complex, possibly stabilised by contact with the inner membrane of the nucleus (31) or the nuclear pore complex (32).

The naturally occurring shorter version of Trm1p, lacking 16 N-terminal amino acids, is the form that is preferentially located in the nucleus (13), where the majority of the tRNA modification events occur. We therefore chose this form as representing the wild-type. Studies on effects of deletions or point mutations in yeast methylating enzymes are scarce and, consequently, very little is known about what parts of the 570 amino acid long Trm1 protein are involved in or influence the methyl transfer activity and which amino acids constitute the active site of the enzyme. Deleting amino acids from the N- and/or the C-terminal ends had adverse effects on the overall structure, as manifested by a change of or a loss of methylating activity. As expected for assays performed in different surroundings, the *in vivo* and *in vitro* data for the respective mutants do not result in the same numerical values; the *in vitro* data measured initial rates of enzymatic activity, while *in vivo* data represent levels of modification obtained in conditions that the living cell itself optimises. However, both types of data demonstrate the same trend with respect to the ability to form methylated G^26. Compared with our wild-type enzyme, N-terminal deletions up to at least Ile44 hardly affected the activity. From a predicted secondary structure of Trm1p (33), amino acids 45–48 are the last in a stretch from 30 to 48 that might form a long α-helix. Since single amino acid shifts at positions 47 and 48 strongly decreased the activity, we conclude that it is the identity of the amino acids at these two positions and not their participation in the C-terminal portion of the hypothetical helix that were instrumental for creating an active enzyme structure.

The fact that the methylation efficiencies were affected by a short deletion as ΔC5aa and that such an enzyme is rather inefficient *in vitro* demonstrated that the utmost parts of the C-terminus in rTrm1p have a greater impact on a functional structure of the enzyme than the utmost parts of the N-terminus. It is not known if these five C-terminal amino acids, where unexpectedly the conserved serine at the very last position could be exchanged for alanine without affecting the enzyme activity, are directly involved in substrate binding, are part of the active site or indirectly influence structures needed for the activity. The fact that antibodies directed towards a synthetic peptide corresponding to the last 15 C-terminal amino acids of Trm1p could block Trm1p activity suggests that the C-terminal region is probably exposed on the surface of the enzyme (18). It is tempting to compare this system, where there are several indications for the native Trm1 enzyme to exist as a complex, with ribonucleotide reductase, in which the dimer is the active form and where forming the dimer requires the utmost seven C-terminal amino acids (34). Whether the C-terminal amino acids in Trm1p might participate in forming a native complex is under study.

Simultaneous deletions at both termini somewhat exceeded additivity of the respective effects observed, especially the inability to perform the dimethylation step, indicating that both ends of the comparatively large Trm1 polypeptide influence the creation of the 3-dimensional structure necessary for methyl group transfer. Thus, these data, together with the fact that the *S.*adenosyl-L-methionine (SAM)-binding site (amino acids 136–152) is located in the N-terminal half and the required Ser467 in the C-terminal half, eliminated the model where the Trm1 activity might be located in either one of the two hypothetical C- and N-terminal domains and show that almost the whole polypeptide is needed for activity.

To date, still very little is known about the mechanism behind methyl group transfer and which parts of methylating enzymes recognise the substrates. The target point for the yeast tRNA (m^2G)methyltransferase, position G26 in tRNA, will only be recognised if certain primary sequence and secondary structure requirements in tRNAs are fulfilled, then it catalyses the transfer of two methyl groups to G26 in tRNA (7,8). The fact that the mono- and dimethylation steps are differently sensitive to terminal deletions is consistent with earlier observations that the transfer of the two methyl groups is a successive process, first a monomethylation, then the step leading to the dimethylated product (7). This observation, together with the biochemical
characteristics of the native Trm1p, could form the basis for possible models for $m^2G_{26}$ formation. In Trm1p only one sequence, I$_{36}$LEALSATGLRAIY_R$_{152}$, could be considered as a theoretical SAM-binding site (35). In order to transfer two methyl groups to one tRNA molecule it could be imagined that each tRNA molecule forms a complex with a monomeric enzyme–SAM complex, which dissociates after transfer of the first methyl group before a new complex is formed with a new enzyme–SAM complex for transfer of the second methyl group. Alternatively, each tRNA could bind two (or more) enzyme–SAM molecules into dimers (multimers) thereby creating fast access to two methyl group donors. In this latter model it is also possible to imagine how a truncated or mutated protein might form a less stable complex with tRNA and still be able to perform, although with difficulty, the first methyl group transfer, while the second methylation step could be more severely disturbed. Now with different versions of the enzyme available in an essentially pure form, attempts to identify the exact role of Ser467 and to elucidate more steps in the complicated formation of $m^2G_{26}$ are currently under way.

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