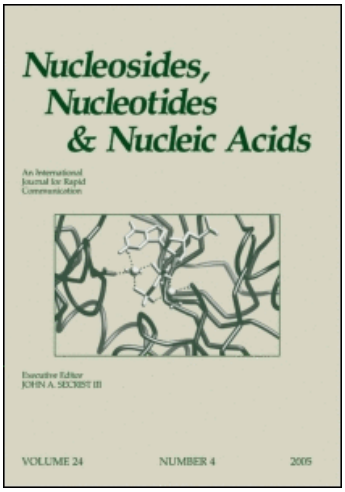

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NMR ANALYSES OF STRUCTURES AND FUNCTIONS OF MODIFIED NUCLEOSIDES IN TRANSFER RIBONUCLEIC ACIDS

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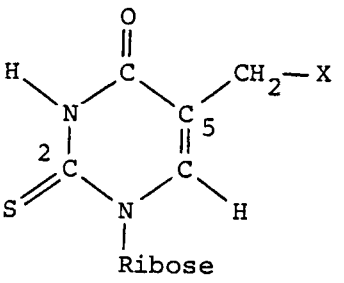
Abstract: By nuclear magnetic resonance spectroscopy, it was found that conformational rigidity or flexibility of modified uridine in the first position of the anticodon of transfer RNA contributes to the rigid or flexible recognition of codons.

In protein biosynthesis, codons (three-nucleotide sequences) of messenger RNA are recognized by the anticodons of transfer RNA (tRNA) species. It was proposed by Crick that "wobble" base pairs are formed as well as the Watson-Crick A:U and G:C base pairs between the third letter of codon and the first letter of anticodon[1]. For example, guanosine in the first position of the anticodon recognizes uridine in addition to cytidine, while cytidine recognizes guanosine only. And a modified nucleoside, inosine (I), was proposed to recognize cytidine, uridine and adenosine[1]. Later, A in the first position of the anticodon (position 34) was found to be always modified to I. Further, a variety of modified nucleosides have also been found in this position[2]. This suggests that the post-transcriptional modification in position 34 is essential for the codon recognition, while many modified nucleosides found so far in this position of tRNA are yet to be identified.

UUU UUC	Phe	UCU UCC UCA UCG	Ser ○	UAU UAC	Tyr	UGU UGC	Cys
UUA UUG	Leu ?			UAA UAG	term	UGA UGG	term Trp
CUU CUC CUA CUG	Leu	CCU CCC CCA CCG	Pro ○	CAU CAC	His	CGU CGC CGA CGG	Arg
				CAA CAG	Gln ●		
AUU AUC AUA ?	Ile	ACU ACC ACA ACG	Thr ○	AAU AAC	Asn	AGU AGC	Ser
AUG	Met			AAA AAG	Lys ●	AGA AGG	Arg ?
GUU GUC GUA GUG	Val ○	GCU GCC GCA GCG	Ala ○	GAU GAC	Asp	GGU GGC GGA GGG	Gly
				GAA GAG	Glu ●		

FIG. 1. Genetic code and the modified uridines in the first position of the anticodon (position 34) of the corresponding tRNA species. Closed circles indicate xm^5s^2U and open circles indicate xo^5U (see FIG. 2).

Uridine in position 34 was proposed to recognize adenosine and guanosine[1]. In fact, uridine in position 34 has been found to be modified, except for few cases such as mitochondrial tRNAs, to either of two types of modified uridines[2] as summarized in FIG. 1. As for tRNAs specific to Gln, Lys and Glu corresponding to two codons terminating in A and G, U(34) is always modified to 5-methyl-2-thiouridine derivative (xm^5s^2U) (FIG. 2). In contrast, 5-hydroxyuridine derivatives (xo^5U) (FIG. 2) have been found in position 34 of tRNAs corresponding to four codons. It has been found that xm^5s^2U recognizes mainly A as the third letter of codon, whereas xo^5U recognizes U in addition to A and G (ref. 2). On the other hand, uridine is able to form base pairs with guanosine, uridine, and cytidine in addition to adenosine, provided that the uracil base is free from conformational restriction due to the backbone structure and therefore can move around from the position in the Watson-Crick U:A pair[1]. Those two types of modifications of U(34), therefore, might regulate the conformational flexibility of the residue.

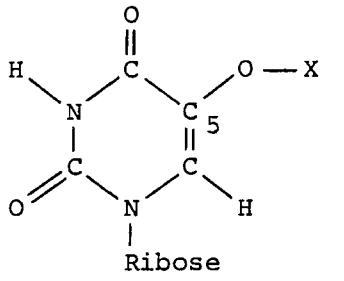


xm^5s^2U

mnm^5s^2U : X = CH_2-NH_2^+-CH_3
5-methylaminomethyl-2-thiouridine
Escherichia coli

$cmnm^5s^2U$: X = CH_2-NH_2^+-CH_2-COO^-
5-carboxymethylaminomethyl-2-thiouridine
Bacillus subtilis

mcm^5s^2U : X = CH_2-CO-O-CH_3
5-methoxycarbonylmethyl-2-thiouridine
yeast, mammals



xo^5U

cmo^5U : X = O-CH_2-COO^-
5-carboxymethoxyuridine
Escherichia coli

mo^5U : X = O-CH_3
5-methoxyuridine
Bacillus subtilis

FIG. 2. Modified uridines in the first position of the anticodon.

Thus, we made a working hypothesis that the post-transcriptional modifications of the first residue of the anticodon regulate the conformational flexibility of this residue to guarantee the suitable flexibility in formation of wobble base pairs[3]. To test such a hypothesis, we analyzed dynamic structures of the modified uridine residues of tRNAs and conformational equilibria of modified uridine nucleosides and nucleotides by nuclear magnetic resonance (NMR) spectroscopy.

The two-dimensional NMR spectroscopy was used to elucidate the dynamic structure properties of tRNA molecules whose molecular weights are about 25,000. Glutamate tRNA from *E. coli* has mnm^5s^2U (FIG. 2) residue in the first position of the anticodon (position 34). The methyl proton resonance of $mnm^5s^2U(34)$ was observed in the methyl proton resonance region of the spectrum, and assigned by comparing the chemical shift with that of the nucleoside. In a two-dimensional nuclear Overhauser effect

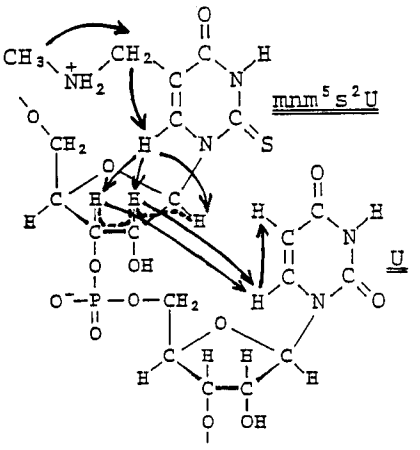


FIG. 3. NOEs (arrows) and spin-couplings (broken lines) observed for $mnm^5s^2U(34)$ and U(35) of *E. coli* glutamate tRNA.

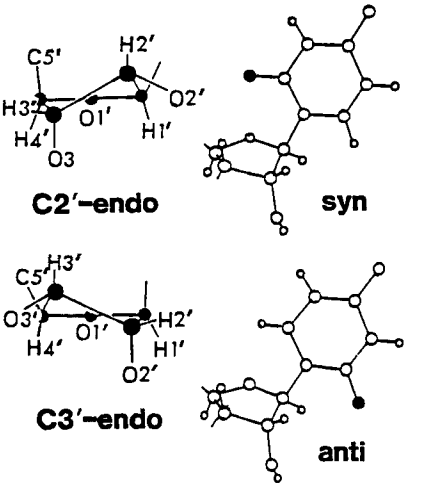


FIG. 4. Local conformations for the ribose ring puckering and the rotation around the *N*-glycosidic bond.

spectroscopy (NOESY)[4] spectrum, a cross peak between the methyl resonance and the methylene resonance of the side chain of $mnm^5s^2U(34)$ was found (FIG. 3). Then, from the NOE connectivity to this methylene protons, the resonance of the H6 proton of the uracil ring of $mnm^5s^2U(34)$ was assigned (FIG. 3). In the region of ribose proton resonances, three protons showed NOE to the H6 proton. By two-dimensional homonuclear Hartmann-Hahn spectroscopy (HOHAHA)[5], these three protons were shown to have spin-coupling among them, and thus identified unambiguously to be the H1', H2' and H3' protons, respectively, of the ribose of $mnm^5s^2U(34)$ (FIG. 3). Further, the resonances of the H5 and H6 protons of U(35), which is the second letter of the anticodon, were identified on the basis of the inter-residue NOE connectivity (FIG. 3). These intra-residue and inter-residue NOE connectivities and the spin-coupling constants of $mnm^5s^2U(34)$ were found to be consistent with a A-type RNA conformation (the *C3'-endo-anti* form) (FIG. 4), even if the residue is situated in a single stranded loop region.

Next, we measured NOESY spectrum of valine and threonine tRNA species from *B. subtilis*. These two tRNA species have $mo^5U(34)$ (FIG. 2), so that the methyl proton resonance was clearly identified. NOE was observed between the methyl protons and the H6 proton of the uracil base of this modified uridine residue. However, NOE between the H6 proton and the ribose protons are very weak. In contrast, by rotating-frame nuclear Overhauser effect spectroscopy (ROESY)[6], NOE connectivities were clearly observed for proton pairs of H6-H1' and H6-H2'. All these indicate that $mo^5U(34)$ is in a mixture of the *syn* and *anti* forms and *C2'-endo* and *C3'-endo* forms, and the interconversion among the conformers is rapid. Therefore, $mo^5U(34)$ residues of *B. subtilis* $tRNA^{Val}$ and $tRNA^{Thr}$ were found to be "flexible" and in an equilibrium of the *C3'-endo* and *C2'-endo* forms. According to our model building analysis, uridine (or modified uridine) in the first position of the anticodon should take the C2'-endo conformation to form a wobble base pair with uridine in the third position of the codon (FIG. 5)[3]. Therefore, the conformational flexibility of $mo^5U(34)$ as found in the present study certainly contribute to the flexible codon recognition. In contrast, $mnm^5s^2U(34)$ of *E.coli* $tRNA^{Glu}$ was found to be "rigid" predominantly in the *C3'-endo* form. Such a conformational rigidity of $mnm^5s^2U(34)$ prevents undesirable wobble base pairs, and therefore contributes to the rigid codon recognition.

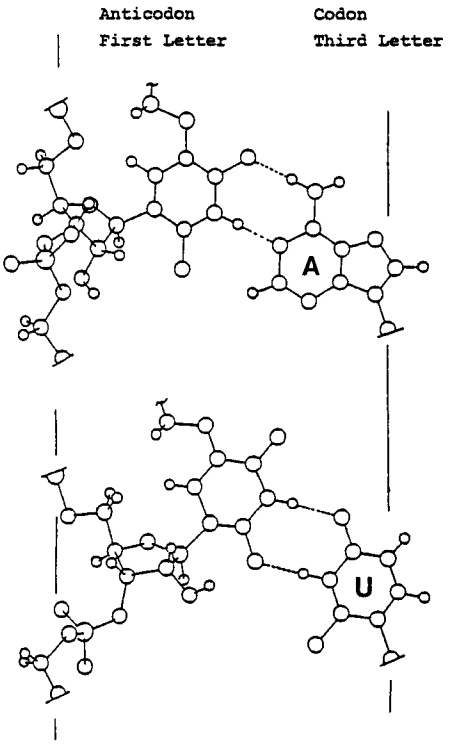


FIG. 5. mo^5U:A and mo^5U:U pairs.

Mechanisms of the conformational flexibility/rigidity of the two types of modified uridine residues were elucidated by ^1H-NMR analyses of conformation equilibria of 5'-mononucleotides (pxm^5s^2U and pxo^5U) and a

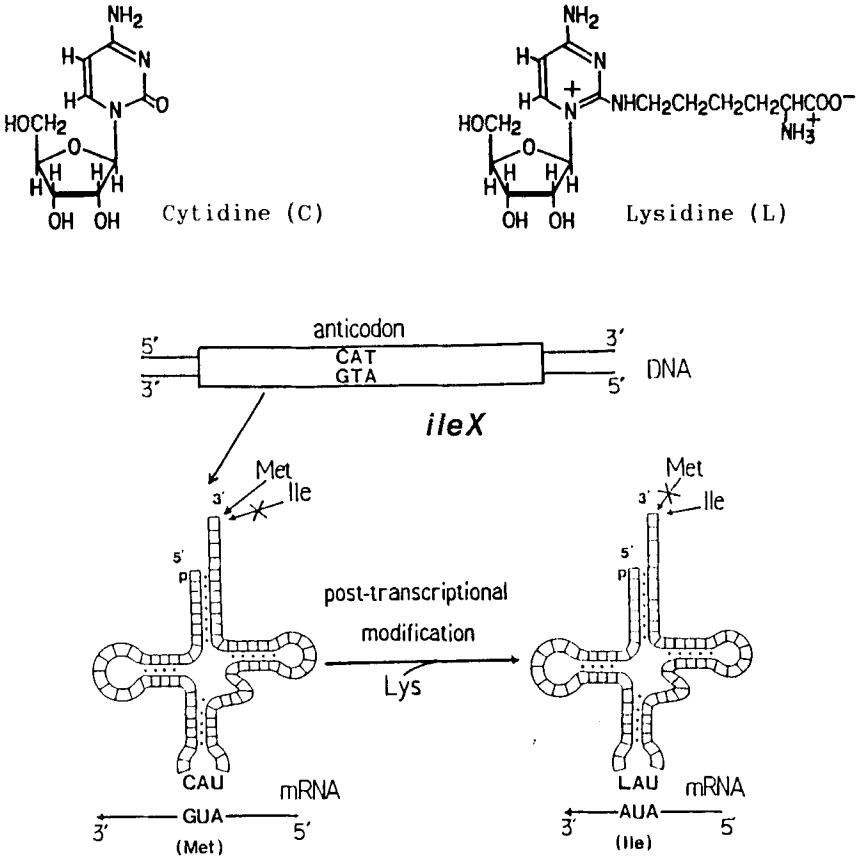


FIG. 6. Structure and functions of lysidine (a novel modified cytidine) in the first position of the anticodon of *E. coli* $tRNA_2^{Ile}$ species.

series of analogs[3,7-10]. The spin-coupling constants between ribose protons were obtained by careful simulation of one-dimensional proton NMR spectra[7]. Then, the enthalpy and entropy differences between the *C2'-endo* form and the *C3'-endo* form were estimated from the temperature dependence of the equilibrium constant[8]. In pxo^5U, it was found that the *C2'-endo* form is much more stable than the *C3'-endo* form[3,9]. Such a conformational preference for the *C2'-endo* form of pxo^5U is due to the interaction between the 5-substituent and the 5'-phosphate, because xo^5U nucleosides prefer the *C3'-endo* form rather than the *C2'-endo* form[3]. In contrast, in pxm^5s^2U, the *C3'-endo* form is extraordinarily more stable than the *C2'-endo* form[3,9]. Such a conformational rigidity was ascribed

primarily to the steric effect: the steric repulsion between the bulky 2-thiocarbonyl group and the 2'-hydroxyl group of the ribose ring is much more strong in the *C2'-endo* form than in the *C3'-endo* form[10]. Further, the 5-substituent was also found to contribute to the stability of the *C3'-endo* form[3]. Consequently, the conformational flexibility and rigidity characteristic to $mo^5U(34)$ and $mnm^5s^2U(34)$, respectively, of whole tRNA molecules are due to the inherent natures of these two types of modified uridines.

In summary, $xm^5s^2U(34)$ is "rigid" and fixed in the *C3'-endo* form so that base pairs are formed stably with A (and G) but never with U in the third position of codon. By contrast, $xo^5U(34)$ is "flexible" enough to take the unusual *C2'-endo* form as well as the *C3'-endo* form so that a base pair with U is formed in addition to those with A and G. Accordingly, the two types of post-transcriptional modifications of U(34) regulate rigidity/flexibility of the anticodon of tRNAs and allow correct and efficient translations of codons. We confirmed this mechanism by finding novel "rigid" modified uridines in position 34 of *E.coli* $tRNA^{Leu}$ and $tRNA^{Arg}$ specific to two codons terminating in A and G (FIG. 1) (to be reported elsewhere).

Finally, mechanisms of characteristic recognition of an isoleucine codon AUA and the methionine codon AUG (FIG. 1) were investigated. An *E. coli* isoleucine tRNA ($tRNA_2^{Ile}$) specific for the codon AUA has an unidentified modified nucleoside (N^+) in the first position of the anticodon[11]. By ^1H-NMR analysis in combination with mass spectrometry and chemical synthesis, we have determined the structure of nucleoside N^+ as 4-amino-2-(N^6-lysino)-1-(β-D-ribofuranosyl)pyrimidinium ("lysidine" or L), a novel type of nucleoside substituted with L-lysine (FIG. 6)[12,13]. We isolated the gene coding for $tRNA_2^{Ile}$ and found that the anticodon is CAT, characteristic of the methionine tRNA gene[14]. Therefore, the modification of C(34) to L(34) prohibits the recognition of G and instead allows the recognition of A as the third letter of the codon. Further, we replaced L(34) of $tRNA_2^{Ile}$ molecule enzymatically by unmodified C(34), which resulted in marked reduction of isoleucine accepting activity and surprisingly in appearance of methionine accepting activity[14]. Thus, the codon specificity and amino acid specificity of this tRNA are both converted by a single post-transcriptional modification of the first position of the anticodon during tRNA maturation (FIG. 6). Therefore, the

post-transcriptional modification in the first position of the anticodon plays essential roles in the functions of tRNA species.

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