Identification of a bifunctional enzyme MnmC involved in the biosynthesis of a hypermodified uridine in the *wobble* position of tRNA

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ABSTRACT

The gene encoding the bifunctional enzyme MnmC that catalyzes the two last steps in the biosynthesis of 5-methylaminomethyl-2-thiouridine (mnm⁵s²U) in tRNA has been previously mapped at about 50 min on the *Escherichia coli* K12 chromosome, but to date the identity of the corresponding enzyme has not been correlated with any of the known open reading frames (ORFs). Using the protein fold-recognition approach, we predicted that the 74-kDa product of the *yfcK* ORF located at 52.6 min and annotated as “putative peptidase” comprises a methyltransferase domain and a FAD-dependent oxidoreductase domain. We have cloned, expressed, and purified the YfcK protein and demonstrated that it catalyzes the formation of mnm⁵s²U in tRNA. Thus, we suggest to rename YfcK as MnmC.

Keywords: tRNA; modified nucleosides; *wobble* position; methyltransferase; flavoprotein

INTRODUCTION

Transfer RNA (tRNA) from all Domains of Life contains a high number of modified nucleosides, whose synthesis is catalyzed post-transcriptionally by specific enzymes (Björk 1995). Some of the modifications are simple and correspond to methylation, isomerization, reduction or deamination of the ordinary nucleosides. However, some nucleosides contain multiple modifications or very complex modifications, and their biosynthesis requires the action of several enzymes.

The hypermodified nucleoside 5-methylaminomethyl-2-thiouridine (mnm⁵s²U) is found in position 34 (first position of the anticodon, also called “wobble” position) of *Escherichia coli* tRNAs specific for glutamate, lysine, and possibly glutamine (Sprinzl et al. 1998). These tRNAs read

split codon boxes (boxes in which the third nucleotide determines which of the two alternative amino acids are encoded). The mnm⁵s²U modification pathway is complex and remains partially unknown. The *E. coli* *mnmA* (formerly *asuE/trmU*) gene product and the cysteine desulfurase IscS are required for the thiolation of position 2 of the uridine, leading to 2-thiouridine (s²U; Sullivan et al. 1985; Kambampati and Lauhon 2003). The *mnmE* (formerly *trmE*) and *mnmG* (formerly *gidA*) gene products are involved in the conversion of s²U to 5-carboxymethylaminomethyl-2-thiouridine (cmnm⁵s²U; Elseviers et al. 1984; Cabedo et al. 1999; Brégeon et al. 2001). However, it is possible that MnmE and MnmG are not the only gene products involved in the formation of cmnm⁵s²U. Finally, cmnm⁵s²U is first demodified to 5-aminomethyl-2-thiouridine (nm⁵s²U) and subsequently methylated in an S-adenosyl-L-methionine (AdoMet) dependent step to mnm⁵s²U. These last two activities (MnmC1 and MnmC2) are carried out by the same enzyme called MnmC (formerly TrmC; Taya and Nishimura 1973; Hagervall et al. 1987).

Methylation is one of the most common modifications and several mutants affected in tRNA methylation have

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been obtained (Björk 1995). On the one hand, most of known tRNA methyltransferases (MTases) have been purified and the corresponding genes have been mapped on the *E. coli* chromosome (Björk 1995). On the other hand, only a few of these tRNA MTase genes have been cloned and unambiguously linked to the open reading frames (ORFs) in the *E. coli* genome. The MTases characterized so far at both the gene and protein level include *trmA*, *trmB*, *trmD*, and *trmH*, involved in the formation of m⁵U54, m⁷G46, m¹G37, and Gm18, respectively (Ny et al. 1988; De Bie et al. 2003; Byström and Björk 1982; Persson et al. 1997). However, other genes encoding tRNA MTases remain to be identified. This is the case for the enzyme MnmC. Early genetic studies located this gene at 50.5 min on the *E. coli* K12 chromosome (Hagervall and Björk 1984). However, despite the *E. coli* chromosome sequence has been published over six years ago (Blattner et al. 1997), it has not yet been found which of the ORFs corresponds to the *mnmC* gene. As a result, the studies of sequence-structure-function relationships of the MnmC enzyme are seriously hampered and the mechanism of mnm⁵s²U formation remains unknown.

RESULTS AND DISCUSSION

As a part of a large-scale project, aiming at identification of the “missing links” between known enzymatic activities and ORFs predicted to encode RNA MTases, we sought to identify the gene encoding the MnmC enzyme. The molecular mass of the purified MnmC protein was estimated as 79 kDa (Hagervall et al. 1987), which corresponds to approximately 710 amino acids. It was reported that the *mnmC* gene is located between the *aroC* and the *hisT* genes (Hagervall and Björk 1984). Thus, we analyzed the sequences of all 10 proteins encoded by genes in this region (nucleotide sequence coordinates 2433656 and 2444408 on the *E. coli* K12 chromosome) using the NCBI Conserved Domain Database RPS-BLAST search utility and the protein fold-recognition approach (see Materials and Methods for details).

The RPS-BLAST search of the Conserved Domain Database (Marchler-Bauer et al. 2003) revealed no significant similarity of any of the uncharacterized ORFs in the analyzed region to known proteins involved in RNA modification or enzymes that utilize AdoMet as a methyl group donor. Nonetheless, the results obtained allowed to divide the sequences into fragments corresponding to putative domains (data not shown). When the fold-recognition analysis was performed via the GeneSilico metaserver interface (Kurowski and Bujnicki 2003), the N-terminal domain of the uncharacterized protein YfcK (amino acids 1–260) was found to be significantly related to the known structures of AdoMet-dependent MTases (SAM-T02 score 3.7×10^{-7} (Karplus et al. 2003), FFAS03 Z-score -25.5 (Rychlewski et al. 2000), Pcons consensus score 1.89 (Lundström et al. 2001). The fold-recognition analysis confirmed (with very

high scores from all servers used) that the C-terminal domain of the YfcK protein (amino acids 261–668) is closely related to glycine/D-amino acid oxidases, FAD-linked oxidoreductases and their relatives (Dym and Eisenberg 2001), which was also reported by RPS-BLAST, but initially did not prompt us to consider its involvement in RNA modification. The molecular features of the uncharacterized protein YfcK (668 amino acids, theoretical molecular weight 74434.16 Da and the presence of two domains with potentially distinct enzymatic activities) suggested it as a plausible candidate for the MnmC enzyme.

Analysis of the sequence alignment of the YfcK protein and its homologs (Fig. 1) reveals conserved sequence regions in both predicted domains. Amino acids in these conserved regions show similarity to the substrate binding and active sites of MTases and FAD-dependent reductases identified by the fold-recognition methods as homologs of the MnmC protein. Based on the fold-recognition alignment, we predict specific functions for a number of conserved amino acids: in the C-terminal domain, G271, G272, G273, S304, R567, S617, and R618 are predicted to be essential for FAD binding and for the oxidation reaction; in the N-terminal domain, E64, E101, D156 are predicted to be involved in the AdoMet binding, while D178, F180, and R237 are likely to be essential for the target base binding and the methyl transfer reaction.

The *E. coli yfcK* ORF was PCR-amplified and cloned into the pET28a expression vector, allowing the production of an N-terminal His-tagged protein (H6YfcK) in *E. coli* (see Materials and Methods). The expression of the recombinant protein was induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) in the *E. coli* strain BL21 (DE3). The His-tagged protein was purified by immobilized metal ion chromatography followed by ion-exchange chromatography. SDS-PAGE analysis of the fractions containing H6YfcK showed that the recombinant protein was purified to near homogeneity (Fig. 2A). The apparent molecular mass of the recombinant protein (78 kDa) was consistent with the theoretical value (76.593 kDa). The purified protein had a yellow color and the spectrum showed maximum absorption peaks at 357 nm and 430 nm (Fig. 2B), in keeping with the presence of a flavin derivative.

In order to obtain undermodified tRNAs that could be substrates of the YfcK enzyme, the *E. coli yfcK* ORF was inactivated by the insertion of an ampicillin resistance (Ap^r) cassette as described in Materials and Methods. The resulting strain was called RD2. To determine whether the recombinant YfcK protein displayed tRNA modification activity, unfractionated tRNA from the RD2 strain was incubated in the presence of the purified H6YfcK protein and ¹⁴C-radiolabeled AdoMet (S-adenosyl-L-[methyl-¹⁴C]methionine). After the incubation, the tRNA was recovered by phenol extraction and ethanol precipitation and further completely hydrolyzed by nuclease P1. The resulting hydrolysate was then analyzed by bidimensional cellulose thin

FIGURE 1. Multiple sequence alignment of *E. coli* YfcK and a representative selection of its homologs selected from the α , β , γ , and ϵ divisions of the Proteobacteria (Ec, *E. coli*; Pa, *Pseudomonas aeruginosa*; Cj, *Campylobacter jejuni*; Rs, *Ralstonia solanacearum*; Cc, *Caulobacter crescentus*). The bottom panel shows the fold-recognition alignment of both domains with the known crystal structures of MTase (1dus) and sarcosine oxidase (1e15). The amino acid residues of *E. coli* YfcK are numbered. The residues predicted to bind AdoMet, participate in the methylation reaction and to catalyze the FAD-dependent oxidation are indicated by A, M, and F, respectively.

FIGURE 2. Purification of the recombinant *E. coli* YfcK protein. (A) SDS-polyacrylamide gel showing purified recombinant YfcK protein. The gel was stained with Coomassie G-250 (Invitrogen). Lane 1, purified H6YfcK protein; lane 2, molecular mass markers in kilodaltons (Pharmacia Biotech). (B) Visible spectrum of purified H6YfcK protein (2 mg protein/ml).

layer chromatography (2D-TLC) followed by autoradiography. The result shown in Figure 3 revealed the presence on the TLC plate of a radioactive compound with migration characteristics identical to those of $\text{mnm}^5\text{s}^2\text{U}$ 5'-phosphate (Taya and Nishimura 1973; Marinus et al. 1975). Total tRNA from the wild-type DY330 strain was used in a control experiment. In this case, no incorporation of ^{14}C in tRNA was observed (result not shown). *Bacillus subtilis* does not possess an MnmC enzyme and the undermodified $\text{cmnm}^5\text{s}^2\text{U}$ and cmnm^5U nucleosides are found in tRNAs of this organisms (Sprinzl et al. 1998). When total tRNA from *B. subtilis* was incubated in the presence of ^{14}C AdoMet and of the purified H6YfcK enzyme, two ^{14}C -labelled modified

nucleosides were formed, corresponding to $\text{mnm}^5\text{s}^2\text{U}$ and mnm^5U (Fig. 3B). This result shows that the YfcK enzyme catalyzes the two steps leading from $\text{cmnm}^5\text{s}^2\text{U}$ to $\text{mnm}^5\text{s}^2\text{U}$ (demodification followed by methylation), and this independently of the presence of a 2-thio group.

In conclusion, the recombinant YfcK protein was shown to catalyze the formation of $\text{mnm}^5\text{s}^2\text{U}$ in tRNA in vitro. In

FIGURE 3. The product of the *E. coli* yfcK ORF catalyzes the formation of $\text{mnm}^5\text{s}^2\text{U}$ in tRNA. Autoradiography of a two-dimensional chromatogram of 5' phosphate nucleosides on a thin layer cellulose plate. Total tRNA from the *E. coli* RD2 strain (A) or from the *B. subtilis* strain 168 (B) was incubated in the presence of [*methyl*- ^{14}C]AdoMet and purified H6YfcK protein as described in Materials and Methods. After a 30-min incubation at 37°C, the tRNA was recovered and digested by nuclease P1, and the resulting nucleotides were analyzed by 2D-TLC as described previously (Keith 1995). Circles in dotted lines show the migration of the four canonical nucleotides used as uv markers.

accordance with previous observations (Hagervall et al. 1987), the first demodification step leading to $\text{nm}^5\text{s}^2\text{U}$ from $\text{cmnm}^5\text{s}^2\text{U}$ occurred independently on the addition of any exogenous cofactor. Therefore we propose that the *yfcK* gene be renamed *mnmc* and the corresponding protein MnmC. Based on the results of our analysis and the knowledge of the mechanism of reactions catalyzed by MTases (Cheng and Blumenthal 1999), and FAD-dependent oxidoreductases (Settembre et al. 2003), we propose a tentative mechanism of the $\text{nm}^5\text{s}^2\text{U}$ formation catalyzed by the MnmC enzyme (Fig. 4).

Sequence searches of the complete genomes revealed that the distribution of the putative orthologs of *E. coli* YfcK comprising the fused oxidoreductase (MnmC-1) and the MTase (MnmC-2) domain is conserved only in γ -Proteobacteria, with a few additional members found in some β -Proteobacteria (*Chromobacterium violaceum*, several genera of Burkholderiales), ϵ -Proteobacterium *C. jejuni*, unclassified Proteobacterium *Magnetococcus* sp. MC-1, and even Spirochaetes (*Leptospira interrogans*). Separate homologs of the MnmC-1 and MnmC-2 domains were found in various bacterial genomes (data not shown), although given the commonness and significant sequence divergence of the MTase and oxidoreductase superfamilies it is difficult to assess with certainty which of them represent remote orthologs of MnmC with “standalone” functions in tRNA:U34 modification and which may be paralogs with

quite distinct functions. Interestingly, in several genomes a potential ortholog of only one domain could be identified with confidence. For instance *Neisseriae* (β -Proteobacteria) possess a close homolog of the MnmC-1 domain but no close homolog of the MnmC-2 domain. On the other hand, *Thermus thermophilus*, Cyanobacteria, and some α -Proteobacteria possess only a close homolog (putative ortholog) of the MnmC-2 domain, while the closest homolog of the MnmC-1 seems to be very strongly diverged and probably corresponds to a paralog with a different function. It is unclear if the development of the tRNA:U34-modification functions MnmC-1 and MnmC-2 by the MTase and oxidoreductase domains predated their fusion that created the bifunctional enzyme and if the “standalone” domains represent the ancestral or derived (shortened) versions. The inference of the origin and the evolutionary history of the MnmC family will require the experimental determination of the function of these “standalone” members of the family and the resolution of the phylogenetic relationships at the level of the oxidoreductase and MTase superfamilies.

MATERIALS AND METHODS

Bioinformatic analyses

Sequence similarity searches were performed with PSI-BLAST (Altschul et al. 1997) and RPS-BLAST (Marchler-Bauer et al. 2003) at the National Center for Biotechnology Information (Bethesda, Maryland, USA; <http://www.ncbi.nlm.nih.gov/BLAST/> and <http://www.ncbi.nlm.nih.gov/Structure/cdd/>). The coverage of RPS-BLAST alignments between the query sequences and conserved protein domains (mostly of unknown function) was used to split the query into putative domains. For these domains a fold-recognition analysis was carried out to identify homologous structures in the Protein Data Bank and thereby predict the three-dimensional structure of the query protein. Sequences were submitted to the GeneSilico protein structure prediction Meta Server (Warsaw, Poland; <http://genesilico.pl/meta/>) and analyzed by a large collection of fold-recognition tools (for the complete list of programs and references see Kurowski and Bujnicki 2003). All results were compared and ranked by the consensus server Pcons (Lundström et al. 2001), yielding the final fold prediction.

Cloning, expression, and purification of the *E. coli* YfcK protein

The *E. coli* *yfcK* ORF was amplified by the polymerase chain reaction (PCR) using *E. coli* genomic DNA as template and forward (5'-CTGAACCATATGAAACACTACTCCATACAACCTGCC-3') and reverse (5'-CTGAACAGATCTTTACCCCGCCTTAACCGCTTTACCCTTCAAC-3') oligonucleotides as primers. The Expand High Fidelity PCR kit (Roche) was used to avoid errors in the amplified product. The primers incorporated an NdeI site before the start codon and a BglII site after the stop codon, such that the amplified sequence could be cloned between the NdeI and BamHI sites of the expression vector pET28a (Novagen), which encodes an N-terminal poly-histidine tag. The sequence of the clone was

FIGURE 4. A tentative mechanism of the enzymatic formation of $\text{nm}^5\text{s}^2\text{U}$ from $\text{cmnm}^5\text{s}^2\text{U}$. (A) FAD-dependent demodification of $\text{cmnm}^5\text{s}^2\text{U}$ to $\text{nm}^5\text{s}^2\text{U}$ involving formation of the MnmC-base complex, release of H^+ , reoxydation of FAD by dioxygen and release of $\text{nm}^5\text{s}^2\text{U}$. (B) Transfer of a methyl group from AdoMet to $\text{nm}^5\text{s}^2\text{U}$ to produce $\text{nm}^5\text{s}^2\text{U}$. Particular groups of atoms are shown in color to illustrate their transfer between different molecules.

entirely checked. The recombinant His-tagged protein (H6YfcK) was expressed in the *E. coli* strain BL-21 (DE3). Transformed cells were grown at 37°C in Luria broth (supplemented with kanamycin at 30 µg/ml) to an optical density at 660 nm of 0.7. At this stage, IPTG (isopropyl-β-D-thiogalactopyranoside) was added to a final concentration of 1 mM to induce recombinant protein expression. Cells were harvested after 3 h incubation at 37°C and resuspended in buffer A (50 mM Tris-HCl, pH 8.0, 200 mM KCl, 10 mM MgCl₂, 10% glycerol) and lysed by sonication. The lysate was cleared by centrifugation (20,000 × g during 10 min) and was applied to a column of Chelating Sepharose Fast Flow (Pharmacia Biotech) charged with Ni²⁺. The column was washed with buffer A supplemented with 5 mM imidazole and the adsorbed material was eluted with a linear gradient (0.05 M up to 0.5 M) of imidazole. The recombinant protein was further purified by anion-exchange chromatography. The partially purified enzyme was dialyzed against buffer B (50 mM Tris-HCl, pH 8.0, 10 mM MgCl₂, 10% glycerol) and was applied on a 6 ml Resource Q column (Pharmacia Biotech) equilibrated with the same buffer. The enzyme was eluted with a linear gradient (0 M up to 0.5 M) of KCl.

Inactivation of the *E. coli* *yfcK* gene

The *E. coli* *yfcK* gene was inactivated by the insertion of an ampicillin resistance (Ap^r) cassette. This was achieved by homologous recombination, depending on bacteriophage λ recombination functions present in the host strain (Yu et al. 2000). A linear DNA fragment in which the β-lactamase gene is flanked by 40 bp corresponding to the 5' and 3' ends of the *yfcK* gene was obtained by PCR using the oligonucleotides 5'-CGCTAAGATCGAGCCACC GCCTGTAAGACGAGTAACCTTACCATTCAAATATGTATCCGC TC-3' and 5'-AGGCGTTTCACGCCGCATCCGGCAAACACTG CGAGAGCAGAAGAGTTGGTAGCTCTTGATC-3' as primers and plasmid pUC18 as the template. The PCR product was used to transform the DY330 strain (Yu et al. 2000), and transformants were selected for ampicillin resistance. The presence of the Ap^r cassette in the *yfcK* gene in the resulting RD2 strain was checked by PCR using oligonucleotides 5'-CAATACCTCTGTAAGTCG CACATAGAG-3', 5'-CGTCTGGAGCAGCATGCCTGATGCGAC-3', and 5'-TGTTGAGATCCAGTTCG-3' as primers (result not shown).

Detection of enzymatic activity

Total tRNA (80 aeg) from the *E. coli* RD2 strain or from the *B. subtilis* strain 168 was incubated in a 300 µl reaction mixture containing 50 mM Tris-Cl (pH 8.0), 20 mM NH₄Cl, 10 µM [methyl-¹⁴C]AdoMet (53 mCi/mmol), and 7.5 µg of the purified H6YfcK protein. After a 30-min incubation at 37°C, the tRNA was recovered and digested by nuclease P1, and the resulting nucleotides were analyzed by two-dimensional thin-layer chromatography on a cellulose plate (Merck) as described previously (Keith 1995), using isobutyric acid:water: conc. ammonium hydroxide (66:33:1, by vol) as the solvent for the first dimension, and conc. HCl:isopropanol:water (17.6:68:14.4, by vol) as the solvent for the second dimension.

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