FEBS Letters 385 (1996) 15–20

NMR evidence for helix geometry modifications by a G–U wobble base pair in the acceptor arm of *E. coli* $tRNA^{Ala}$

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Received 19 February 1996

Abstract A ribooligonucleotide duplex representing the acceptor stem of *E. coli* RNA^{Ala} with a G3–U70 wobble base pair, which is the main identity element for the recognition by the alanine-tRNA synthetase, has been characterized by 2D-NMR, as having two sequence variants with a regular Watson-Crick G3–C70 and an I3–U70 wobble pair, respectively. As compared to a regular A-RNA, the G–U base pair gives rise to variations of the local helix geometry which are reflected in distinct local chemical shift changes. Structural differences between the duplex possessing an I3–U70 base pair and the wild-type G3–U70 sequence have also been found. The nucleotides in the ubiquitous single-stranded NCCA terminus display a surprisingly high degree of stacking order, especially between A73, C74, and C75.

Key words: NMR; RNA; Wobble base pair; Identity element; Helix geometry

1. Introduction

To fulfill their specific role in protein biosynthesis, tRNAs are aminoacylated by their aminoacyl-tRNA synthetases (ARS). For correct aminoacylation, the corresponding ARS has to discriminate between different tRNAs which are very similar with respect to secondary and tertiary structure. Hence, each tRNA must be equipped with distinct identity features, enabling the corresponding ARS to correctly recognize this tRNA. Often these identity elements are formed by single nucleotides or base pairs (for reviews, see [1,2]). Very frequently they are provided by the anticodon or are located in the acceptor stem. Probably the most extensively characterized and best-known example for the latter type is given by the G3–U70 wobble base pair in the $tRNA^{Ala}$ from *E. coli* and eukaryotes. It was demonstrated in different studies [3,4] that this G–U pair represents the major identity element of the $tRNA^{Ala}$ recognition by alanine-tRNA synthetase. This led Schimmel and coworkers to construct truncated tRNA molecules in which the G–U recognition element is still preserved. Indeed, such 'microhelices' consisting of only seven base pairs were shown to be efficiently aminoacylated with alanine [5].

If the G3–U70 base pair is replaced by G–C or I–U, no aminoacylation can be detected [6,7]. Likewise, no aminoacylation was observed if the G–U base pair was shifted by one position giving a G3–C70/G4–U69 duplex, demonstrating the importance of the specific location of the G–U wobble pair in the sequence.

McClain and coworkers were able to demonstrate by an in vivo experiment that suppressor $tRNA^{Ala}$ is aminoacylated by alanine-tRNA synthetase and reconstitutes bacterial growth on minimal medium if its anticodon matches an amber mutation in the *E. coli* TrpA-gene and it contains a mismatch base pair such as G–A, C–A or U–U at position 3–70 [4,8]. Aminoacylation was observed when suppressor $tRNA^{Ala}$ contained A3–U70/G4–U69 but not when it contained G3–C70/G4–U69.

These biochemical observations suggest an influence of the G–U base pair on the local structure of the acceptor stem helix. It could be assumed that the wobble pair in the acceptor stem of $tRNA^{Ala}$ induces specific modifications of the helix geometry in comparison to sequences containing regular Watson-Crick pairs. There are reports from NMR studies [9,10] that there is a pronounced stacking of the G–U base pair preferably to the 3′-side of the G and the 5′-side of the U, respectively. However, analysis of the available X-ray structural data [11], which suggest that the stacking of the G–U pair varies depending on the sequence context, calls for qualification of this general assertion of the NMR studies.

In the present work we studied the influence of the G3–U70 wobble pair on helical geometry in the acceptor stem of the $tRNA^{Ala}$ from *E. coli*. A short helix corresponding to the acceptor stem of this tRNA (Fig. 1) was prepared by chemical synthesis and its structure as derived from a 2D-NMR analysis was compared to mutant sequences containing, instead of the G3–U70 identity element, either a regular Watson-Crick G3–C70 or a wobble I3–U70 base pair.

2. Materials and methods

2.1. Oligoribonucleotide synthesis

The oligoribonucleotides were chemically synthesized on a Gene Assembler Plus (Pharmacia) by the H-phosphonate method as described previously [12]. The 5′- and 2′-hydroxy groups of the ribose were protected by 4,4′-dimethoxytrityl and *tert*-butyldimethylsilyl protecting groups, respectively, the exocyclic amines of purines by dimethylaminomethylene protection groups [12,13]. RNA synthons were synthesized as specified by Arnold et al. [13]. The oligoribonucleotides were purified by HPLC on a Vydac C4 column. The purity of the samples was analyzed by PAGE in the presence of 7 M urea [13].

For the 2D-NMR measurements 7–10 mg of RNA (single-strand concentrations ca. 2–3 mM) were dissolved in 0.5 ml D_2O buffer solution containing 100 mM NaCl, 10 mM sodium phosphate, pH 6.5. Small amounts of 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) served as an internal chemical shift reference.

RNA annealing was achieved by heating the samples to 80°C for 5 min and subsequently cooling to room temperature.

2.2. NMR spectroscopy

All NMR measurements were performed either at a proton NMR resonance frequency of 500 MHz on an AM500 spectrometer or at 400 MHz on an AMX400 spectrometer (both Bruker, Karlsruhe, Germany) using standard pulse sequences. 4K data points in t_2 (in the DQF-COSY spectra from which the scalar coupling constants

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PII S0014-5793(96)00339-0

were derived also 8K) and 512 t_1 increments were normally used in the phase-sensitive homonuclear proton 2D-NMR experiments, giving a 4K × 1K 2D-NMR spectrum after Fourier transform. Spectra were apodized by π/3-shifted squared sine functions (NOESY spectra) or π/16-shifted sine functions (COSY), in both dimensions. Fourier transforms were carried out on an ASPECT 3000 computer, a Hewlett-Packard HP9755 and SGI Iris Indigo workstation. Data processing and spectra analysis were performed using the NDEE program package developed by F. Herrmann (Bayreuth).

Usually spectral widths of 9 ppm (4504.5 Hz) with 500 MHz and of 10 ppm (4000 Hz) for 400 MHz experiments, and relaxation delays of 1.2 s were employed. Presaturation was used to suppress the residual HDO resonance peak.

DQF-^{1}H-^{1}H COSY spectra were recorded with ^{31}P broadband decoupling. Heteronuclear ^{1}H-^{13}C COSY was performed at a proton resonance frequency of 400 MHz employing a sweep width of 16000 Hz in the ^{13}C dimension. According to a $^1J_{CH}$ scalar coupling of 200 Hz the delay time has been set to 2.5 ms. NOESY spectra were recorded for mixing times of 80 ms, 200 ms, 300 ms and 400 ms.

3. Results

3.1. Resonance assignment of the acceptor stem duplex (18mer/GU)

The expected cross peak patterns for a (nearly) regular RNA duplex [14-17] were indeed observed in the NOESY spectra at large mixing times (≥300 ms). However, at short mixing time (80 ms) H1′–H6/H8 interactions were not detected at the available signal-to-noise ratio.

The imino resonances of the duplex containing a G3–U70 (Fig. 1) have been assigned previously [18]. In Fig. 2 the portion of the 400 MHz NOESY spectrum that contains the NOE cross peaks between H1′/H5 (5.0–6.2 ppm) and aromatic H8/H6/H2 proton resonances (7.0–8.5 ppm) is displayed. The assignment path for the pyrimidine-rich strand, comprising the nucleotides U66 through A76, is given. This connectivity extends even up to the end of the single-stranded terminus (A76). The assignment given in Fig. 2 was confirmed using a ^{1}H-^{13}C COSY experiment. Remarkably, the 2′ and 3′ ribose resonances (4.145 and 4.138 ppm, respectively) of U70, i.e. of the wobble pair nucleotide, appear at an unusually high field for a residue in the interior of a helical stem. Such values normally are typical for 3′-terminal nucleotides [19].

Of particular interest is the finding that there are several aromatic–aromatic H5–H5, H6–H5, H6–H6 NOE interactions between C71 and C72, and H5–H5, H6–H5 NOE contacts between C69 and U70, whereas there are none between U70 and C71 (cf. Fig. 3). Since the H6 and H5 chemical shifts of the bases U70 and C71 do not differ much (Fig. 2), the corresponding cross peaks would appear close to the diagonal and hence could not be identified conclusively. Interestingly, in the spectrum of the duplex containing 5-methyluridine at positions 6 and 70 (18mer/GU), there is no cross peak due to m^{5}U70H6–C71H6 interaction, which would be detectable because of the distinctly greater separation of the aromatic proton shifts from the 5-methyl substitution (data not shown).

A compilation of all observed inter-nucleotide NOE contacts is given in Fig. 3. Especially remarkable is the pronounced stacking between the two cytidines, C74 and C75, of the invariant single-stranded CCA terminus, and base stacking is even observed between C75 and the 3′-terminal A76.

The number of NOE connections permits a qualitative survey of the extent of stacking between the individual bases within each strand. The data are compatible with an A-RNA helix which is modified in the vicinity of the G3–U70 base pair. One-dimensional proton-decoupled ^{31}P NMR measurements revealed that all the ^{31}P resonances fall into a fairly narrow spectral region with a width of about 1.3 ppm (data not shown). This can be taken as an indication for a backbone geometry which is not too strongly distorted as compared to a regular A-RNA.

The COSY and DQF-COSY spectra show H1′–H2′ scalar couplings (indicative of partial population of 2′-endo sugar conformation [14-17]) for the nucleotide A7 (ca. 3.3 Hz), which is part of a terminal base pair, and A76, C75, C74 (4.8, 3.2, and 3.2 Hz, respectively), which are located in the 3′-single-stranded end.

To study the influence of the terminal A76 on the single-stranded ACCA end in more detail, a duplex lacking this nucleotide (17mer/GU) was synthesized and a NOESY spectrum under the same conditions as for the 18mer/GU (mixing time 300 ms, 303 K) was recorded. The lack of the A76 should affect the chemical shifts of the nearest neighbor C75 and its next-nearest neighbor C74 if A76 is at least partially stacked upon C75. As expected, noticeable downfield shifts of H5 and H6 resonances are only observed for the residues C75 and C74 (ca. 0.31 and 0.15 ppm for the H5 signals of C75 and C74, respectively, ≈0.20 and 0.14 ppm for the corresponding H6 resonances).

3.2. Acceptor stem duplex with a G3–C70 base pair (18mer/GC)

In order to study the effect of the G–U base pair on local helical geometry, we synthesized a duplex in which the G3–U70 pair (18mer/GU) was replaced by a regular G3–C70 Watson-Crick pair (18mer/GC). Such a duplex is not a substrate for the *E. coli* alanine-tRNA synthetase [6].

The chemical shift differences for aromatic and H1′ protons between 18mer/GU and 18mer/GC duplexes at 303 K are shown in Fig. 4A. A positive difference indicates an upfield shift of the respective resonance in the 18mer/GC as compared to the 18mer/GU. Whereas at both helix ends the shift alterations hardly exceed 0.03 ppm, there are dramatic differences

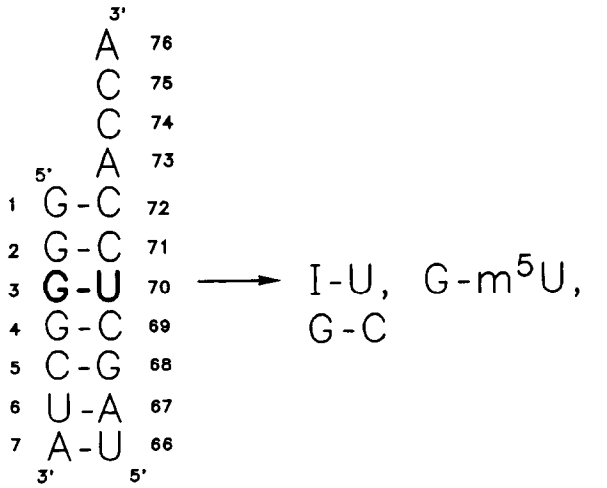

Fig. 1. Sequence of the acceptor stem duplex derived from the *E. coli* tRNA^{Ala} (18mer/GU). Heptamers with guanosine-3 and inosine-3, and undecamers with uridine-70, 5-methyl-uridine-70, and cytidine-70, respectively, have been prepared by chemical synthesis (see section 2) from which four duplexes have been assembled and studied by NMR.

around the 3–70 base pair. At positions 70 and 71 there are differences of more than 0.2 ppm for the H5 protons and more than 0.1 ppm for the H6 protons. It could be supposed, at least for position 70, that these alterations can be attributed to intrinsic chemical shift differences between cytidine and uridine. However, a detailed analysis of oligoribonucleotide chemical shifts [20] shows that theoretically a downfield shift of 0.166 ppm of the C70H5 proton should be expected as compared to the U70H5 proton, and only a minor upfield shift of 0.017 ppm for the C70H6 proton.

The difference between chemical shifts of the aromatic protons H6, H8, H5, and H2 in the disordered, unstacked state (for a temperature of 70°C; [20]) and in the helical duplex directly reflects the extent of shielding of each nucleotide due to the stacking in the duplex form [21]. The values of these shift differences for the H5 protons of U70 and C70 amount to +0.056 and +0.451 ppm, respectively. For the H5 protons of C71 in the 18mer/GU and the 18mer/GC, they are +0.201 and 0.484 ppm, respectively. These findings are clearly compatible with a better stacking between C70 and C71 in the 18mer/GC than between U70 and C71 in the 18mer/GU. Though the shielding strength of cytidine is to some extent better than that of uridine [22], it cannot completely account for the observed shift differences for the C71 between the 18mer/GC and the 18mer/GU duplexes.

3.3. *Acceptor stem duplex with an I3–U70 base pair (18mer/IU)*

The $tRNA^{Ala}$-derived acceptor stem duplex containing an I3–U70 base pair (18mer/IU) instead of the natural G3–U70 base pair (18mer/GU) is not aminoacylated by the Ala-tRNA synthetase [6]. This is remarkable since inosine and guanosine differ from each other by only one amino group which is absent in inosine. The 2-amino group of G3 is not involved in hydrogen bonding with U70. Instead, it points into the minor groove and can participate — along with the O2′ ribose

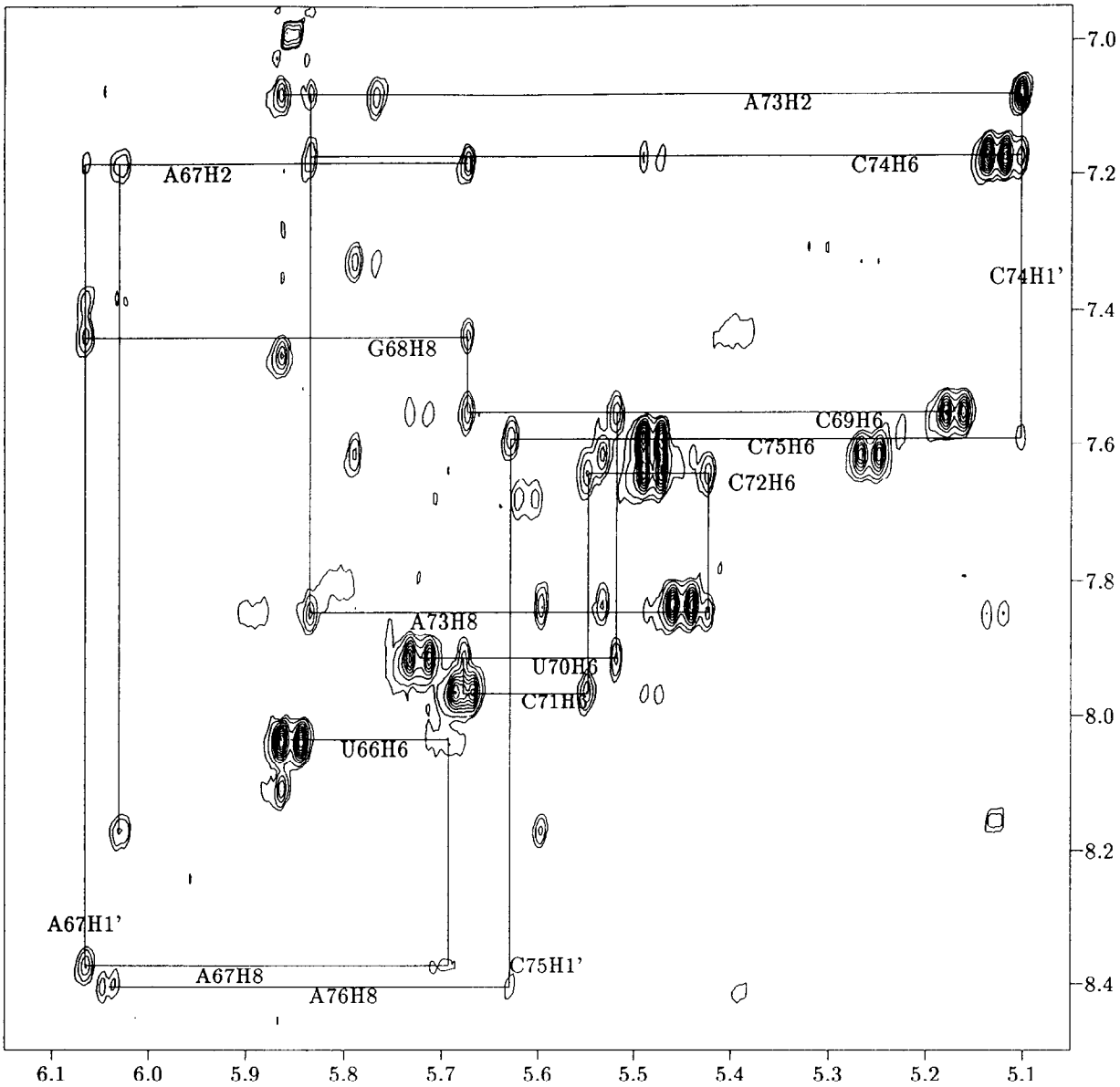

Fig. 2. H1′/H5–H6/H8/H2 portion of the 400 MHz NOESY spectrum; mixing time 300 ms; T=303 K. The assignment path for the 11-nucleotide (pyrimidine-rich) strand is indicated.

hydroxyl oxygen — in hydrogen bonding to a water molecule [23].

The question arises whether the lack of substrate properties of the 18mer/IU for the alanine-tRNA synthetase is a result of structural changes induced by the inosine, or simply due to the lack of the 2-amino group at inosine.

The differences between chemical shifts of aromatic and H1′ protons of 18mer/GU and 18mer/IU have been evaluated and are plotted in Fig. 4B. Obviously, there are significant deviations only on the short, purine-rich strand at positions 3 and 4. As to the strong downfield shifts of H8 and H1′ of I3, it must be kept in mind that there is already a difference in the intrinsic chemical shifts for 5′-GMP and 5′-IMP of −0.37 and −0.26 ppm, respectively [24], reducing the effective chemical shift alterations corrected for the intrinsic shift differences to ca. −0.09 ppm and +0.03 ppm, respectively, for the H8 and H1′ protons. To account for the shift alterations, especially concerning the H1′ and H8 resonances of G4, local structure variations, possibly associated with the lack of the stabilizing water molecule held between the amino group of guanosine and the 2′-OH group of uracil [23], have to be considered.

4. Discussion

To derive a high-precision structure a considerably larger number of reliable distance and dihedral angle restraints than could be deduced from our spectra is required. To this end, ^{13}C and ^{15}N labelling and heteronuclear three-dimensional NMR methods are of great use and for larger molecules (with more than ca. 20 nucleotides) probably even indispensable (cf. [25]). Nevertheless, the analysis of the chemical shift data of the original sequence and two sequence variants suggests differences in the stacking geometries between a 'regular' duplex (with G3–C70 base pair) and the G3–U70 duplex. The very least that can be inferred from a tentative relaxation matrix analysis and molecular dynamics calculation (on the basis of only 90 NOESY cross peaks which could be safely integrated) is an assumption of a destacking between the bases of U70 and C71.

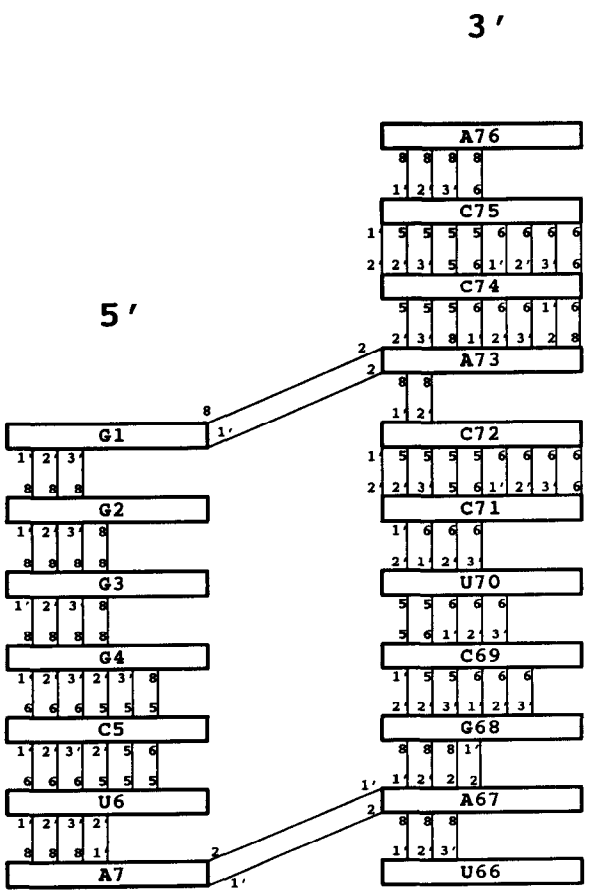

Fig. 3. Compilation of the observed inter-nucleotide NOESY contacts in the *E. coli* $tRNA^{Ala}$-derived acceptor stem duplex (18mer/GU) for mixing time of 300 ms at 303 K.

The helical secondary structure of RNA and DNA is characterized by regularly stacked bases. Here, chemical shift — especially of the aromatic protons — can be used to obtain additional information about local structural and conformational alterations of the regular helical geometry, since the main cause of the considerable (up to more than 1 ppm) shift differences between coiled (denatured) and ordered (helical duplex) states is the shielding effects of the stacked bases due to their strong ring currents [21,22,26]. The chemical shifts are rather sensitive to changes of the local geometry [21]. Hence, it makes sense to compare sequences which differ from each other at only one position, with respect to the chemical shift changes introduced by these replacements. Moreover, the existence or absence of certain cross peaks and the comparison of the intensities of cross peaks originating from equivalent interactions (e.g. H1′–H6/H8, etc.) can be used for structural evaluation, providing qualitative information about local deviations from the regular structure. Using this type of NOE information, compiled in Fig. 3, along with the chemical shift variations upon the base replacements (Fig. 4), a qualitative model of the duplex can be derived which already reflects the essential features of the molecular structure.

The experimental data suggest an essentially regular stacking compatible with A-RNA geometry, even for the four nucleotides in the single-stranded terminus. In particular, there is pronounced stacking between residues A73, C74 and C75. Even the last nucleotide, A76, seems to be at least partly stacked upon C75, though with a reduced degree of order due to its inherently greater conformational freedom. The assumption of an enhanced mobility of A76 is further supported by the detection of a relatively large scalar H1′–H2′ coupling of nearly 5 Hz, suggesting a considerable population of 2′-endo conformation. A certain amount of 2′-endo sugar puckering is also found for C75 and C74. Interestingly, this base stacking between C75 and A76 is removed upon interaction of the aminoacylated tRNA with certain proteins, such as EF-Tu [27] and aminoacyl-tRNA synthetases [28]. Moreover, the detection of an A73H2–G1H8 cross peak suggests a remarkable degree of inter-strand stacking of A73 over G1 that can explain the significant duplex stabilization effect of even a single dangling 3′-adenine over a terminal G–C base pair [18,29]. Such a displacement of the A73 base towards the G1 of the opposite strand also accounts for the lack of the C72H6–A73H8 cross peak. Additionally, the difference of the chemical shifts (H8, H1′, H2′, H3′) for G1 between 18mer/GU at 303 K and 276 K is smaller than 0.02 ppm, whereas it is distinctly greater for the other terminal nucleotides (up to 0.10 ppm).

The marked upfield shift of H5 and H6 proton resonances of C71 and C70 in the 18mer/GC duplex can be explained in

terms of a better stacking (i.e. increased base plane overlap) between C70 and C71 as compared to C71/U70 in the 18mer/GU duplex. A similar behaviour was also observed for another duplex variant with a regular A3–U70 base pair (data not shown) where the U70 H5 proton resonates ca. 0.34 ppm upfield from the resonance position of the same proton in the G3–U70 duplex.

Thus, the structure of the 18mer/GU can be approximately visualized by a distortion of the regular A-RNA helix, assumed to be adopted by the 18mer/GC duplex, in such a way that the base C71 is displaced in the 18mer/GU duplex from the position which it occupies in the regular 18mer/GC helix.

The helix is possibly flexible and/or deformable at the U70 site, thus creating a favourable interaction locus for the alanine-specific aminoacyl-tRNA synthetase (ARS). Probably, the structural discontinuity/irregularity introduced by U70 at this location is recognized by the cognate ARS [8]. This has been suggested by McClain et al. [8] who extensively investigated the contribution of the G3–U70 base pair to the acceptor identity of *E. coli* $tRNA^{Ala}$ using a large number of synthetic amber suppressor tRNAs in *E. coli*. From these experiments it was concluded that the G3–U70 pair induces an irregularity in the helix structure which forms the most important recognition feature for the cognate synthetase. In particular, other wobble base pairs at the same location as, e.g. G–A, A–C, C–A, which likewise should give rise to modifications of the local helical geometry, did not significantly affect the acceptor identity in $tRNA^{Ala}$ [8].

Since G–U base pairs are capable in certain sequence contexts of creating specific binding sites for divalent cations, in particular manganese [18,30,31], it could be asked whether these binding sights play a role in the recognition by the ARS. The helix geometry alterations introduced by the G–U pair could favour the specific manganese ion binding.

The relative changes of the helix geometry in the 18mer/IU duplex in comparison to the wild-type 18mer/GU molecule seem — at least on the pyrimidine-rich 11mer strand — to be less pronounced than the differences between the 18mer/GC variant and the wild-type G–U duplex. However, clear upfield shift changes still remain for the H8 and H1′ resonances of G4, which are all the more remarkable as the shielding effect of the preceding inosine-3 is less than that of a guanosine-3 [32]. Accordingly, downfield shifts for the G4 signals would instead be expected if there were no structural alterations. These structural alterations in the 18mer/IU duplex could be associated with a water molecule hydrogen-bonded between the 2-amino group of G3 and the ribose O2′ of U70 [23], which might also be of importance in the recognition of $tRNA^{Ala}$ by Ala-tRNA synthetase, as has been deduced from systematic aminoacylation studies of several $microhelix^{Ala}$ variants. It may also be that the structural modifications induced by the G–U pair serve to position the 2-amino group of G3 in such a way as to ensure optimum interaction with (and hence recognition by) specific groups of the synthetase.

Acknowledgements: We thank Drs. P. Rösch and A. Ejchart (Bayreuth) for kind support with their NMR equipment, and Drs. W.H. McClain (Madison, Wisconsin) and P. Bayer (Bayreuth) for discussions. This work was supported by the Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 213 (Projekt D4).

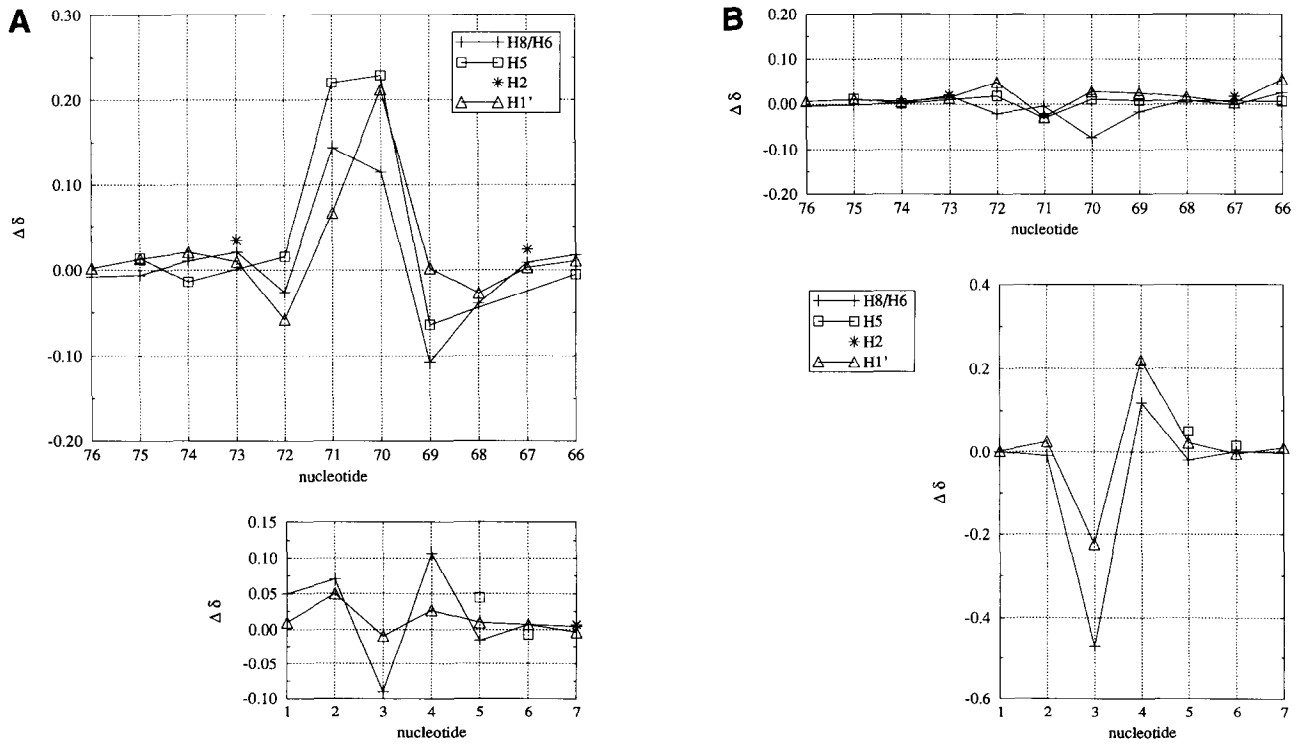

Fig. 4. (A) Differences [Δδ (in ppm)=δ(18mer/GU)−δ(18mer/GC)] between the chemical shifts in the 18mer/GU and the 18mer/GC duplex for aromatic and H1′ proton resonances at T=303 K, given separately for both strands. (B) Differences [Δδ (in ppm)=δ(18mer/GU)−δ(18mer/IU)] of the aromatic and H1′ proton chemical shifts between 18mer/GU and 18mer/IU duplexes at 303 K. Correction for the intrinsic chemical shift difference yields values of −0.09 ppm for the I3/G3H8 resonance and +0.03 ppm for the G3/I3H1′ resonance (see text).

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