5-[[(Carboxymethyl)amino]methyl]uridine Is Found in the Anticodon of Yeast Mitochondrial tRNAs Recognizing Two-Codon Families Ending in a Purine†

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ABSTRACT: The modified nucleoside (U*) present in the wobble position of *Saccharomyces cerevisiae* mitochondrial $tRNA^{Leu}$ and $tRNA^{Trp}$ was isolated by thin-layer chromatography and HPLC. Its chromatographic, UV spectral, and mass spectrometric properties were shown to be identical with those of 5-[[(carboxymethyl)amino]methyl]uridine ($cmnm^5U$). This nucleoside found in yeast mitochondrial tRNAs reading two-codon families ending in a purine permits the selective recognition of A and G in the third codon position.

Mitochondrial protein synthesis uses a restricted number of mitochondrial (mt) DNA-coded tRNAs (e.g., 24 tRNA genes in *Saccharomyces cerevisiae*), which is far below the minimal number of tRNA species, i.e., 32, necessary to translate all the codons of the genetic code according to the wobble hypothesis [for a review, see Dirheimer and Martin (1990)]. From determination of their primary structures, it appeared that mt tRNAs use a simplified mechanism for codon reading, which minimizes the number of required tRNA species (Heckman et al., 1980; Martin et al., 1983; Sibler et al., 1986). Such an expanded codon recognition pattern is due to the presence of an unmodified uridine in the first position of the anticodon in each of the single tRNAs that decodes all four triplets of a given four-codon family in the genetic code. The presence of nonmodified uridine in mt tRNAs contrasts with the situation in both procaryotic and eucaryotic cytoplasmic tRNAs, in which a uridine in the wobble position is always modified (Dirheimer, 1983; Björk, 1984). In contrast, two-codon boxes ending in a purine are read by mt tRNAs having an unknown derivative of U in the wobble position; this modification is thought to be the major mechanism for preventing the misreading of two-codon families ending in a pyrimidine (Heckman et al., 1980; Dirheimer & Martin, 1990).

In *S. cerevisiae* mitochondria, a uridine derivative, referred to as U*, has been detected in the anticodon of mt $tRNA^{Trp}$, $tRNA^{Arg\text{-}1}$, $tRNA^{Leu}$, and $tRNA^{Lys}$ (Sibler et al., 1980, 1986). By two-dimensional thin-layer chromatography (TLC) using different solvent systems, pU* showed migration properties identical with those of 5-[[(carboxymethyl)amino]methyl]-uridine 5′-phosphate ($pcmnm^5U$) (Martin et al., 1983; Sibler et al., 1986), which had been previously identified in the first position of the anticodon of $tRNA^{Gly}$ of *Bacillus subtilis* (Murao & Ishikura, 1978). The structure determination of this modified residue rests on its UV spectra, thin-layer chromatographic mobility, susceptibility to RNases, and conventional mass spectrometric analyses (Murao & Ishikura, 1978). In this paper, we confirm by HPLC, UV spectral, and thermospray mass spectrometric analyses the structural identity of the U* in mt $tRNA^{Leu}$ and $tRNA^{Trp}$ with $cmnm^5U$.

Experimental Procedures

Purification of mt tRNAs. Preparation of *S. cerevisiae* mitochondria, extraction of total mt tRNA, and isolation of mt $tRNA^{Trp}$, $tRNA^{Arg\text{-}1}$, $tRNA^{Leu}$, and $tRNA^{Lys}$ were as previously described (Martin et al., 1977; Sibler et al., 1980, 1986).

TLC Isolation of pU.* The 5′-monophosphate of U* was prepared by total hydrolysis of tRNA in the following conditions: 1 A_{260nm} of the $tRNA^{Leu}$ (or $tRNA^{Trp}$) was treated with 0.25 μg of P1 nuclease in 50 mM ammonium acetate, pH 5.3, at 37 °C for 2 h. It was localized and purified by two-dimensional thin-layer chromatography using the following solvent systems: (A) isobutyric acid/H_2O/25% NH_4OH (66:33:1 v/v); (B) 2-propanol/concentrated HCl/H_2O (68:17.6:14.4 v/v) (Sibler et al., 1980). By use of these solvents, the residue pU* was shown to migrate in close vicinity to pG [see Figure 3 in Sibler et al. (1986)], and therefore the sample obtained by preparative TLC was contaminated with pG. The spot corresponding to pU* was scraped off, eluted with 200 μL of water, and lyophilized.

Preparation of U.* Dephosphorylation of pU* was achieved by incubating the sample (in 200 μL of water) with 7.5 units of *Escherichia coli* alkaline phosphatase (type III, Sigma Chemical Co., St. Louis, MO) in 75 μL of 0.25 M $(NH_4)_2SO_4$ and 50 μL of 0.5 M Tris buffer, pH 8.2, at 37 °C for 1 h (Gehrke et al., 1982).

HPLC-UV. A Perkin-Elmer series 4 liquid chromatograph (Norwalk, CT) and Supelcosil C-18DB 250 × 4.6 mm liquid chromatography column (Bellefonte, PA) were used for the purification and isolation of U*. An HP 1040A HPLC detector system (Avondale, PA) was used for monitoring the eluates and obtaining their UV spectra. The signals were monitored at 210 ± 2, 254 ± 2, 280 ± 2, and 260 ± 40 nm to detect all the UV-absorbing peaks. The chromatographic conditions for the first elution step of the standard ribo-

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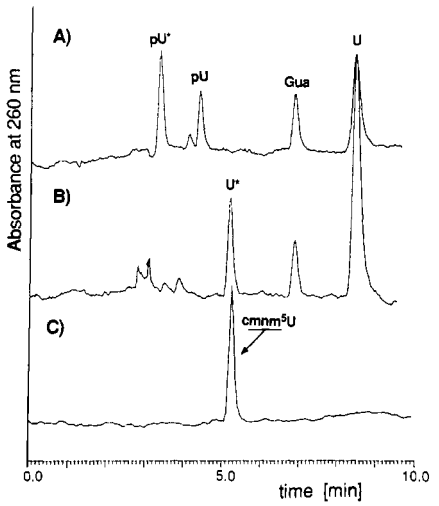

FIGURE 1: HPLC identification and isolation of pU* and U* in yeast mt $tRNA^{Leu}$. The P1 nuclease hydrolysate of the tRNA (1 A_{260nm}) was partially purified by TLC as described in Sibler et al. (1980). 5% of the total TLC eluate was injected (A) before alkaline phosphatase treatment and (B) after alkaline phosphatase treatment. (C) Elution profile of the reference sample of $cmnm^5U$ (ca. 15 ng). For experimental parameters of chromatography and enzymatic hydrolysis conditions, see Gehrke and Kuo (1989).

nucleoside HPLC protocol as described by Gehrke and Kuo (1989) was used for the isolation of this unknown.

Thermospray Liquid Chromatography Mass Spectrometry (LC/MS). Thermospray mass spectra of authentic $cmnm^5U$ and of nucleosides from enzymatic hydrolysis of mt $tRNA^{Leu}$ were acquired by using the method of Edmonds et al. (1985), in which eluants separated by reversed-phase HPLC are passed through a UV detector and then directly into a mass spectrometer. Chromatography was carried out on a Beckman 322M liquid chromatograph (San Jose, CA) and a Waters 440 UV absorbance monitor (Milford, MA) by using the gradient system of Buck et al. (1983) designed for nucleoside separations. The mass spectrometer was a noncommercial quadrupole instrument, previously described (Edmonds et al., 1985). The thermospray vaporizer exit temperature was maintained in the range 250–280 °C, with a vapor temperature at the point of ion sampling of 290 °C. The mass spectrum of authentic $cmnm^5U$ was determined by conventional scanning; analysis of mt $tRNA^{Leu}$ was done by selected ion monitoring.

Reference Sample. Authentic 5-[[(carboxymethyl)-amino]methyl]uridine used as reference was a kind gift of Dr. H. Ishikura (Tochigi-ken, Japan).

RESULTS

The pU* partially purified by TLC after P1 nuclease digestion of mitochondrial $tRNA^{Leu}$ or $tRNA^{Trp}$ (Sibler et al., 1980, 1986) was examined by HPLC to investigate all the UV-absorbing peaks in the sample and to locate the elution position to unknown pU*. Five major UV peaks were observed (Figure 1A). By use of our established reference retention times and HPLC-UV spectra, four peaks were identified as uridine 5′-monophosphate (pU), uridine (U), guanine (Gua), and guanosine 5′-monophosphate (pG). The pG peak is not shown in the figure and eluted at 20.1 min. The presence of pU in the TLC sample is due to partial cleavage of the C5 side chain of pU* which is unstable at acidic pH (TLC solvent B is acidic, pH <1). The presence of pG results from contamination because pU* and pG have similar migration properties in the solvent system used (see Experimental Procedures). The presence of guanine is explained by cleavage of the glycosyl linkage of pG which is known to occur to a certain extent and pH ~1 (Michelson, 1963). Finally, the presence of uridine can only be explained by acid-catalyzed dephosphorylation of pU.

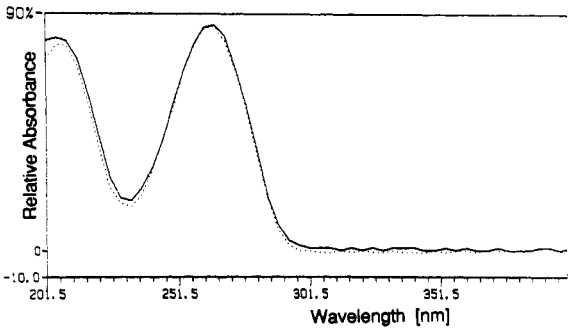

FIGURE 2: Comparison of UV spectra of U* isolated from mt $tRNA^{Leu}$ (—) and of an authentic sample of $cmnm^5U$ (···).

On the basis of the characteristics of chromatography retention time, UV spectrum, and change in retention time upon hydrolysis with alkaline phosphatase, the unknown nucleotide pU* was identified. It eluted 1.2 min earlier than the pU peak, which indicates that it is more hydrophilic than pU. To verify that this unknown peak is phosphorylated, the sample solution was incubated with alkaline phosphatase. Five microliters of the resulting hydrolysate was analyzed by HPLC under the same conditions as for Figure 1A. The chromatogram presented in Figure 1B revealed a new peak at 5.2 min with the same UV spectrum and peak area as for the pU* peak in Figure 1A. The increased retention time is a result of an increase in hydrophobic properties of the compound upon removal of the phosphate group. The HPLC-UV spectrum of the unknown peak U* (Figure 2) is very similar to that of uridine, but the λ_{max} of the unknown is shifted 4 nm to the red (266 nm instead of 262 nm as for uridine). The HPLC-UV spectral data indicate that this unknown has a uridine-like UV chromophore and a modification which is attached to one of the pyrimidine ring carbons. Also, the atom in the modification group that is bonded to the pyrimidine ring is a carbon atom not oxygen, nitrogen, or sulfur. These data strongly suggest that the unknown is a modified uridine (U*) and that the modification is most likely a polar group containing a hydroxyl, amino, or carboxyl and has a methylene group between the polar group and the pyrimidine ring. The position of the modification is on, most likely, carbon 5 of the uridine (Gehrke & Kuo, 1990). A combination of the results from the characteristic chromatography retention, the UV spectra, and enzymatic reaction confirmed that the peak at 5.2 min in Figure 1B is a modified uridine with polar group modification (U*). The comparison of its chromatographic retention and UV spectral properties with those of an authentic sample of 5-[[(carboxymethyl)amino]methyl]uridine ($cmnm^5U$) was done. This reference modified nucleoside gave an identical retention time (Figure 1C) and UV spectrum (Figure 2) as for the peak U* in Figure 1B, thus indicating that the U* is most probably $cmnm^5U$. This was confirmed by the LC/MS technique.

The ion used to test specifically for the presence of $cmnm^5U$ in mt $tRNA^{Leu}$ by LC/MS was established from the mass spectrum of authentic $cmnm^5U$. The principal ions for which structural assignments were made were m/z 76 ($^+H_3NCH_2CO_2H$), 142 ($BH_2^+CH_2CO_2$), 150 ([ribose-H]·NH_4^+), 274 ($MH^+CH_2CO_2$), 332 (<1% relative intensity; MH^+). The MH^+ and BH_2^+ ions, corresponding to the protonated nucleoside and protonated free base, which are usually prominent ions (Edmonds et al., 1985) are in the case of $cmnm^5U$ very low in abundance. This behavior is charac-

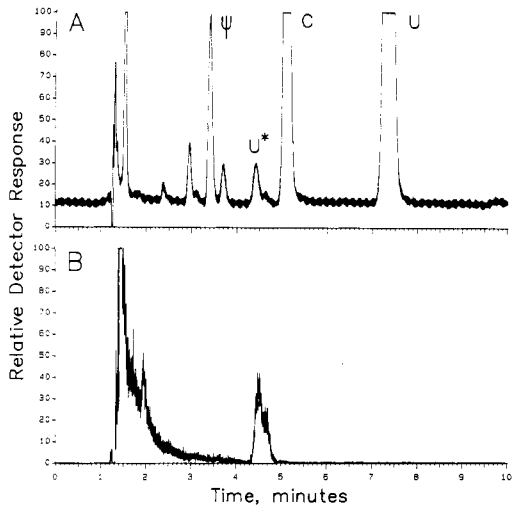

FIGURE 3: Analysis of cmnm^{5}U in an enzymatic digest of 6 μg of mt tRNA^{Leu}. Portion of HPLC chromatogram with (A) UV absorbance detection at 254 nm and (B) MS detection of m/z 274 ion characteristic of cmnm^{5}U. Six-second offset in time between UV and MS channels is due to transit time of the effluent from the UV detector to the mass spectrometer.

teristic of nucleosides that contain basic side chains, such as queuosine (Phillipson et al., 1987), in which side-chain nitrogen protonation leads to facile cleavage of the side chain.

The m/z 274 ion was chosen for measurement of cmnm^{5}U in mt tRNA^{Leu} because this mass value is not significant in the spectra of other nucleosides or of HPLC solvents in the elution region of nucleosides. Greatly increased selectivity in the identification of cmnm^{5}U is afforded by mass spectrometric detection compared with UV absorbance, because isolation of cmnm^{5}U or its HPLC resolution from other constituents is not necessary.

Figure 3A shows UV detection at 254 nm of nucleosides from 6 μg of mt tRNA^{Leu}, using the reversed-phase system of Buck et al. (1983). Components eluting at 1.5, 2.9, 3.7, and 4.6 min are due to non-nucleoside UV-absorbing substances. Figure 3B shows mass spectrometric detection using m/z 274, which corresponds to the elution time of component U* in Figure 3A, eluting 43 s before cytidine. The m/z 274 signals shown in Figure 3B between 1.5 and 2.5 min are due to extraneous materials typically associated with the void volume and are clearly separated from the elution of nucleosides, which begin with pseudouridine at 3.4 min. The retention time of authentic cmnm^{5}U when mixed with cytidine showed a satisfactory retention time comparison of 42 s before cytidine (data not shown). The chromatographic shoulder exhibited in the thermospray response for m/z 274 (Figure 3B) is believed to be due to sample adsorption on the heated vaporizer tip, which was also observed in some runs of authentic cmnm^{5}U, and was previously shown to be characteristic of the related nucleoside cmnm^{5}s^{2}U (Hagervall et al., 1987).

In conclusion, the LC/MS data demonstrated that the peak eluting at 4.4 min is cmnm^{5}U (M_r = 331), the structure of which is shown in Figure 4.

Discussion

Among the 17 *S. cerevisiae* mitochondrial tRNAs we have sequenced, tRNA^{Gly}, tRNA^{Pro}, tRNA^{Ser-2}, and tRNA^{Thr-1}, which decode four-codon families (GGN, CCN, UCN, and CUN, respectively), all contain an unmodified U in the first position of the anticodon. In contrast, the mt tRNA^{Arg-1}, tRNA^{Leu}, tRNA^{Lys}, and tRNA^{Trp}, which recognize two-codon families ending in a purine (AGR, UUR, AAR, and UGR, respectively), all contain a modified U in the wobble position

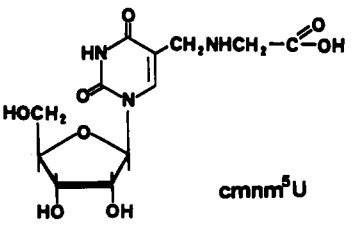

FIGURE 4: Structure of 5-[[(carboxymethyl)amino]methyl]uridine (cmnm^{5}U).

(Martin et al., 1983; Sibler, et al., 1980, 1986). Moreover, this modified uridine, referred to as U*, appeared to be the same in these four tRNAs. The data reported here demonstrate that U* is 5-[[(carboxymethyl)amino]methyl]uridine (cmnm^{5}U).

Yokoyama et al. (1985) have proposed that codon recognition is regulated by the modification of uridine in the wobble position of tRNA through the control of the rigidity/flexibility of this residue. On the basis of proton NMR analyses, these authors have shown that there are primarily two types of modified uridines in the first position of anticodon, which can be distinguished by their conformational characteristics: (i) 5-hydroxyuridine derivatives (xo^{5}U) such as mo^{5}U and cmo^{5}U; and (ii) 5-methyl-2-thiouridine derivatives (xm^{5}s^{2}U) such as mnm^{5}s^{2}U and mcm^{5}s^{2}U. The xo^{5}U derivatives found in the anticodon of nonmitochondrial tRNAs decoding four-codon families adopt preferentially the C2′-endo form which renders the residue more flexible, thus allowing the pairing with U in addition to A and G. In contrast, mnm^{5}s^{2}U found in certain tRNAs decoding two-codon families ending in a purine takes the C3′-endo form exclusively; such a conformation is stabilized by the effect of the 5-methylaminomethyl group and the steric interaction between the bulky 2-thiocarbonyl group and the 2′-OH group of the ribose (Miyazawa & Yokoyama, 1985; Yokoyama et al., 1985). This confers rigidity to the first position of anticodon, allowing a stable pairing only with A as the third position of codon.

The first position of the anticodon of the *E. coli* minor tRNA^{Leu-4} species is occupied by the 2′-O-methylated derivative of cmnm^{5}U (Yokoyama et al., (1987); this residue, though lacking the stabilizing 2-thiocarbonyl group, takes the C3′-endo form (S. Yokoyama, personal communication). We therefore suggest that the cmnm^{5}U found in mt tRNAs should also be preferentially in the C3′-endo form. This would enhance the rigidity of the anticodon, so as to prohibit the misrecognition of codons terminating in a pyrimidine. In fact, in in vitro protein synthesis, the *E. coli* tRNA^{Leu-4} recognizes both the codons UUA and UUG for leucine, but never the codons UUU and UUC for phenylalanine (Yokoyama et al., 1987).

Finally, the conformational rigidity of cmnm^{5}U would also not favor the pairing with G (Hillen et al., 1978); this could explain the strong bias against utilization of G in the third codon position of mixed codon families ending in a purine in yeast mitochondrial protein coding genes (Sibler et al., 1986).

A modified uridine has also been found in the wobble position of several mitochondrial tRNAs originating from organisms others than yeast. This is the case of *Neurospora crassa* and mammals. In *N. crassa* mitochondria, Heckman et al. (1980) found it in glutamine, leucine 1, and tryptophan tRNAs; it is relatively unstable and has properties similar to those of cmnm^{5}U. In the wobble position of rat liver mt tRNA^{Lys}, Randerath et al. (1981) have found a modified U that has a chromatographic behavior very similar to or identical with that of cmnm^{5}U, strongly suggesting structural

identity. Thus, the presence of cmnm^{5}U in mitochondrial tRNAs reading two-codon families ending in a purine seems to be an evolutionarily conserved mechanism to prevent misreading of pyrimidine as the third position of codon.

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Reduced DNA Flexibility in Complexes with a Type II DNA Binding Protein†

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ABSTRACT: We studied internal molecular motions in *Bacillus subtilis* phage SPO1 DNA using the time-resolved fluorescence polarization anisotropy (FPA) of intercalated ethidium. The torsional flexibility of this (hydroxymethyl)uracil-containing DNA is very similar to that of naturally occurring thymine-containing DNAs, as judged from fits of the time-resolved FPA decay to an elastic DNA model. Binding of transcription factor 1 (TF1), a type II procaryotic DNA binding protein encoded by the phage SPO1, enhances the FPA, indicating a substantial decrease in the average DNA torsional flexibility in the DNA–TF1 complex. The FPA increase is correlated with a reduced ethidium binding affinity. The effects can be noticed at TF1 binding ratios less than 1 TF1 dimer/500 DNA base pairs, and the measured torsional rigidity at high TF1 binding ratios (1 TF1 dimer/15–20 DNA base pairs) is about 7 times greater than in the absence of TF1. On the basis of a discussion of various mechanisms for the observed effect we argue that it is due to protein-induced DNA bending at low binding densities although other explanations are also possible. This interpretation might have implications for understanding the biological function of TF1.

The type II DNA binding proteins (DBPII)[1] are a family of small (monomer MW ≈ 10000) basic procaryotic proteins (Drlica & Rouviere-Yaniv, 1987) that have a high degree of sequence homology. DBPII have been shown to induce DNA bending and compaction, and it has been inferred that this might be one of the mechanisms by which they function biologically. For instance, the DBPII from *Escherichia coli* (the HU protein) share some common properties with eukaryotic

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[1] Abbreviations: DBPII, type II DNA binding proteins; IHF, integration host factor; TF1, transcription factor 1; hmUra, (hydroxymethyl)uracil; FPA, fluorescence polarization anisotropy; bp, base pair; ARF, amplitude reduction factor; EDTA, ethylenediaminetetraacetic acid.

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