The C-A mismatch base pair and the single-strand terminus in the *E. coli* initiator $tRNA^{fMet}$ acceptor stem adopt unusual conformations

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Abstract Acceptor stem variants of $tRNA^{fMet}$ (*Escherichia coli*) have been characterized by nuclear magnetic resonance. The wild type contains a C1-A72 mismatch pair which is crucial for its biological function. For comparison, the mismatch was replaced by regular pairs U1-A72 and C1-G72. Further variants contain an altered discriminator base, G73, or a G1-C72/U73 combination. The stems of variants U1-A72/A73 and C1-G72/A73 have A-RNA geometry, which extends essentially to the single-strand terminus. C1-A72/G73 variant and wild type are structurally almost identical. C1 and A72 adopt peculiar conformations with C1 being largely destacked with respect to G2, while A73 stacks upon C1. The unique arrangement of the mismatch causes a distinctly different orientation of the single-strand terminus compared to variants with regular 1–72 base pairs, and to formyltransferase-complexed $tRNA^{fMet}$.

Key words: Initiator tRNA; Acceptor stem; C-A base pair; tRNA identity; Nuclear magnetic resonance

1. Introduction

For the initiation of protein biosynthesis in eubacteria an initiator tRNA is used which is aminoacylated with *N*-formylated methionine (fMet) [1]. Elongator Met-$tRNA^{Met}$ is bound by elongation factor Tu (EF-Tu) and transported to the ribosomal A-site. By contrast, initiator Met-$tRNA^{fMet}$ interacts with methionyl-tRNA-formyltransferase (MTF) which formylates methionine at its α-amino group. Therefore, recognition of initiator Met-$tRNA^{fMet}$ by MTF represents a crucial step in the initiation process. Recognition elements of $tRNA^{fMet}$ for MTF are mainly located in the acceptor stem [2,3]. The wild type contains a C1-A72 mismatch pair. Mutant studies clearly demonstrated that a thermodynamically 'weak' first base pair in the acceptor stem of $tRNA^{fMet}$ is required for efficient formylation, whereas 'strong' first base pairs prevent formylation almost completely [4,5]. Other base pairs in the acceptor stem affect the rate of formylation to a lesser extent. Replacement of the discriminator base A73 in wild type by a pyrimidine base does not affect the kinetics of formylation, whereas substitution for guanosine lowers the k_{cat}/K_M eight-fold [6]. Therefore, an alteration of the local structure due to the replacement was suspected [6]. A $tRNA^{fMet}$ mutant with a G1-C72 base pair and U73 discriminator nucleotide also represents a comparatively poor substrate for MTF [6]. In a nuclear magnetic resonance (NMR) study of a corresponding acceptor stem-loop construct a 'folded-back' structure of the single-stranded end was suggested [7]. Generalizing this finding, such a 'fold-back' arrangement of the single-stranded end was also proposed for the wild type acceptor stem, thus representing a distinct recognition signal for the MTF [7].

To infer conclusions about the structural requirements in the acceptor stem of the $tRNA^{fMet}$ for recognition by MTF, we characterized wild type *Escherichia coli* initiator $tRNA^{fMet}$ acceptor stem conformation by NMR. In addition, the conformations of sequence variants with a mutated first base pair and/or altered discriminator bases (Fig. 1) were compared to the structure of the wild type acceptor stem. Of particular interest was, of course, the arrangement of the unique C1-A72 pair and its influence upon the conformation of the single-stranded terminus.

2. Materials and methods

2.1. RNA synthesis and purification

RNA was synthesized using the H-phosphonate method [8] on a synthesizer 'Gene Assembler Plus' (Pharmacia). Transversion of the last base pair G7-C66 to C7-G66 in all cases was necessary to avoid formation of undesired, very stable G-quartet tetraplex structures [9]. High performance liquid chromatography (Beckman) purification was performed with DEAE 500-7 anion exchange columns (Macherey-Nagel) under denaturing conditions [10] with a KCl gradient. Products were desalted on a Biogel P6 (Bio-Rad) column. Equimolar amounts of single-stranded RNA were annealed by heating to 80°C and subsequent cooling to room temperature. NMR samples contained about 1 mM double-stranded RNA in 0.5 ml D_2O or D_2O:H_2O (1:9 v/v) buffer solution with 100 mM NaCl and 10 mM phosphate at pH 6.5.

2.2. NMR measurements

Spectra were recorded on a DRX-500 spectrometer (Bruker) at a proton frequency of 500.13 MHz. Temperature adjustment was performed by cryostat (Haake) or thermostat (Bruker). As an internal reference 0.5 mM DSS (2,2-dimethyl-2-silapentane-5-sulfonate) was used. Water signals were suppressed by presaturation or 1331 binomial sequence [11]. NOESY (mixing times of 80, 150 and 350 ms), DQF-COSY and Clean-TOCSY spectra were recorded at different temperatures (289, 297, 303 K) in phase-sensitive mode in F1 with 4096×512 (F2×F1) data points using standard pulse sequences. ^1H-^{13}C HMQC spectra were recorded with 2048×512 (F2×F1) data points and spectral widths of 130 ppm for ^{13}C and 10 ppm for ^1H. Phosphorus spectra were recorded with proton decoupling, 85% phosphorus acid was used as external reference. Fourier transformation and data processing was performed on workstations O2 and Indigo^2 (SGI) with the NDEE NMR software (Spin-up). Apodization was done by multiplication with exponential (1D) or squared π/2 shifted sinebell (2D) functions.

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2.3. Structural model calculations

Restrained molecular dynamics (RMD) calculations for the wild type acceptor stem were performed with XPLOR [12] on an O2 workstation (SGI). The dynamics protocol was adapted from the one described by Varani et al. [13]. A force field optimized for nucleic acids was employed in the calculations [14] with all parameters involving hydrogen bonds being taken from the CHARMM force field. A total of 180 measured NOEs were used (10 per nucleotide). NOE-derived distance restraints were classified similar to the scheme employed by Dallas and Moore [15]. Since the resonance distribution in the ^{31}P spectra was narrow [16] the conformation of the backbone was assumed to be regular with allowed torsion angle variations of $\pm 20°$ for all nucleotides in base pairs 2–7. Sugar puckering was restrained either to 3′-*endo* or 2′-*endo*, respectively, according to the absence or presence of ribose H1′–H2′ scalar coupling. Since a NOESY cross-peak pattern typical of A-helical geometry was found for the stem region between base pairs 2 and 7, such a conformation was initially used for the RMD [17,18]. A structural model was calculated by averaging 18 out of 30 low energy structures with no NOE and dihedral angle violations (rmsd 1.1 Å). Analysis of the structures was performed with the program CURVES [19]. Figs. 3–5 and 7 were generated with the INSIGHTII 98.0 program (MSI).

3. Results

3.1. NMR-based structural comparison of the acceptor stem variants

For all acceptor stems six imino resonances due to regular G-C Watson–Crick base pairs 2–7 have been observed. No imino (as well as amino) signal is contributed by a first mismatch pair C1-A72. However, even in the U1-A72 variant no imino resonance originating from the first base pair can be detected. By contrast, imino resonances originating from the first base pairs are found for the variants with a C1-G72 or G1-C72 base pair. In NOESY spectra at 350 ms mixing time, all possible intra-nucleotide and inter-nucleotide (5′→3′) H1′–H6/H8 cross-peaks for base pairs 2–7 are observed, enabling a continuous assignment trail for both strands. Moreover, medium to strong intra-nucleotide H3′–H6/8 cross-peaks were observed corroborating the assumption of an A-helical geometry. All possible H2′–H6/H8 inter-nucleotide NOE contacts are still detectable even in the 80 ms NOESY spectra suggesting an essentially A-helical arrangement. For the terminal nucleotides A76 and C75 values of the scalar coupling constants $J^{1'2'}$ of 4.9 and 3.7 Hz, respectively, were determined. This is typical for the 3′-end of tRNAs [20] and

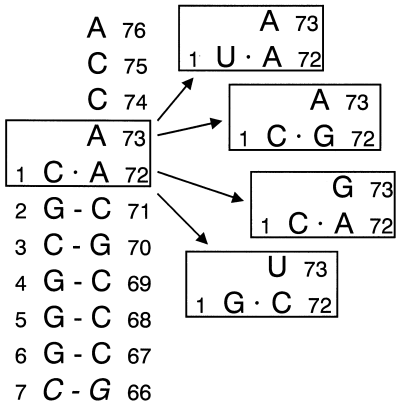

Fig. 1. Wild type acceptor stem of duplex $tRNA^{fMet}$ (*E. coli*) as used in the NMR studies. The altered nucleotides in the variants are shown in the boxes. Base pair G7-C66 (wild type) is transverted to C7-G66. The numbering is according to nomenclature generally employed for tRNA [38].

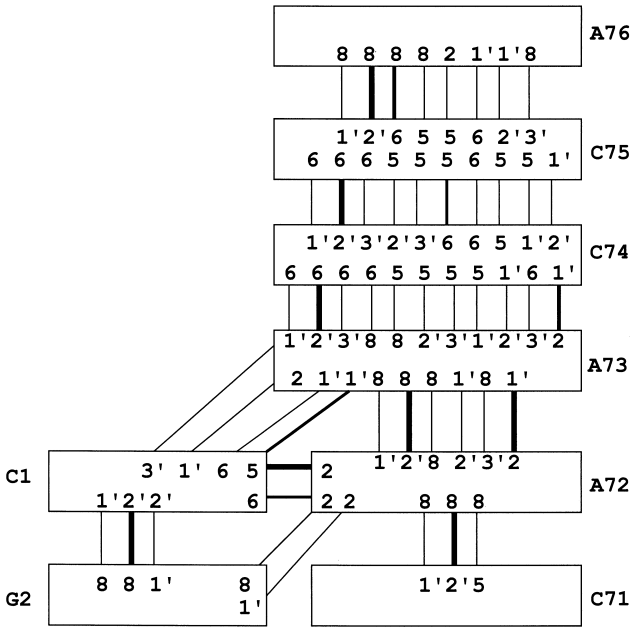

Fig. 2. Inter-nucleotide NOE contact scheme of non-exchangeable proton resonances of the wild type C1-A72/A73 $tRNA^{fMet}$ acceptor stem as derived from NOESY spectra with different mixing times. The line thickness visualizes the intensity of the corresponding cross-peaks.

indicates 2′-*endo* populations of the corresponding ribose pucker of 57 and 40%, respectively [21]. Interestingly, in the G1-C72/U73 variant additional H1′–H2′ COSY cross-peaks for U73 and C74 with coupling constants of 3.7 Hz were found. Moreover, C1 of the wild type and its G73 variant also feature a scalar coupling constant of about 3.7 Hz. For the remaining nucleotides in all variants no H1′–H2′ couplings could be detected. This indicates predominantly 3′-*endo* puckered riboses. A unique feature of the NOESY spectra of both the wild type $tRNA^{fMet}$ acceptor stem and its G73 variant is the unusual cross-peak pattern around C1 (Fig. 2). In particular, this includes a very intense A72H2-C1H5 cross-peak (which is still observed in the 80 ms NOESY spectrum), cross-peaks C1H6-A72H2 (medium), A73H1′-C1H5 (medium) and A73H1′-C1H3′ (weak). The same, unusual cross-peak pattern around C1 is likewise present in the spectra of the C1-A72/G73 variant. The ^{13}C shift of A72C2 (149.4 ppm) indicates that A72 is definitely not protonated at N1 [22–24].

The NOESY cross-peak pattern for the G1-C72/U73 variant significantly differs from the one for the wild type and its G73 variant. Several cross-peaks between A76 and G1 were detected which suggests a bending back of the single-strand such that A76 stacks somehow above G1 of the first base pair, though the stacking order might be low due to high conformational flexibility of this arrangement. Among the respective cross-peaks are G1H1′-A76H1′ (medium), A76H8-G1H8 (weak), A76H1′-G1H8 (very weak), and C75H3′-A76H2 (medium). These observations essentially agree with the findings reported by Puglisi et al. [7], and corroborate the fold-back arrangement of the single-strand terminus postulated by them.

3.2. Conformation of the C1-A72 mismatch base pair of the wild type

A superposition of eight out of 18 selected individual struc-

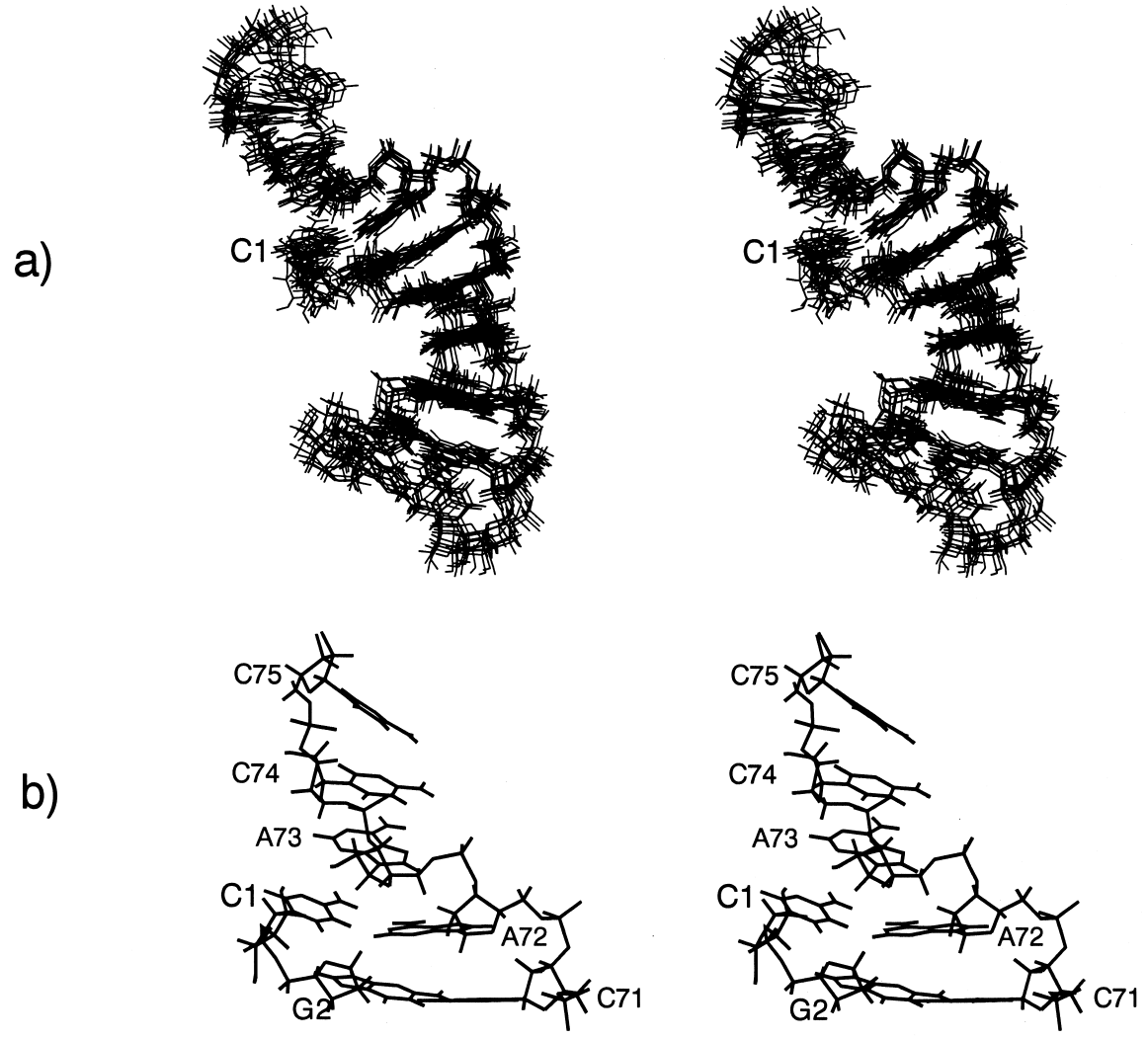

Fig. 3. Stereo representations of (a) the superposition of eight out of 18 selected structures of the wild type C1-A72/A73 $tRNA^{fMet}$ acceptor stem and (b) the structural model calculated from the average of the selected structures comprising mismatch pair C1-A72, base pair G2-C71, discriminator nucleotide A73 and nucleotides C74 and C75.

tures calculated from the NMR data (cf. Section 2) is presented in Fig. 3a. The last base pair (C7-G66) and the terminal nucleotide A76 (rmsd of 1.88 Å) mark the most flexible regions of the molecule. Accordingly, they display the highest degree of conformational disorder. Remarkably, the precision in the other regions, in particular the one of special interest (base pairs 2 and 1, nucleotides A73–C75), is much better as indicated by rmsd values of, for example, 1.34 Å for C1. The other nucleotides G2, C71, A72, A73 and C74 are characterized by low rmsd values of <1 Å, which compare well with those of the residues in the interior of the helical stem. A stereo representation of the acceptor stem region around the mismatch pair is exhibited in Fig. 3b, and from different viewing angles in Fig. 4. Whereas C75 stacks fairly well above C74 (Fig. 4), A76 (not shown in Figs. 3b and 4) is largely destacked with respect to C75. It is obvious that A72 of the mismatch stacks reasonably well upon the preceding nucleotide C71 of base pair 2 which is still fairly well ordered (Figs. 4 and 5). However, C1 stacks poorly above G2, and the base plane of C1 makes an angle of about 30° with the plane of the underlying base of G2. Moreover, A73 stacks quite well above C1 rather than above A72 (Figs. 4 and 5). This is an extraordinary interstrand stacking. The C1 of the mismatch is close to high-*anti* conformation ($\chi=-102°$). It acts in a way as a 'wedge' which forces the single-strand terminus as a whole to tilt (Fig. 4). The sugar–phosphate backbone makes a sharp bend between residues A72 and A73 (see also Fig. 7).

The 5′-terminal nucleotide C1 adopts a conformation completely different from that in an 'ordinary' C-A mismatch pair with an N1-protonated adenine as found in the interior of A-RNA double strands [24,25] (see Fig. 5, bottom). In the $tRNA^{fMet}$ acceptor stem, C1 and A72 are located at the end of a regular Watson–Crick duplex, and act rather as independent nucleotides making no recognizable direct contacts via functional groups. In particular, there is no evidence for any hydrogen bonding.

3.3. Chemical shift difference analysis

A qualitative comparison between the wild type acceptor stem of $tRNA^{fMet}$ and three sequence variants was performed (Fig. 6). In all cases, intrinsic chemical shift differences for the corresponding protons have been accounted for [26]. For the

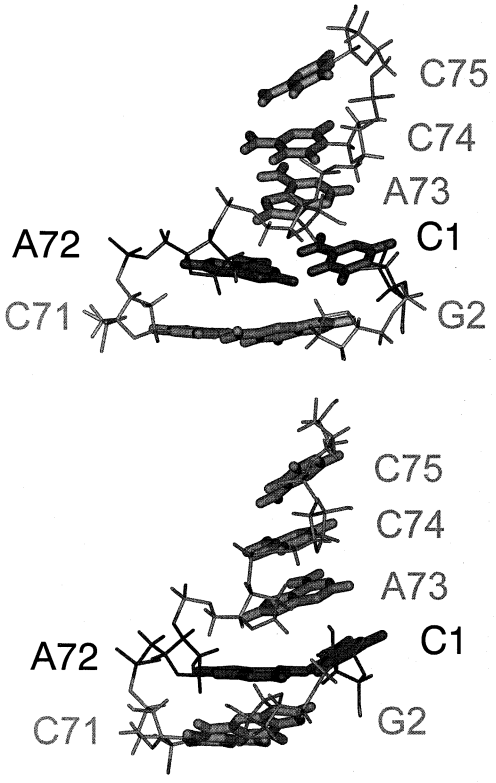

Fig. 4. Side views of the wild type acceptor stem structural model comprising mismatch pair C1-A72, base pair G2-C71, discriminator nucleotide A73 and nucleotides C74 and C75.

variant where merely the discriminator nucleotide (A73) has been replaced by G73, the only significant shift changes occur in the immediate neighborhood of the replaced nucleotide 73. This can easily be attributed to the distinctly diminished shielding strength of G as compared to A [27] which gives rise to downfield shifts of the aromatic and H1′ resonances of C74 (and to a lesser extent of A72 and C75) in the G73 variant. A markedly different behavior is found for the variants with regular 1–72 Watson–Crick base pairs in place of the C1-A72 mismatch. In both variants, the aromatic proton (H5/H6) resonances of nucleotide 1 in the variants are clearly shifted to lower field, indicating decreased shielding in comparison to the wild type acceptor stem. By contrast, the H8 signals of G2 in the Watson–Crick variants are shifted upfield. In the opposite strand, significant downfield shifts are observed for the H5 and H6 protons of C71 in the Watson–Crick variants, whereas those of A72/C72 and A73 are shifted upfield (Fig. 6b,c) to roughly the same extent. Generally, in the wild type acceptor stem, the aromatic bases of C1 and C71 are better shielded whereas the bases of A72 and A73 at the same time are deshielded as compared to 1–72 Watson–Crick variants. This can be explained in terms of stacking interactions of C1 with A73 (instead of G2), and is fully compatible with the NOE-based structural model.

4. Discussion

The NMR data – both the NOE contact pattern and the chemical shift data – indicate that there are no significant structural differences between wild type and the G73 variant of the $tRNA^{fMet}$ acceptor stem. The decrease in formylation efficiency (reduction of k_{cat}/K_M) by about one order of magnitude for a G73 mutant of $tRNA^{fMet}$ [4,6] can be ascribed to an unfavorable contact between the 2-amino group of G73 and the peptide backbone of loop 1 of MTF (amino acid residues alanine 40 and glycine 41) [28].

The structural model of the wild type $tRNA^{fMet}$ acceptor stem presented here on the basis of NMR data deviates from the conformation of this part of the $tRNA^{fMet}$ molecule determined by X-ray structure analysis [29] – mainly with respect to the C1-A72 mismatch and the single-strand terminus. According to the latter report, the single-strand terminus "curves over the minor groove of the acceptor helix, bringing phosphate 76 close to phosphates 3 and 4 of the opposite strand". In addition, nucleotides A72 and C1 are separated by a much greater distance than in our structural model. The exact reason for these structural differences remains elusive. They might be associated with crystal packing effects.

The NMR data for the G1-C72/U73 variant suggest a folded-back single-strand terminus in such a way that A76 stacks upon G1 (cf. Section 3.1; see also Puglisi et al. [7]). It has been discussed in the literature that such a 'hairpin' structure could be recognized by MTF [7], and that the wild type $tRNA^{fMet}$ stem likewise adopt this fold-back conformation [7]. However, the structural model of the acceptor stem of wild type $tRNA^{fMet}$ derived from NMR data in this work does not support the assumption of a folded-back single-strand terminus.

The Watson–Crick variants C1-G72/A73 and U1-A72/A73 are in an A-helical conformation, their single-stranded ends continue the helix almost regularly. This is in agreement with the findings obtained for elongator tRNAs like $tRNA^{Ala}$ [20,30], $tRNA^{Phe}$ [31], and $tRNA^{Asp}$ [32], and moreover for

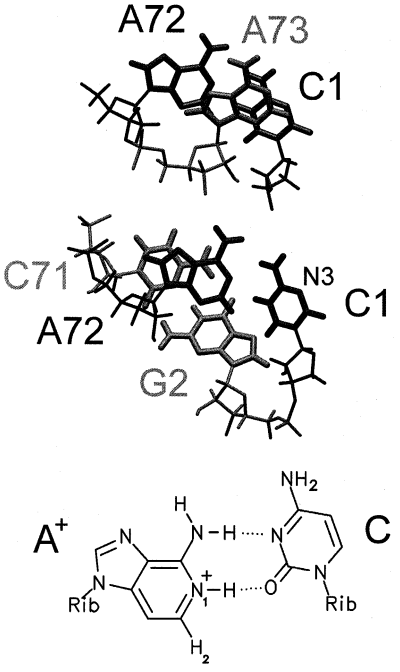

Fig. 5. Down views from the single-stranded end of the wild type acceptor stem structural model comprising the C1-A72 mismatch and A73 (top) or G2-C71 (middle). For comparison, a schematic representation of a protonated C-A^+ wobble base pair (from [24]) is presented (bottom) with the adenine orientation being the same as for A72.

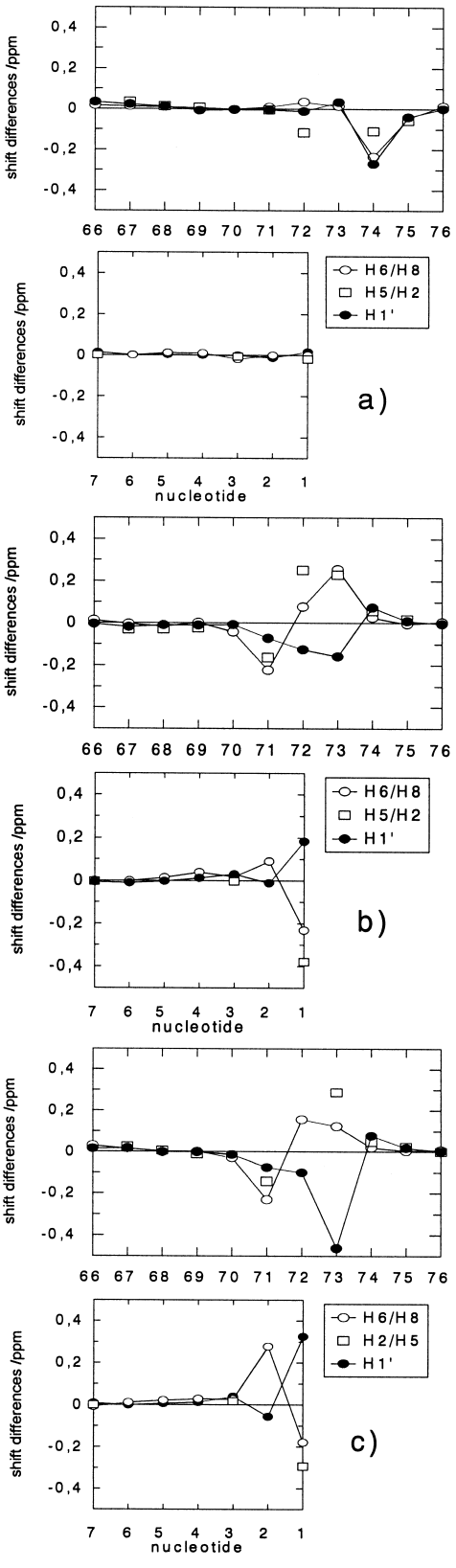

Fig. 6. Proton chemical shift differences between wild type C1-A72/A73 and variants of $tRNA^{fMet}$ acceptor stems ($\Delta\delta = \delta^{wild\ type} - \delta^{variant}$) for aromatic and anomeric protons: variants (a) C1-A72/G73, (b) U1-A72/A73, and (c) C1-G72/A73.

fMet-$tRNA^{fMet}$ complexed with MTF [28]. In the latter complex, C1 is rejected out of the helical stem by the action of loop 1 of the enzyme (remarkably, the base pairing of the second base pair G2-C71 is not affected) [28]. Whereas the degree of stacking between the bases in the single strand of the acceptor stem according to the structural model presented here does not differ much from that seen in the before-mentioned elongator tRNAs and MTF-complexed $tRNA^{fMet}$, the unusual inter-strand stacking between C1 and A73 causes a distinctly different orientation of the single-strand terminus as a whole (Fig. 7). Therefore, upon complexation a reorientation of the single-stranded end has to occur. Probably, the apparent lack of any interaction between C1 and A72 in conjunction with the absence of stacking of C1 upon bases of the preceding base pair G2-C71 favors an easy displacement of C1 out of the helix stem on the one hand, and of the single-strand terminus (A73–A76) as a whole on the other hand. In this way, optimal interaction with the contact regions of the MTF is enabled [28]. A certain degree of conformational flexibility, or deformability, may be required to warrant an induced fit, as considered for the case of the recognition of $tRNA^{Ala}$ by its cognate aminoacyl-tRNA synthetase [24]. More generally, a 'mutual accommodation' between the tRNA acceptor stem and the MTF binding surfaces may take place, in the manner discussed before by Draper [33]. An enhancement of the local conformational flexibility or deformability by introduction of variant mismatch pairs might be important also in other cases of tRNA-protein recognition. This can be exemplified by the $tRNA^{Phe}$ interaction with its cognate synthetase [34]. This recognition is dramatically deteriorated by the replacement of an original C2-G71 pair (which is no 'identity element') for a G2-C71 base pair. Possibly due to the increased local adaptability brought about by an additional variant G3-U70 pair the simultaneous introduction of the G2-C71 is tolerated. By this, an exact positioning of the terminal A76 at the correct aminoacylation site in the synthetase may be enabled which might not be feasible with the structurally more rigid G2-C71/G3-C70 combination.

The altered orientation of the single-strand terminus of $tRNA^{fMet}$ distinctly affects the relative position of the nucleotide A76 with reference to the body of the tRNA, which is obviously important for the interaction of charged elongator tRNAs with EF-Tu [35,36]. In this way, the unique C-A pair of $tRNA^{fMet}$ and the concomitant single-strand orientation could contribute essentially to the discrimination (or favor rejection) of fMet-$tRNA^{fMet}$ by EF-Tu.

In native tRNAs, a 5′-phosphate group at the 5′-terminal nucleotide 1 is present (see, e.g. [37]). In the chemically synthesized microhelices employed in our studies, this 5′-phosphate group is lacking. It could be argued that its absence might have a significant impact upon the arrangement of the nucleotides of the first mismatch base pair C1-A72. Though this cannot be completely excluded there is at least indirect evidence that makes this assumption seem less likely. Analysis of a variant of the $tRNA^{fMet}$ acceptor stem lacking the whole single-stranded end (data not shown) revealed that for the remaining nucleotides most of the inter-nucleotide NOE cross-peaks (including the strong, characteristic C1H5-A72H2 contact) are still present, though less intense than in the full acceptor stem. Though the presence of the single-strand terminus seems to exert a stabilizing influence, the arrangement of the C1-A72 mispair is vastly conserved. Fur-

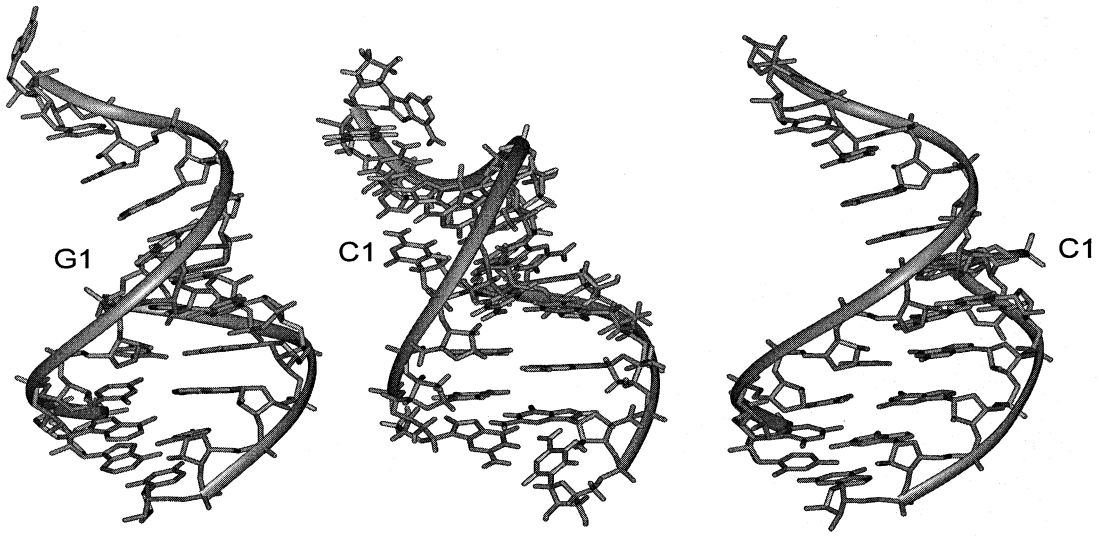

Fig. 7. Comparison of the sugar–phosphate backbones (ribbon representation) of the acceptor stems of the yeast $tRNA^{Asp}$ from X-ray analysis [32] (left), of the $tRNA^{fMet}$ (middle, present work), and of MTF-complexed $tRNA^{fMet}$ [28] (right).

thermore, we studied acceptor stems derived from $tRNA^{Ala}$ with single-strand extensions on the 5′-terminus of mature tRNAs serving as minimal substrates for RNase P (to be published). There, too, a C-A apposition arises by the nucleotides C-1 and A73 in the single-strand termini on the 5′- and 3′-sides of the precursor-tRNA microhelices, respectively. Though the conformational order in the 5′-extension of the acceptor stem is distinctly less than in the 3′-single strand nevertheless again a C1H5-A72H2 cross-peak (among others) was detected.

These findings suggest that the nucleotide arrangement derived for the before-described $tRNA^{fMet}$ C1-A72 mismatch seems to be generally adopted if neither the C nor the A in the mispair becomes protonated, independent of the absence or presence of phosphate groups (both free and in phosphodiester linkages).

In the case of the chemically synthesized G1-C72/U73 variant with the fold-back arrangement of the single-strand terminus (see Section 3.1), the lack of the 5′-phosphate at G1 does not seem to alter the overall conformation in comparison to the structure derived for a (T7 polymerase-synthesized) RNA sequence possessing such a 5′-phosphate [7]. In both studies very similar cross-peak patterns (and, accordingly, conformations of the acceptor stems) have been found. This again could corroborate the assumption that the 5′-phosphate does not have a significant impact on the arrangement of both the nucleotides in the first base pair, and the conformation of the single-strand terminus.

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