Heteronuclear NMR Studies of the Interactions of ^{15}N-Labeled Methionine-Specific Transfer RNAs with Methionyl-tRNA Transformylase†

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ABSTRACT: In *Escherichia coli* the methionylated initiator methionyl-tRNA ($tRNA_f^{Met}$) is formylated on the aminoacyl moiety by the enzyme methionyl-tRNA transformylase. The methionylated elongator methionyl-tRNA ($tRNA_m^{Met}$) is not modified in this way. In order to gain structural information about this specific recognition, solution NMR studies were carried out. To be able to identify changes that were occurring in the tRNA molecule on interaction with the methionyl-tRNA transformylase, the imino protons involved in secondary and tertiary base pairing in the $tRNA_f^{Met}$ and $tRNA_m^{Met}$ molecules first had to be assigned to specific resonances in the NMR spectra. A combination of 2D NOESY, 2D HMQC, and 3D NOESY−HMQC spectra were used on uniformly ^{15}N-labeled samples. After assignment of the base pairs of the tRNA, the two forms of tRNA were separately mixed with transformylase in a 1:1 molar ratio. The HMQC spectra of both the $tRNA_m^{Met}$ and the $tRNA_f^{Met}$ showed general broadening, but in the $tRNA_f^{Met}$ HMQC spectra a decrease in the intensity of several resonances was also observed. These resonances had been assigned to the acceptor stem of the tRNA, confirming site-directed mutagenesis experiments that it is the acceptor stem of the tRNA which is important in conferring the specificity for the transformylase. The loss of intensity of the acceptor stem resonances suggests that this part of $tRNA_f^{Met}$ melts upon binding to the enzyme.

Transfer RNA molecules carry specific amino acids to the ribosome for incorporation into the polypeptide chain during translation. During this process the tRNA molecule is recognized by a number of proteins. This recognition is often specific to the particular tRNA molecule especially in the case of the initiator $tRNA^{Met}$. The initiator $tRNA^{Met}$ and elongator $tRNA^{Met}$ are aminoacylated by the same methionyl-tRNA synthetase but play very different roles in translation (Figure 1). The initiator $tRNA^{Met}$ is used exclusively for the start of protein synthesis whereas the elongator $tRNA^{Met}$ is used to insert methionine into the growing polypeptide chain. In prokaryotes and eukaryote organelles, the initiator tRNA once methionylated is formylated on this methionyl moiety by the enzyme $tRNA^{Met}$ transformylase (formylase; Dickerman *et al.*, 1967; Blanquet *et al.*, 1984). This formylation appears to be a requirement for the recognition of the tRNA by IF2 during the initiation of protein synthesis. All other tRNAs including the elongator $tRNA^{Met}$ are not substrates for this enzyme and so are not formylated, allowing their binding to EF-Tu. The two forms of $tRNA^{Met}$ are also distinguished by the peptidyl-tRNA hydrolase (Kossel & Rajbhandary, 1968).

The specific recognition of the $tRNA_f^{Met}$ by the *Escherichia coli* formylase, a 34 kDa monomeric enzyme, has previously been studied by site-directed mutagenesis of the two forms of $tRNA^{Met}$ (Lee *et al.*, 1991; Guillon *et al.*, 1992). It was shown that the nucleotides governing the recognition of the tRNA by the formylase are in the acceptor stem of the tRNA molecule. The presence of a GC base pair between positions 1 and 72, such as is found in all elongator tRNAs, acts as a negative determinant for the recognition of the tRNA by formylase, whereas the nucleotides A73, G2C71, C3G70, and G4C69 of the acceptor stem of the $tRNA_f^{Met}$ are positive determinants.

More structural information can be gained on tRNAs and their interactions with transformylase using the technique of solution NMR spectroscopy. This has previously been studied for the interaction between tRNA and elongation factor Tu (Heerschap *et al.*, 1986). The imino protons of the tRNA which are involved in secondary and tertiary base pairing have slow exchange times with water and hence are visible in NMR spectra. These resonances also occur downfield (11−15 ppm), well separated from other proton resonances of the molecule and are therefore easily identifiable. Previously, one-dimensional and two-dimensional NOE[1] methods have been used to assign some of the imino protons involved in base pairing in various species of tRNA molecule (Hare & Reid, 1982a; Roy & Redfield, 1983; Hyde & Reid, 1985; Hare *et al.*, 1985). However, many of the peaks in this region overlap, making complete assignment difficult. To overcome this problem, some experiments have been carried out with tRNA regioselectively labeled with [^{15}N]uracil (Griffey & Poulter, 1982; Griffey *et al.*, 1982, Choi & Redfield, 1992) to help to assign the UA base pairs.

Our approach, however, was to uniformly ^{15}N label the entire tRNA molecule *in vivo* by growing strains of *E. coli* which overproduce the $tRNA_f^{Met}$ and $tRNA_m^{Met}$ on ^{15}N-labeled medium. These ^{15}N-labeled samples then allow the imino resonances of the tRNA to be more readily assigned.

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[1] Abbreviations: formylase, 10-formyltetrahydrofolate:L-methionyl-$tRNA_f^{Met}$ formyltransferase (EC 2.1.2.9); HMQC, heteronuclear multiple-quantum correlation; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser enhancement spectroscopy; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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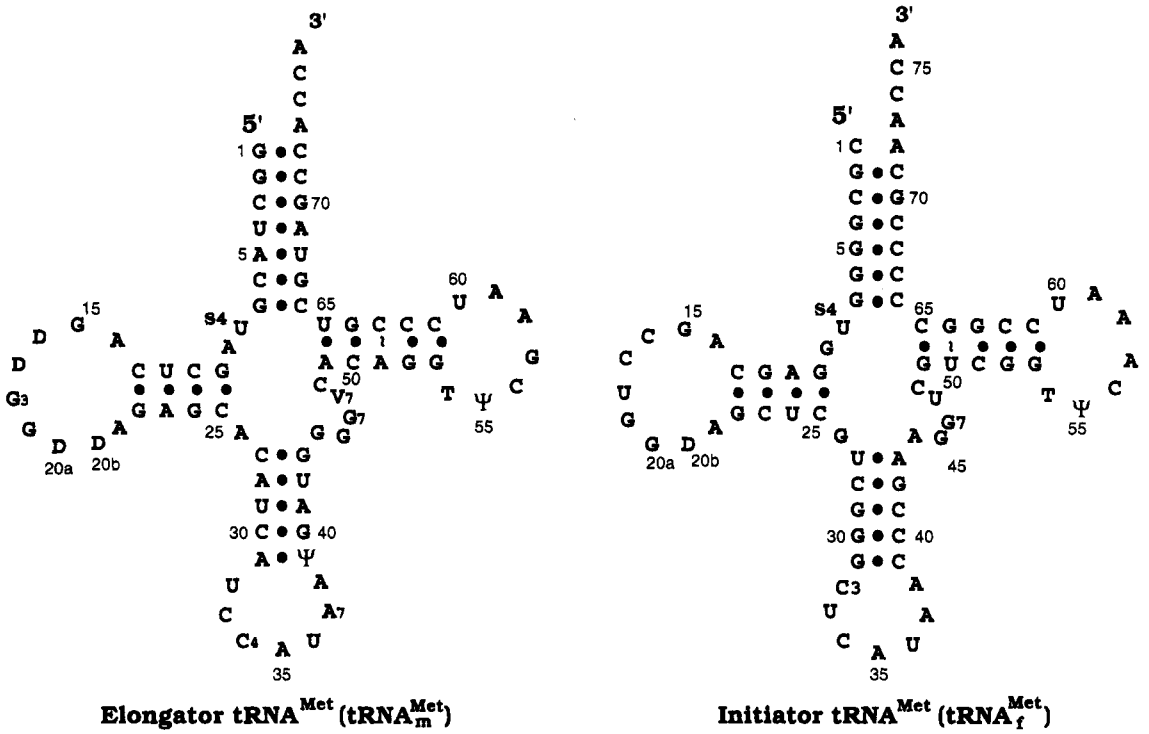

FIGURE 1: Cloverleaf representation of the nucleotide sequences of $tRNA_f^{Met}$ and $tRNA_m^{Met}$. The numbering of tRNA bases is according to Steinberg *et al* (1993).

Table 1: $^1H-^{15}N$ HMQC Spectral Changes in tRNA Variants

variant	information obtained
$tRNA_m^{Met}$ G69C4	base pair U4A69 from wild-type $tRNA_m^{Met}$ disappears base pair A5U68 shifts
$tRNA_f^{Met}$ G3C70	base pair C3G70 from wild-type $tRNA_f^{Met}$ shifts G2C71 and G4C69 shift but cannot be distinguished
$tRNA_f^{Met}$ G1C72	a new GC resonance appears, assigned to G1C72 previously assigned C3G70 shifts allows identification of G2C71 and G4C69, as only the former shifts in this variant
$tRNA_{fasm}^{Met}$	U4A69 and A5U68 from $tRNA_m^{Met}$ disappear four GC resonances are shifted and can therefore be assigned to the acceptor stem of $tRNA_m^{Met}$

The ^{15}N-labeling not only allows a more complete assignment of the imino resonances but also allows the effect of the unlabeled formylase interacting with the tRNA to be observed through changes in these imino resonances, without complicating the spectrum. The specific base pairs in the tRNA molecule which are in contact with the transformylase can be identified as those which have resonances which shift or decrease in intensity when this interaction takes place. A similar approach has also been used for $tRNA^{Ile}$ and isoleucyl-tRNA synthetase (Niimi *et al.*, 1993).

MATERIALS AND METHODS

(i) Preparation of tRNA. Unlabeled tRNA was produced from overexpressing strains of *E. coli* JM101 grown in 2×TY medium supplemented with 100 μg of ampicillin/mL and was purified as described by Meinnel *et al.* (1988), with a further anion-exchange step as described by Guillon *et al.* (1992a). ^{15}N-Labeled tRNA was obtained by growing these strains in M9 salts containing ^{15}N-labeled celltone-N powder (5 g/L) (Isotec Inc.) and 4 mM $^{15}NH_4Cl$ (Isotec Inc.) supplemented with 100 μg of ampicillin/mL and 0.2% glucose (w/v). The ^{15}N-labeled tRNA was purified as above. A total of 500−600 A_{260} of purified tRNA were routinely obtained from 1 L of culture. The methionine acceptance of the purified tRNA was 1000−1200 pmol per A_{260}. This level of purification was found to be sufficient to produce good quality NMR spectra. Wild-type $tRNA_f^{Met}$ and $tRNA_m^{Met}$ were labeled and purified, as well as previously described single base-pair variants $tRNA_f^{Met}$ G1C72 (Guillon *et al.*, 1992a), $tRNA_f^{Met}$ G3C70 and $tRNA_m^{Met}$ G69C4 (Meinnel *et al.*, 1993), and the chimeric $tRNA_{fasm}^{Met}$ in which the acceptor stem of $tRNA_f^{Met}$ has been transferred onto $tRNA_m^{Met}$ (Guillon *et al.*, 1992a) (Table 1).

For NMR measurements tRNA lyophilized from water was dissolved, to a concentration of 0.4−2 mM, in a solution of 10 mM sodium phosphate (pH 6.3) containing 10 mM magnesium chloride, 100 mM sodium chloride, and 10% ^2H_2O.

(ii) Preparation of Formylase. Formylase was overexpressed in *E. coli* JM101 containing the plasmid pUC-*fmt* and grown overnight on 2×TY medium supplemented with 100 μg of ampicillin/mL. pUC-*fmt* is a multistep derivative of pBS936 (Guillon *et al.*, 1992b) in which the *fmt* gene is placed under the control of the *lac* promoter and the *cII* translational signals (E. Schmitt and Y. Mechulam, this laboratory, unpublished data). After cells were disrupted by sonication, the extract was submitted to 0.6% streptomycin precipitation and 45−70% ammonium sulfate precipitations.

After dialysis of the pellet against a buffer (buffer A) containing 10 mM potassium phosphate (pH 7.1), 10 mM 2-mercaptoethanol, and 100 mM potassium chloride, the formylase was purified on a Superdex-75 preparative grade gel filtration column (1.6 × 50 cm; Pharmacia) eluted with the same buffer. Fractions containing the formylase were identified by SDS−PAGE and purified further by loading on a Hi-load Q column (1.5 × 10 cm; Pharmacia) and eluting with a gradient of 0−25% buffer B (200 mL) over 100 min. Buffer B was as above but contained in addition 1 M NaCl. After this step the enzyme was essentially pure, as monitored by SDS−PAGE. Yields of formylase were approximately 20 mg/L medium. Samples of the enzyme were lyophilized from ammonium bicarbonate and dissolved in solutions of 0.4 mM tRNA, 10 mM sodium phosphate (pH 6.3), 10 mM magnesium chloride, 100 mM sodium chloride, and 10% ^2H_2O to give a stoichiometric 1:1 molar ratio of formylase to tRNA. The formylase was still active after lyophilization.

(iii) NMR Spectroscopy. 2D and 3D NMR spectra were recorded at temperatures of 285−310 K with a Bruker AMX600 spectrometer. 2D spectra were acquired using time-proportional phase incrementation for quadrature detection in the ω_1 dimension (Marion & Wüthrich, 1983). 2D proton NOESY spectra with mixing times of 120 ms were recorded with 512 t_1 increments each with 2048 data points, over a spectral width of 12600 Hz, with the carrier on the water frequency. The solvent signal was suppressed by replacing the three $\pi/2$ pulses by "jump and return" (JR) sequences (Plateau & Gueron, 1982) with the delay optimized for maximum intensity in the middle of the imino proton window. Data were zero-filled to 1024 points in the t_1 dimension. Appropriate sine-bell window functions were applied in both dimensions prior to Fourier transformation, and the resulting spectra were submitted to polynomial baseline correction, using the GifA software (Delsuc, 1989).

All 2D [^{1}H−^{15}N] HMQC spectra (Bax *et al.*, 1983) were recorded at 298 K with spectral widths of 6300 and 3165 Hz in the proton and nitrogen dimensions, respectively. The number of transients accumulated per increment varied between 128 and 1024, depending on the tRNA concentration. Water suppression was achieved by replacing proton pulses by JR sequences, as previously described (Bax *et al.*, 1990), and protons were decoupled from ^{15}N during acquisition by a GARP sequence (Shaka *et al.*, 1985). A total of 128 t_1 experiments were acquired, each with 2048 data points. Data processing was as above, except that data were zero-filled to 512 points in the t_1 dimension.

3D NOESY−HMQC spectra (Marion *et al.*, 1989) with NOESY mixing times of 80 ms were recorded with all four ^{1}H pulses replaced with JR sequences to avoid water excitation. The size of the data matrix was 20 complex × 80 complex × 512 real. The spectral widths were 3165 Hz (^{15}N dimension), 12 600 Hz (^{1}H dimension), and 6300 Hz (imino ^{1}H dimension). Data were zero-filled and processed as above, resulting in a real matrix of 64 × 256 × 512.

RESULTS

(i) Production of Labeled tRNA. Using the tRNA expression system described by Meinnel *et al.* (1988), we have designed culture conditions which allow the production of ^{15}N-labeled tRNA. NMR quantities of purified tRNA can be obtained from liter-scale culture. Satisfactory yields of tRNA could only be obtained when the cells were growing rapidly. This is presumably because most of the tRNA processing and modifying enzymes are growth-rate regulated [reviewed by Bjork (1987)]. The cells were therefore grown on medium supplemented with labeled cell lysate, as growth of the overproducing strain on minimal salts produced much lower yields of tRNA. 2D NOESY spectra of these overproduced tRNAs reveal patterns of cross-peaks characteristic of several modified bases (not shown), including ribothymidine (rT), 4-thiouracil (s^4U), dihydrouridine (D), pseudouridine (ψ), ribofuranosylpurinylcarbamoylthreonine (t^6A), and aminocarboxypropyluridine (acp^3U).

(ii) Assignment of tRNA Spectra. A number of NMR techniques were used to assign the imino protons of the two forms of $tRNA^{Met}$. We aimed to assign as many as these imino protons as possible so as to be able to use the assignment to look for changes in the resonances on complex formation with the formylase. Each Watson−Crick base pair contains one imino proton, the UN3H in the case of the UA base pairs and the GN1H in the case of the GC base pairs. The uniform labeling of the tRNA molecules resolved the imino proton peaks in the ^{15}N dimension and allowed the easy differentiation between UA (^{15}N shifts 156.8−179.4 for U N3) and GC (^{15}N shifts 142.9−148.2 for G N1) base pairs. 2D and 3D NOESY spectra of the samples of tRNA were used to obtain some assignments. Small NOEs between the hydrogen bonds of adjacent base pairs allowed assignment of neighboring base pairs from a known starting point. The three-dimensional crystal structures of various tRNAs have been studied (Robertus *et al.*, 1974; Woo *et al*, 1980; Moras *et al.*, 1980), and the base pairs close enough to give NOEs between their imino protons can be seen in these structures. Variants of the two forms of $tRNA^{Met}$ were also used to obtain some extra assignments in the acceptor stem, as this part of the tRNA is of crucial importance for formylase recognition. Specific base-pair modifications in the variant could be correlated to changes appearing in the resonances of the HMQC spectra of the variant compared with the wild-type tRNA.

$tRNA_m^{Met}$ Assignments. Certain modified bases in the tRNA have unusual chemical shifts and therefore give a starting point for assignment of other resonances by the use of NOEs. The imino proton of s^4U8, which makes a tertiary interaction with A14, has a characteristic chemical shift because of the deshielding effect of the sulfur which causes this to be downfield in the proton spectrum (Table 2) and has been previously assigned (Reid *et al.*, 1975; Daniel & Cohn, 1975; Hurd & Reid, 1979). This is also true for the corresponding ^{15}N resonance which is shifted down to 179.2 ppm in the nitrogen dimension (Figure 2, Table 2). The s^4U8A14 imino proton gives an NOE to a GC base pair which can be assigned as the adjacent G22C13 in the dihydrouridine stem. G22C13 has an NOE in turn to an AU base pair (A23U12) which has an NOE to G24C11, and this has an NOE to G10C25. Hence all the base pairs of the dihydrouridine stem can be assigned by following consecutive NOEs from the U8A14 starting point. These connections could be clearly seen on a classical 2D homonuclear NOESY (not shown), and also on a 3D NOESY−HMQC (Figure 3), although some of the cross-peaks had a weaker intensity in the latter, because of the short apparent T_1 of the exchangeable protons in a 26 kDa RNA molecule. The ^{15}N-labeling allows the distinction between UA and GC base pairs to be

Table 2: $tRNA_m^{Met}$ Assignments[a]

^1H shift (ppm)	^{15}N shift (ppm)	assignment	^1H shift (ppm)	^{15}N shift (ppm)	assignment
14.82	179.2	U8A14	12.79	144.8	G53C61
13.99	157.8	U12A23	12.71	144.8	G52C62
13.82	159.4	U29A41*	12.53	145.8	G10C25
13.67	159.4	U42A28*	12.30	143.9	p
13.75	156.8	rT54A58	12.10	143.9	G70C3/q
13.37	158.8	U4A69/U68A5*	11.92	143.1	G67C6
13.31	158.6	U4A69/U68A5*	11.75	143.3	G40C30*
13.65	145.2	G24C11	11.53	156.8	ψ55N3
13.37	145.4	G1C72	11.06	154.5	u
13.30	148.2	G43C27*	10.49	142.9	v
13.06	145.6	C71G2/k	9.50	142.9	w
12.98	145.0	G22C13			

[a] ^1H and ^{15}N chemical shifts of the imino protons from the HMQC spectrum of $tRNA_m^{Met}$ are recorded at 298 K. The ^1H chemical shifts are referenced to (trimethylsilyl)propionate and the ^{15}N chemical shifts to liquid NH_3. Assignments marked with an asterisk are only tentative.

made easily (Figure 2). This was further confirmed by the observation of NOESY cross-peaks between the U imino protons and the aromatic H2 from the corresponding adenine base. Another unusual base in the tRNA is ribothymidine, which is present in the ribothymidine arm of the tRNA and forms a tertiary base pair to A58. The imino proton of ribothymidine can be easily assigned, as it gives a characteristic NOE to the 5-methyl group of this unique base in $tRNA_m^{Met}$ sequence (Tropp & Redfield, 1981). Both the imino proton and the 5-methyl group of rT54A58 give a sequential NOESY cross-peak to the pseudouridine ψ55 imino proton which also has a characteristic proton chemical shift. The identified rT54A58 imino proton has a further NOE to a GC base pair, assigned to the adjacent G53C61 which has an NOE to a further GC base pair G52C62. Hence most of the ribothymidine stem is assigned.

The acceptor stem has no unusual bases to use a starting point for assignment. To identify base pairs in the acceptor stem, the HMQC spectrum of a ^{15}N-labeled variant in which the acceptor stem was replaced with that of the initiator $tRNA_f^{Met}$ was used (Table 1). This allows the identification of resonances belonging to the acceptor stem. The resonances which disappear in the HMQC spectrum of this molecule can be assigned to the acceptor stem of the $tRNA_m^{Met}$ whereas those that appear belong to the acceptor stem of the $tRNA_f^{Met}$. Among these resonances are the two UA base pairs of the acceptor stem, which disappear in the variant spectrum, having been replaced by GCs. These two UA resonances are almost superimposed and can be identified as U4A69 and U68A5 in the $tRNA_m^{Met}$, but they cannot be distinguished from one another (Figure 2). This group of acceptor stem resonances also contains three GCs, i, k, q (Figure 2), which give NOEs to each other, such that i has an NOE to k, k to i and q, and q to k. These three resonances must therefore be G1C72, G2C71, and G70C3 or G70C3, G2C71, and G1C72. G2C71 can be assigned as k, being in the middle of these two sequences, but the other two are indistinguishable. In the HMQC spectrum of variant $tRNA_m^{Met}$ G79C4 the resonance corresponding to q is shifted to a greater extent than that corresponding to i, suggesting that q is closer to the mutated base pair than i. Hence we tentatively assign G1C72 to i and G70C3 to q. This is supported by the analysis of the HMQC spectrum of methionylated $tRNA_m^{Met}$, in which only the i resonance, assigned to G1C72, is slightly shifted. Finally, the peak belonging to U4A69 and U68A5 shows an NOE to a further GC also identified as being in the acceptor stem (from the acceptor stem variant) which we therefore assign as G67C6. The acceptor stem is therefore assigned in this way.

Two further UA resonances can be seen in the spectrum of the $tRNA_m^{Met}$ which probably belong to the anticodon stem. Each of these has a further NOE to an unassigned GC base pair. These two GCs could be the two GCs on either side of these UAs in the anticodon stem. So we have the pattern GC, UA, UA, GC. The orientation of the sequence however is not possible to determine, there being no further NOE connections. However, a cross-peak is seen from one of the GC imino protons to the H2A of its neighboring UA base pair, whereas no such NOE is seen from the other GC. Assuming a regular A helix conformation, this distance between the G imino proton and the sequential H2A should be 0.5 Å shorter for the $^{5'}GA^{3'}$ sequence, occurring for G40C30 and U29A41, than for the $^{5'}UG^{3'}$ sequence, corresponding to U42A28 and G43C27 (Wüthrich, 1986), and so the orientation of this sequence can be tentatively assigned.

$tRNA_f^{Met}$ Assignments. The $tRNA_f^{Met}$ sequence has a very high GC content, and the imino proton resonances are clustered in a narrower chemical shift window (Figure 4, Table 3). Consequently, there is much more overlap in the GC region of the spectrum, which is therefore harder to assign. Again the s^4U8A14 base pair is easily assignable and shows an NOE to a GC, assigned as G22C13. However, this base pair shows no further NOEs, preventing further assignment of the dihydrouridine stem.

Ribothymidine, again characterized by the NOE between the imino proton and the methyl group, can be used as a starting point for assignment of the ribothymidine arm. The rT54A58 imino proton has an NOE to ψ55 and also to another GC base pair assigned as G53C61. G53C61 has an NOE to the next base pair G52C62. Also present in this part of the molecule is a GU wobble base pair. In such a base pair the imino proton of the U has a very strong NOE to that of the G. This is easily recognized in the NOESY spectrum, making the assignments of G64 and U50 straightforward. These two resonances also show a further two NOEs to the base pairs on either side of the G64U50 wobble base pair. One of these is G63C51; the other, G49C65. One of these resonances shows a further NOE to another GC which is not that of G52C62, previously assigned. We hence deduce that these base pairs are G49C65 and G7C66, assuming that the base stacking continues into the acceptor stem from the ribothymidine arm (Hare & Reid, 1982b) while the other connection from the GU is G63C51, with no further connecting NOEs.

Again the acceptor stem is more difficult, having no easy starting point, so again variants with changes in the acceptor stem were used to assign these resonances. A variant in which the G70C3 base pair was mutated to G3C70 helped to assign resonances for base pairs 2, 3, and 4, all of which shift in this variant (Figure 5, Table 1). NOEs from G70C3 to G2C71 and G4C69 can also be seen in the 2D NOESY spectrum, but it is not possible to distinguish between base pairs 2 and 4. The variant in which A72 is mutated to G, allowing the terminal G72C1 base pair to be formed, has an extra resonance in its spectrum corresponding to the newly formed base pair, and two peaks are shifted. The shifted resonances correspond to the already assigned G70C3 and

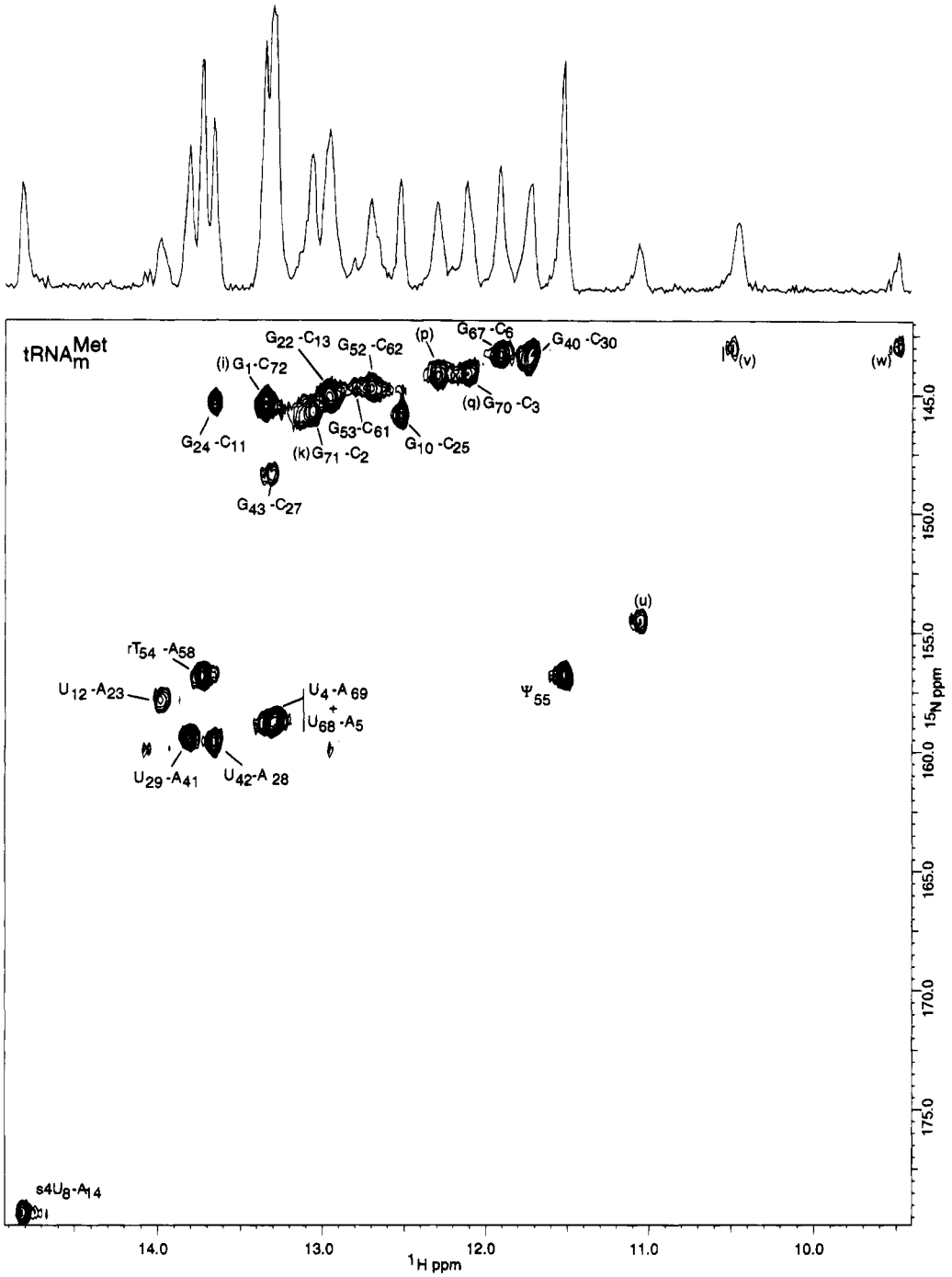

FIGURE 2: HMQC spectrum of ^{15}N-labeled tRNA$_m^{Met}$ (0.4 mM) recorded at 298 K with assigned base pairs labeled. Imino protons originate from the first base given in the labels. The top trace is a skyline projection of the spectrum along the nitrogen dimension.

one of the two resonances seen shifted in the G3C70 mutant. This is assigned as G2C71, since it is closer to the new G72C1 base pair and therefore more likely to be shifted. These resonances of the acceptor stem are also in similar positions to those resonances which are shifted in the acceptor stem switch mutant.

Two further unassigned GC peaks in the tRNA$_f^{Met}$ spectrum, h and k (Figure 4), have NOEs to each other. These could therefore be two of the adjacent GCs in the anticodon stem or G5C68 and G6C67 which are also unassigned.

(iii) Interactions with Transformylase. Transfer RNA samples (25 kDa) were mixed with formylase (34 kDa) to give a stoichiometric 1:1 ratio in 10 mM sodium phosphate (pH 6.3), 10 mM magnesium chloride, and 100 mM sodium chloride. Under these conditions, this enzyme is known to have full activity and specificity (Kahn *et al.*, 1980). Interestingly, formylase can bind both tRNA$_f^{Met}$ and tRNA$_m^{Met}$, with or without methionine attached to the 3′ ribose; however, it only formylates the initiator tRNA. The dissociation constants for both uncharged tRNAs are in the micromolar range (Kahn *et al.*, 1980); therefore, with sample concentrations of 0.4 mM, more than 90% of the tRNA must be complexed with the formylase. The affinity value also implies that the lifetime of the complex is expected to be greater than 100 ms if association is diffusion controlled. The HMQC spectra of the tRNA were compared with those in which the tRNA was mixed with formylase. In all cases a general broadening of 20−30 Hz of all the peaks was seen,

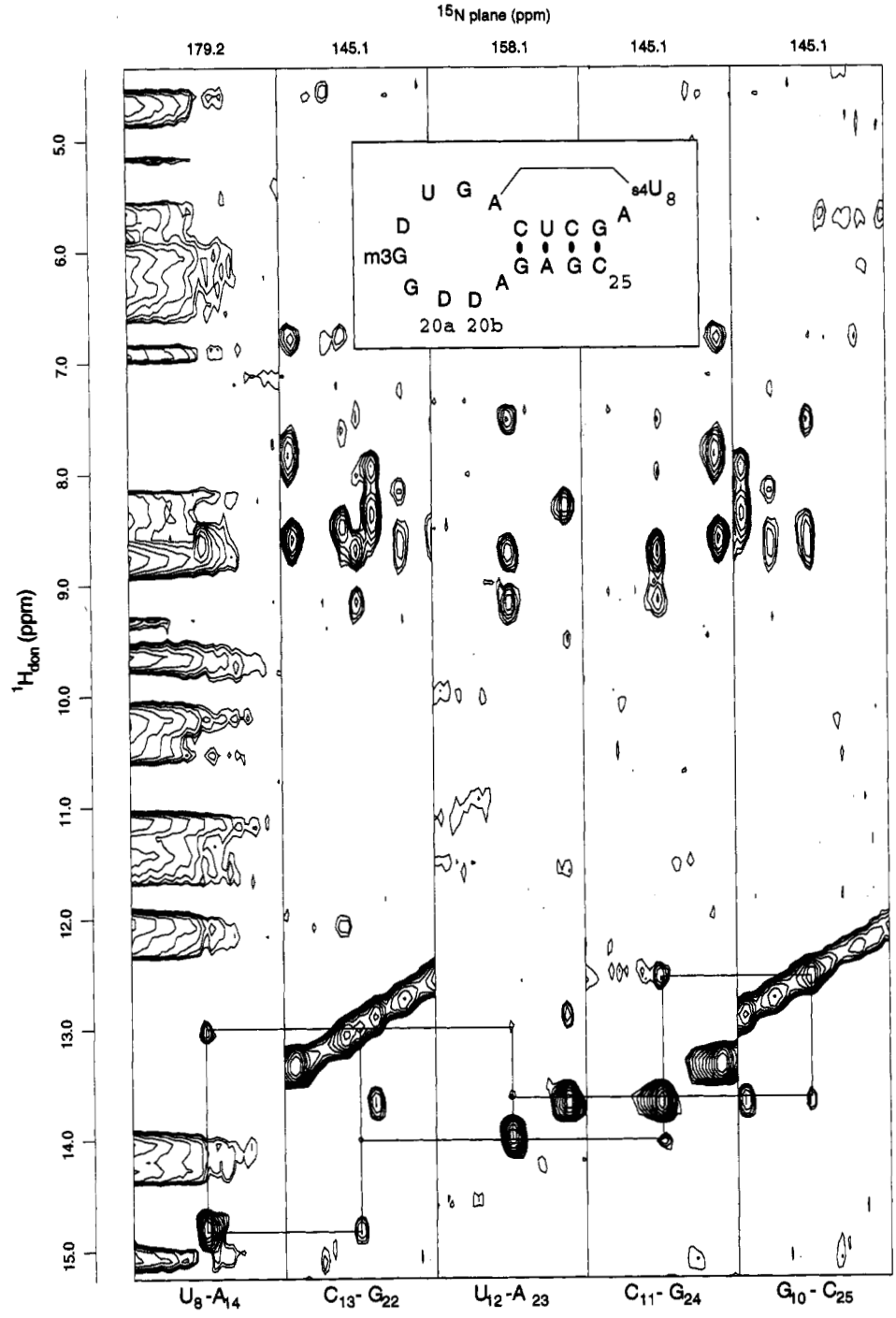

FIGURE 3: 3D NOESY−HMQC spectrum of $tRNA_m^{Met}$ showing the sequential assignment of the dihydrouridine arm (sequence inset). The sequential walk for the $^1H-^1H$ NOEs of the imino protons is shown starting from the s^4U8A14 base pair, which is used as a starting point due to its unusual ^1H and ^{15}N chemical shifts. The vertical strips are extracted at the ^{15}N frequency of the nitrogen atom to which the imino proton is attached.

thus confirming the formation of the complex. Because of the significant reduction in the apparent T_1 of all exchangeable protons upon formation of a 59 kDa complex, the number of transients accumulated per increment had to be increased 4−8-fold to compensate for the loss of signal due to relaxation during the preparation and evolution times of the HMQC experiment. After the spectra were acquired, the tRNAs were separated from the enzyme and submitted to electrophoresis on a sequencing 16% acrylamide−urea gel. Seventy percent of the tRNA remained intact after 24 h at 298 K, although some degradation of the tRNA was observed, presumably due to ribonucleases contaminating the formylase. The primary degradation products were fragments corresponding to half the tRNA length, resulting from cleavages in the anticodon loop. No significant change was observed in the 1D spectrum over the time of the experiment.

As described above in the $tRNA_m^{Met}$ HMQC spectrum the peaks were broadened when formylase was added, but uniformly, and no significant shifts or changes in the relative intensities of the peaks could be observed (Figure 6). The relative intensities of peaks in the bound form with respect to their counterparts in the uncomplexed form were calculated by integration; the values thus obtained ranged from 83% to 118%. In particular, the peaks corresponding to base

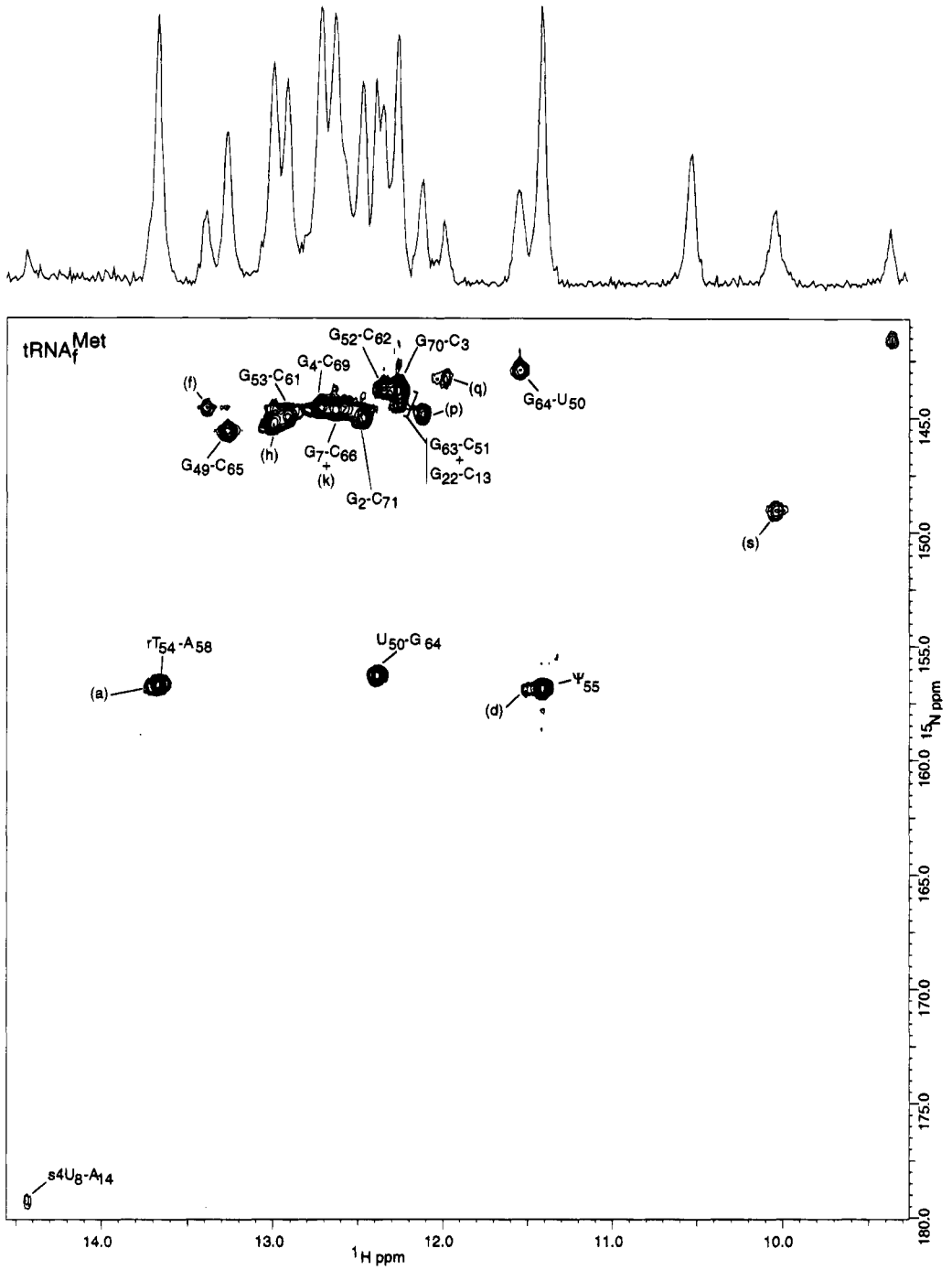

FIGURE 4: HMQC spectrum of ^{15}N-labeled tRNA$_f^{Met}$ (0.4 mM) recorded at 298 K with assigned base pairs labeled. Imino protons originate from the first base given in the labels. The top trace is a skyline projection of the spectrum along the nitrogen dimension.

pairs 1, 2, and 3 fell within that range, with relative intensities of 87%, 101%, and 117%.

In the case of the tRNA$_f^{Met}$ complexed to formylase, five GC peaks were strongly decreased in intensity in addition to this uniform broadening (shown in Figure 7). Two peaks could be identified as resonances which had previously been assigned to the acceptor stem of the molecule, specifically those belonging to base pairs G70C3 and G2C71, and the third one corresponds to the position where G7C66 is located. This resonance is, however, very close to G4C69, so that it is not possible to tell which of these two is actually effected. In the case of G70C3 and (G4C69 + G7C66), the observed loss of intensity is unfortunately partially masked by overlaps with other peaks. However, this experiment was repeated a second time with different samples of both labeled tRNA$_f^{Met}$ and formylase and a third time with labeled methionylated tRNA$_f^{Met}$ and formylase (not shown), and in all cases similar specific losses of intensity on the same peaks were again observed. In addition to these assigned resonances, two other unidentified GC imino resonances almost disappeared. Measurement of the relative intensities of the imino resonances in the bound and free form gave values ranging from 81% to 125%, for all peaks except the five above GCs. The relative intensities of base pairs 2, 3, and 7 (or possibly 4) were 44%, 55%, and 56%, respectively, and those of the other two unassigned GCs were less than 15%. The corresponding resonances of the acceptor stem of the tRNA$_m^{Met}$ molecule were not affected in this way.

Table 3: $tRMA_f^{Met}$ Assignments[a]

^1H shift (ppm)	^{15}N shift (ppm)	assignment	^1H shift (ppm)	^{15}N shift (ppm)	assignment
14.44	179.4	U8A14	12.65	144.5	G7C66/k
13.69	156.3	a/rT54A58	12.49	144.8	G2C71
12.39	156.2	U50	12.35	143.5	G52C62
11.51	156.8	d	12.27	143.7	G70C3
11.42	156.8	ψ55N3	12.27	144.3	G22C13/G63C51
13.40	144.5	f	12.13	144.8	p
13.28	145.6	G49C65	11.99	143.1	q
13.01	145.2	h	11.56	142.9	G64
12.93	144.7	G53C61	10.05	149.0	s
12.72	144.3	G4C69			

[a] ^1H and ^{15}N chemical shifts of the imino protons from the HMQC spectrum of $tRNA_f^{Met}$ are recorded at 298 K. The ^1H chemical shifts are referenced to (trimethylsilyl)propionate and the ^{15}N chemical shifts to liquid NH_3.

The presence of mispaired bases, C1 and A72, at the tip of the acceptor stem is known to be the primary determinant for formylation. We therefore investigated the behavior of the variant $tRNA_f^{Met}$ G1C72 (Table 1) for which the single base-pair substitution causes a strong decrease (by more than 5 orders of magnitude) of the formylation efficiency (k_{cat}/K_m; Guillon *et al*, 1992). Again, a uniform broadening is observed when formylase was added, but the specific effects on individual imino resonances are no longer seen (not shown). Finally, the interaction of the formylase with the variant of $tRNA_m^{Met}$ in which the acceptor stem had been changed for that of $tRNA_f^{Met}$ was also studied. This variant possesses the determinants for formylation and is almost as good a substrate for the enzyme as the native $tRNA_f^{Met}$. Among the four GC resonances which appear in this spectrum when compared to that of $tRNA_m^{Met}$ and which could thus be clearly assigned to the acceptor stem, two disappeared almost completely upon formylase binding and a third one was strongly decreased (Figure 8). Overlap with other GC imino peaks prevented a reliable analysis of the fourth GC.

DISCUSSION

This work describes the NMR solution studies of the binding of various methionine isoaccepting tRNAs to the formylase. The imino proton resonances have been used as structural probes, which required their prior spectral assignment. Taking advantage of the availability of recombinant plasmids containing engineered tRNA genes, a novel strategy was devised for the imino proton resonance assignment. It combines uniform isotope enrichment *in vivo*, with the use of variants obtained by site-directed mutagenesis. The uniform ^{15}N-labeling offers two main advantages: an increased resolution of the imino peaks in a second dimension and the editing of protein signals when recording spectra of the complexes. This allowed the identification of individual imino resonances of tRNA bound to formylase where the overall size of the complex is approximately 59 kDa.

We have thus assigned the resonances of many of the imino protons involved in secondary and tertiary base pairing in the $tRNA_f^{Met}$ and $tRNA_m^{Met}$ molecules from *E. coli*, focusing on the acceptor stem which is the primary site of interaction with formylase. One GC resonance (p, Figure 2) remains unassigned in the $tRNA_m^{Met}$ spectrum, which could correspond to either G64C50 or G7C66. Two other weak unassigned UA resonances also appear in the spectrum

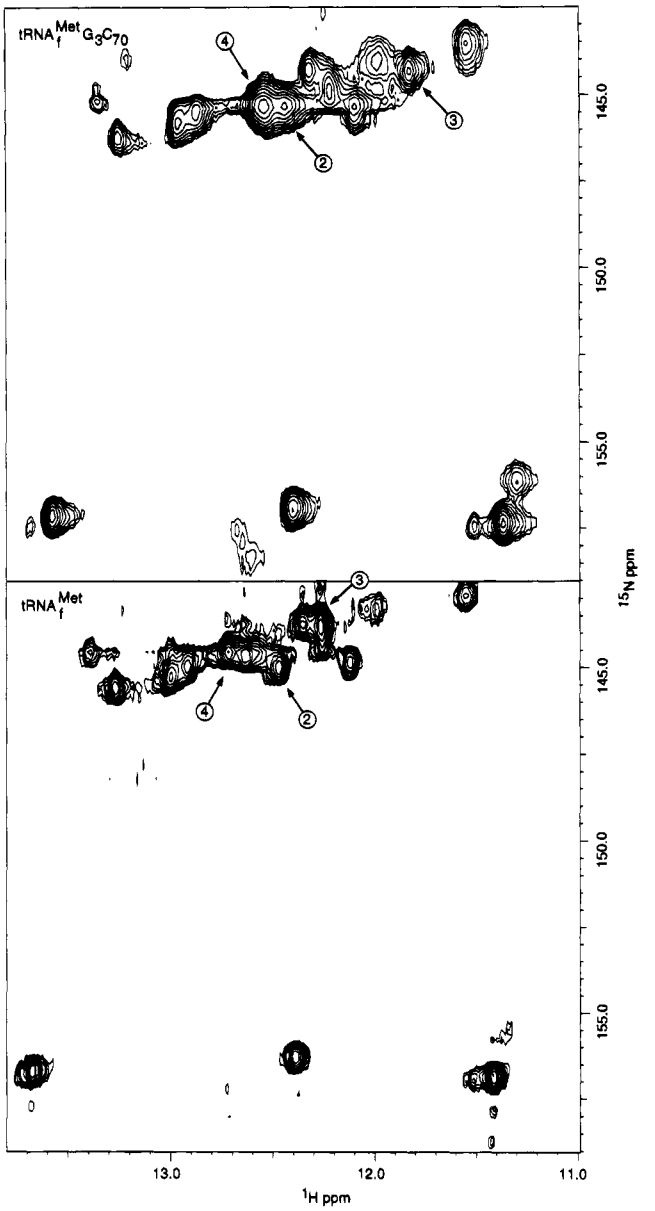

FIGURE 5: Regions of the HMQC spectra of $tRNA_f^{Met}$ (0.4 mM) and of a variant of $tRNA_f^{Met}$ with a change of G70C3 to G3C70. Shifts in the resonances of the variant spectrum in comparison to the wild-type spectrum allow the assignment of the base pairs G70C3, G2C71, and G4C69. The arrows indicate the positions of the affected imino protons, and numbers refer to the base−pair number.

which could correspond to ψ39A31 and U65A49, both of which are at the end of stems and therefore might have weaker base pairs. In the $tRNA_f^{Met}$ spectrum there are two unassigned UA base pairs. Griffey *et al.* (1983) reported some assignments of $tRNA_f^{Met}$ regioselectivity labeled with [3-^{15}N]uracil. Their assignments for rT54, U50G64, ψ55, and s4U8A14 agree with ours. They further tentatively assigned U24A11 and U27A43, which could correspond to peaks a and d, respectively, on Figure 4. However, we have no evidence to confirm these. The anticodon stem of $tRNA_f^{Met}$ remains to be assigned and should contain most of the unidentified GC resonances.

Methionyl-tRNA transformylase binds a wide range of both aminoacylated and nonaminoacylated tRNAs with high affinity, although it only formylates charged $tRNA_f^{Met}$. This suggests that although the enzyme binds both $tRNA_m^{Met}$ and

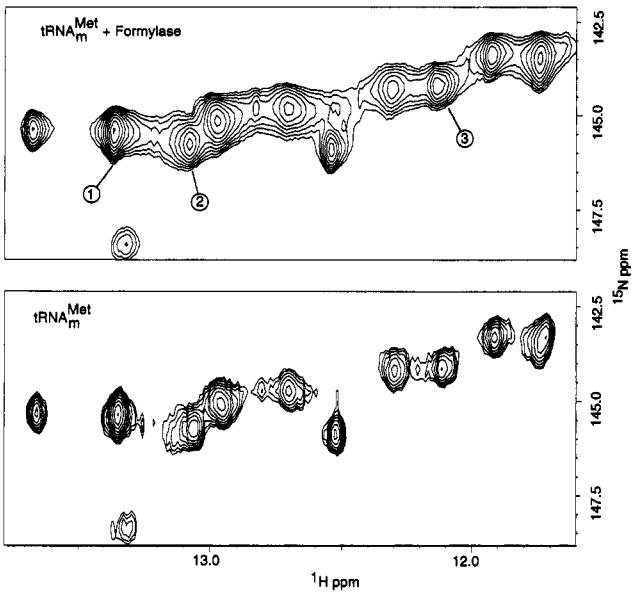

FIGURE 6: Comparison of the GC imino proton region of the HMQC spectra of $tRNA_m^{Met}$ (0.4 mM) with and without formylase. The molar ratio of $tRNA_m^{Met}$:formylase was 1:1. Base pairs 1, 2, and 3 from the acceptor stem are indicated by arrows.

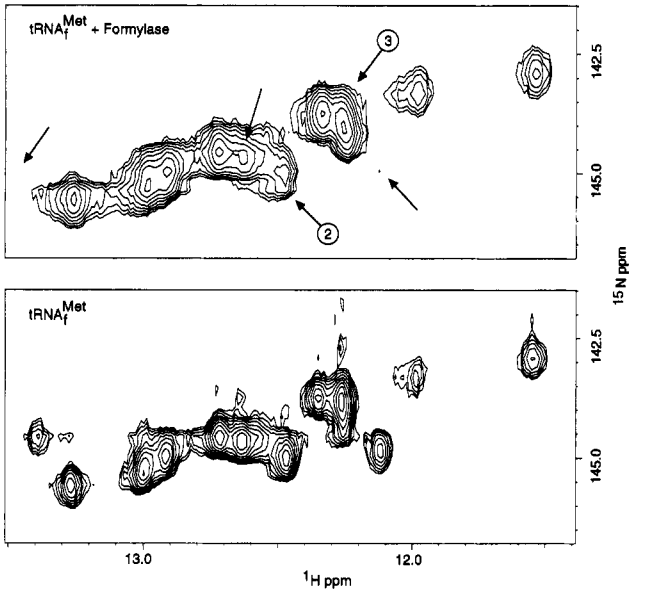

FIGURE 7: Comparison of the GC imino proton regions of the HMQC spectra of $tRNA_f^{Met}$ (0.4 mM) with and without formylase. The molar ratio of $tRNA_f^{Met}$:formylase was 1:1. Base pairs 2 and 3 from the acceptor stem are indicated. Three other resonances also show changes in intensity when formylase is added and are indicated by arrows. One of these is located in the central massif at the position to base pair 7, very close to base pair 4.

$tRNA_f^{Met}$ with similar affinities, some specific interaction is required for catalysis which takes place only with the latter. It has been clearly documented that the specificity of methionyl-tRNA transformylase relies solely upon recognition of a limited number of nucleotide determinants all located within the acceptor stem of initiator $tRNA_f^{Met}$ (Lee *et al.*, 1991; Guillon *et al.*, 1992). In agreement with these results, we report that, upon formylase binding to $tRNA_f^{Met}$, the intensities of several peaks corresponding to base pairs in the acceptor stem and two unassigned peaks are specifically decreased. An explanation of these data may be that

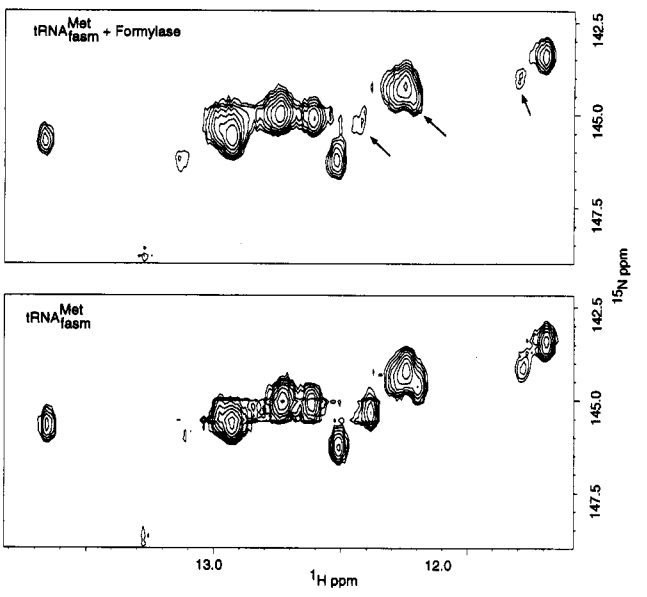

FIGURE 8: Comparison of the GC imino proton regions of the HMQC spectra of the fasm variant (acceptor stem switch) (0.4 mM) with and without formylase. Three of the four resonances assigned to the acceptor stem, indicated by arrows, are greatly decreased in intensity.

the loss of intensity of the imino proton resonances of the acceptor stem is due to basic groups of the formylase coming close to the base pairs and catalyzing the exchange of these protons with water. This proton-catalyzed exchange would only occur for tRNA species which are substrates of the formylase, suggesting that the enzyme binds initiator and elongator tRNA differently. Alternatively, this loss of intensity could be due to the partial melting of the corresponding base pairs. These two possibilities do not exclude each other, and both weakening of the acceptor stem base pairs and protein-catalyzed exchange could account for this effect. In an attempt to distinguish between these two effects, we have investigated imino proton exchange in $tRNA_f^{Met}$, by adding ammonia as a catalyst (Leroy *et al.*, 1985). Resonances f and p on Figure 4, which disappear upon formylase binding, can be individually resolved on a 1D spectrum. Upon addition of increasing concentrations of ammonia to free $tRNA_f^{Met}$, up to 300 mM NH_3, these two resonances remain largely unaffected, whereas about two-thirds of the imino protons are broadened beyond detection (not shown). This suggests that the corresponding base pairs are stable and have a long lifetime and that their disappearance from the spectrum of $tRNA_f^{Met}$ complexed with formylase cannot be accounted for solely by exchange effects.

The idea that the acceptor stem is being melted is supported by the observation that the presence of a mismatch or weak base pair (i.e., UA or GU) at position 1−72 is a necessary condition for the formylation reaction to take place (Guillon *et al.*, 1992a). This requirement suggests that a melting of the top of the acceptor stem is involved, such as that which has been observed in the crystal structure of glutaminyl-tRNA synthetase complexed with $tRNA^{Gln}$, where base pair 1−72 is disrupted (Rould *et al.*, 1989). This is also suggested by data obtained for binding of formylase to $tRNA_f^{Met}$ at low salt concentrations (Kahn *et al.*, 1980). At concentrations of 5 mM KCl, 10 molecules of formylase bind 1 molecule of tRNA. This situation would only be possible if the tRNA has been denatured to a single strand such that

the formylase molecules can bind along it and suggests that the formylase has the ability to unwind the tRNA in some way. The loss in intensity of some of the resonances assigned to the acceptor stem in $tRNA_f^{Met}$ could therefore be due to the formylase actually unwinding the first base pairs such that the corresponding imino protons are exposed to exchange with the solvent. In this context it is tempting to propose that the two unidentified GC peaks that disappear upon binding to the formylase correspond to G5C68 and G6C67, which are presently unassigned. From our results, obtained at high ionic strength, it is clear that this only occurs when the tRNA is a substrate of the enzyme, i.e., with $tRNA_f^{Met}$ and $tRNA_{fasm}^{Met}$, both of which have a mismatch at position 1−72. Both $tRNA_m^{Met}$ and $tRNA_f^{Met}$ G1C72 do not show any such change in the imino proton peak intensities, indicating that the presence of a strong base pair at the tip of the acceptor stem prevents this effect. This suggests that melting of the acceptor stem could be required for the 3′-CCA terminus carrying the esterified methionine to reach the active site of the formylase, thus explaining why most tRNA species, which have a GC or CG base pair at position 1−72, are not substrates of formylase, although they bind to the enzyme.

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