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Site-specific incorporation of arginine analogs into proteins using arginyl-tRNA synthetase

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ABSTRACT

Arginine analogs were incorporated site-specifically into proteins using an *in vitro* translation system. In this system, mRNAs containing a CGGG codon were translated by an aminoacyl-tRNA$_{CCCG}$, which was charged with arginine analogs using yeast arginyl-tRNA synthetase. N^G-monomethyl-L-arginine, L-citrulline and L-homoarginine were incorporated successfully into proteins using this method. The influence of arginine monomethylation in histone H3 on the acetylation of lysine residues by histone acetyltransferase hGCN5 was investigated, and the results demonstrated that K9 acetylation was suppressed by the methylation of R8 and R17 but not by R26 methylation. K18 acetylation was not affected by the methylation of R8, R17 and R26. This site-specific modification strategy provides a way to explore the roles of post-translational modifications in the absence of heterogeneity due to other modifications.

1. Introduction

Proteins have various functions, which are determined not only by their amino acid sequences but also by post-translational modifications that serve to modulate protein activity, such as acetylation, methylation and phosphorylation. Arginine modifications, such as monomethylation and dimethylation by arginine methyltransferase [1,2], and citrullination by arginine deiminase [3], have been reported for many proteins. However, the roles played by arginine modifications are not well understood, and in order to investigate these roles *in vitro*, a protein arginine modification method is necessary. Although modification enzymes can be used for this purpose, there are limitations to their use because it is necessary to obtain or prepare an enzyme for each type of modification and because the enzyme does not always modify only a single site of a protein efficiently.

In this study, we attempted to introduce arginine analogs, such as methylarginine and citrulline, into proteins at specific positions using an *Escherichia coli* cell-free translation system with an expanded genetic code [4–6]. A yeast tRNA^{Arg}-derived tRNA mutant bearing a CCCG four-base anticodon (tRNA$_{CCCG}$) was prepared and charged with arginine analogs using yeast arginyl-tRNA synthetase (ArgRS). Several different mRNAs containing a CGGG four-base codon were translated by the cell-free translation system using the aminoacyl-tRNA$_{CCCG}$ (aa-tRNA$_{CCCG}$).

This expanded translation system was used to synthesize histone H3 proteins containing a N^G-monomethyl-L-arginine (mArg) at position 8, 17, or 26. It is known that the arginine residues (R8, R17 and R26) of histone H3 are the target sites of arginine methyltransferase [7,8], and that post-translational modifications of histone N-termini, such as methylation and acetylation, play a crucial role in transcriptional regulation and chromatin structure [7]. However, the impact of each modified group on other modifications is mostly unknown. In this study, we have examined the influence of arginine (R8, R17 or R26) methylation on the acetylation of lysine residues (K9 and K18) catalyzed by the histone acetyltransferase hGCN5 [7,9,10] using site-specifically methylated histone H3.

2. Materials and methods

2.1. Preparation of yeast tRNA^{Arg}-derived tRNA possessing a CCCG four-base anticodon

An anticodon-mutant of yeast cytosolic tRNA^{Arg2} bearing a CCCG anticodon (tRNA$_{CCCG}$) was prepared by transcription as follows: to generate DNA templates for transcription, primer extension was performed using two primers (1 μM of each), 5′-ccgggtaatacgactcactatagctcgcgtggcgtaatggcaacgcgtct-3′ and 5′-tggcactcacgatgggggtcgaacccataatcttctgattcgggagtcagacgcgttgccattacg-3′, in a 100 μl reaction mixture containing 0.2 mM dNTPs, 25 units KOD Dash DNA polymerase (Toyobo, Japan), and 10 μl of 10X Buffer #1, with the following temperature

Abbreviations: ArgRS, arginyl-tRNA synthetase; tRNA$_{CCCG}$, tRNA mutant bearing a CCCG four-base anticodon; aa-tRNA, aminoacyl-tRNA; mArg, N^G-monomethyl-L-arginine; Cit, L-citrulline; hArg, L-homoarginine.

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program: 94 °C for 60 s, and three cycles of 94 °C for 30 s, 55 °C for 2 s, and 72 °C for 30 s. The primers were designed to complement each other at the 3′ end (about 20 nucleotides), and the resultant double-stranded DNA was collected by precipitation with 2-propanol. The transcription reaction was performed at 37 °C for 4 h in a reaction mixture that contained 40 mM Tris–HCl (pH 8.0), 24 mM $MgCl_2$, 5 mM DTT, 2 mM spermidine, 0.01% Triton X-100, 10 mM GMP, 2 mM ATP, 2 mM GTP, 2 mM CTP, 2 mM UTP, 1.8 units/ml pyrophosphatase (SIGMA), 750 units/ml T7 RNA polymerase (Takara, Japan), and 200 nM of the DNA template. The tRNA transcripts were purified using a 10% denaturing polyacrylamide gel.

2.2. Aminoacylation of the $tRNA_{CCCG}$ with arginine analogs

His-tagged yeast ArgRS was expressed using an ArgRS-expression vector and purified by Ni^{2+}-NTA agarose (Qiagen) column chromatography as described previously [11]. The $tRNA_{CCCG}$ was aminoacylated at 30 °C for 20 min in a reaction mixture containing 50 mM HEPES–KOH (pH 7.6), 15 mM $MgCl_2$, 10 mM ATP, 30 mM KCl, 1 mM DTT, 0.5–7.5 mM arginine analog, 30 μM tRNA and 1.1 μM yeast ArgRS. For use in translation, aa-$tRNA_{CCCG}$ was extracted from the reaction mixture using phenol:chloroform:isoamyl alcohol (25:24:1, pH 5.2) (Nakarai Tesque Co. Ltd., Japan) and purified by ethanol precipitation. The aa-tRNA pellet was redissolved in 1 mM AcOK (pH 4.5) at a concentration of 100 μM, and stored at −80 °C.

2.3. Measurement of aminoacylation efficiency by gel shift analysis

The aminoacylation efficiency was evaluated by gel shift analysis with the prokaryotic elongation factor, EF-Tu [11]. When aminoacyl- and unacylated tRNAs are mixed with EF-Tu/GTP, aa-tRNAs can be separated from unacylated tRNAs on a native gel due to the fact that EF-Tu binds only to aa-tRNAs. The $tRNA_{CCCG}$ was aminoacylated at 30 °C for 20 min in the presence of the *E. coli* EF-Tu E215A mutant ($EF\text{-}Tu^{E215A}$), which binds more tightly to aa-tRNAs than does wild-type EF-Tu. The aminoacylation reaction mixture contained 50 mM HEPES–KOH (pH 7.6), 15 mM $MgCl_2$, 10 mM ATP, 30 mM KCl, 1 mM DTT, 0.5–7.5 mM arginine analog, 5 μM tRNA, 1.1 μM yeast ArgRS, 0.5 mM GTP, and 10 μM EF-Tu. GSA sample buffer (1 μl) containing 0.05% bromo phenol blue and 50% glycerol was added to 4 μl of the reaction mixture. Electrophoresis of samples was performed using 8% polyacrylamide gels at 4 °C in a buffer containing 50 mM Tris–HCl (pH 6.8), 65 mM NH_4OAc and 10 mM $Mg(OAc)_2$. Gels were stained with SYBR Gold (Invitrogen, US), and fluorescence images of SYBR Gold-stained gels (l_{ex} = 488 nm; l_{em} = 545–565 nm) were obtained using an FMBIO III-SC01 imaging system (Hitachi, Japan). The band intensity was evaluated using ImageJ Ver.1.34s (National Institutes of Health, USA, http://rsb.info.nih.gov/ij/). The gel-shift assay was repeated twice and reproducibility of the results was confirmed.

2.4. In vitro translation of a mutated streptavidin mRNA and Western blot analysis

A mutated streptavidin mRNA containing a CGGG codon at the 83rd position was prepared as described previously [4]. A T7-tag sequence was also encoded at the N-terminus of this streptavidin mRNA so that the protein could be detected using an anti-T7 antibody. *In vitro* translation and Western blot analysis were performed as described previously [4]. Briefly, an *in vitro* translation mixture (10 μl) containing 2 μl of *E. coli* S30 Extract for Linear Templates (Promega), 55 mM HEPES-KOH (pH 7.5), 210 mM potassium glutamate, 6.9 mM ammonium acetate, 1.7 mM dithiothreitol, 1.2 mM ATP, 0.28 mM GTP, 26 mM phosphoenolpyruvate, 1 mM spermidine, 1.9% polyethyleneglycol-8000, 35 μg/ml folinic acid, 12 mM magnesium acetate, 0.1 mM of each amino acid, 8 μg mRNA and 10 μM aa-tRNA was incubated at 37 °C for 1 h. The reaction mixture was separated on a 15% SDS–polyacrylamide gel, and proteins were transferred to a polyvinylidine fluoride (PVDF) membrane (Bio-Rad) and Western blotted using an anti-T7-tag monoclonal antibody (Merck) and the ProtoBlot II AP system (Promega). The efficiency of the four-base decoding was estimated by comparing the band intensity of the full-length product with those of serial dilutions of wild-type streptavidin expressed *in vitro*. The band intensity was evaluated using ImageJ Ver.1.34s.

2.5. In vitro translation of mRNAs encoding oligopeptides using the E. coli PURE system and mass spectrometric analysis

An mRNAs encoding a FLAG-tagged (underlined) oligopeptide with the amino acid sequence *N*-fMDYKDDDDKLKLXLTHKETAAAKFERQHMDS-*C* (where X is an amino acid encoded by the CGGG codon) was prepared by transcription from a DNA template generated by PCR according to Sando et al. [12] with the following minor modifications: the PCR was performed in 100 μl of a reaction mixture containing 100 pmol of the forward primer (5′-gaaattaatacgactcactatagggagaccacaacggtttccctctagaaataatttgtttaactttaagaaggagatataccaatggac-3′), 100 pmol of the reverse primer (5′-tattcattagctgtccatgtgctg-3′), 0.2 pmol of the synthetic DNA template (5′-aaggagatataccaatggactacaaggacgacgacgacaagctaaaactgCGGGctgacgcataaagaaaccgctgctgctaaattcgaacgccagcacatggacagctaa-3′), 0.2 mM of each dNTP, 1 mM of $MgSO_4$, 10 μl of 10 × KOD-plus Buffer, and 2 μl of KOD-plus DNA polymerase (Toyobo, Japan). The resulting double-stranded template DNA contained a T7 promoter, an SD sequence (ribosomal binding site), and the oligopeptide-coding sequence. The mRNAs were obtained by T7 transcription of this template and purified as described previously [4].

The mRNAs were translated using PURESYSTEM Classic II (Wako, Japan) [13]. Reactions (10 μl) were started by the addition of 0.2 μg/μl mRNA and 20 μM aa-tRNA, and incubated for 1 h at 37 °C. The synthesized oligopeptides were purified using an anti-FLAG M2 affinity gel (Sigma) as described [12]. After affinity purification, the oligopeptide solution was desalted and concentrated using $ZipTip_{C18}$ silica resin (Millipore) according to the manufacturer's instructions, and then eluted with matrix solution (10 mg/ml α-cyano-4-hydroxycinnamic acid dissolved in a 1:1 mixture of water and acetonitrile containing 0.1% trifluoroacetic acid) directly onto a MALDI target plate. MALDI-TOF mass spectra were measured on a Voyager-DE Pro instrument (Applied Biosystems) in the reflector mode. A chemically synthesized peptide (*N*-MDYKDDDDKLKLYLTHKETAAAKFERQHMDS-*C*) was used as an external standard for mass calibration.

2.6. Preparation of histone H3 protein containing an arginine analog and acetylation of the modified histone H3

Histone H3 mRNA and mutated histone H3 mRNAs each containing a CGGG codon at the 8th, 17th or 26th position were prepared by transcription from DNA templates generated by PCR. The PCR was performed using a pETT7 primer (5′-ccgcgaaattaatacgactc-3′), a pETterm primer (5′-gctagttattgctcagcgg-3′) and template plasmids. The template plasmids contained a gene encoding histone H3 or its mutant (see Supporting Information) inserted between the *Nco*I and *Hind*III sites of pET-28b. The mRNAs were obtained by T7 transcription and purified as described [4].

Histone H3 proteins containing arginine analogs were synthesized at 37 °C for 1 h by *in vitro* translation (50 μl) using *E. coli* S30 Extract for Linear Templates (Promega) as described above. Then, the translation product was purified using a Microcon