Sequence Dependence of Stability for Coaxial Stacking of RNA Helixes with Watson–Crick Base Paired Interfaces†

Amy E. Walter and Douglas H. Turner*

Department of Chemistry, University of Rochester, Rochester, New York 14627-0216

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ABSTRACT: Thermodynamic parameters from UV melting studies are reported for the helix–helix interfaces of coaxially stacked helixes in RNA. The model system consists of a short oligomer binding to a four-nucleotide overhang at the end of a hairpin stem, creating the helix–helix interface. Interfaces containing Watson–Crick base pairs are approximately 1 kcal/mol more stable than the corresponding nearest neighbor interaction in an uninterrupted helix. Thus the sequence dependence of stability for coaxially stacked interfaces is similar to that for regular helixes. This provides experimental evidence for an assumption that has been shown to improve predictions of RNA secondary structure [Walter, A. E., Turner, D. H., Kim, J., Lyttle, M. H., Muller, P., Mathews, D. H., & Zuker, M. (1994) *Proc. Natl. Acad. Sci. U.S.A.* (in press)]. The results should also be useful for modeling three-dimensional structures of RNA.

Interest in RNA has grown enormously in recent years because of the many functions of RNA that have been discovered (Watson et al., 1987). The functions of RNA are dependent upon the structure of RNA. RNA structure prediction has improved greatly in the last few years (Turner et al., 1988; Jaeger et al., 1989; Walter et al., 1994), due largely to improved thermodynamic parameters (Freier et al., 1986a; Turner et al., 1987). There is little knowledge, however, of the interactions that stabilize multibranched loops, so predictions of these loops are relatively poor (Zuker et al., 1991). As more information is acquired about multibranched loops, predictions should improve.

One common motif in multibranched loops is coaxial stacking of helixes. This motif is seen in crystal structures of tRNAs (Kim et al., 1974; Robertus et al., 1974; Westhof et al., 1985; Biou et al., 1994) and is essential for the three-dimensional shape of tRNA. Coaxial stacking of helixes is also proposed in modeling studies of group I introns (Kim & Cech, 1987; Michel & Westhof, 1990) and rRNA (Gutell et al., 1994; Laing & Draper, 1994). An NMR study of a model system for adjacent helixes in a group I intron has shown coaxial stacking (Chastain & Tinoco, 1992).

It has been shown previously that a model system composed of an oligomer binding to a four-nucleotide overhang on a hairpin stem can provide thermodynamic parameters for helix–helix interfaces (Walter et al., 1994). Here this model system is used to explore the sequence dependence of stability for interfaces with Watson–Crick base pairs.

MATERIALS AND METHODS

Oligoribonucleotides. RNA oligomers were synthesized on an Applied Biosystems DNA/RNA 392 synthesizer using 2′-*tert*-butyldimethylsilyl-protected RNA phosphoramidites that were synthesized in our lab and had the base-protecting groups phenoxyacetyl for A and G and acetyl for C (Usman et al., 1987; Ogilvie et al., 1988; Wu et al., 1989). Oligomers were deblocked in 3:1, v/v, ammonia/ethanol for 3 h at 55 °C and then overnight at room temperature. The 2′-*tert*-butyldimethylsilyl groups were removed by adding freshly made 1 M triethylammonium hydrogen fluoride in pyridine and incubating the reaction mixture for 48 h at 55 °C. Oligoribonucleotides were desalted with Sep-pak C-18 cartridges (Waters). Hairpins were purified on denaturing 20% polyacrylamide gels. Bands were visualized under ultraviolet light, and the least mobile band was cut out and eluted by a crush and soak procedure. The hairpin was ethanol precipitated and further desalted with Sep-pak cartridges (Waters). Purity was checked by 5′-^{32}P-labeling with T4 polynucleotide kinase and was >95%. Short oligomers were purified by thin-layer chromatography (Baker Si500F plates) in 55:35:10, v/v/v, *n*-propanol/ammonium hydroxide/water (Chou et al., 1989). Final purification and desalting of the oligomers were performed by use of a Sep-pak cartridge. Purity was checked by HPLC on a C8 RP column (Beckman) and was >95%.

Melting Experiments. The buffer used for melting experiments was 1 M NaCl, 10 mM sodium cacodylate, and 0.5 mM Na_2EDTA. Absorbance versus temperature curves were measured at 280 nm on a Gilford 250 spectrophotometer with a heating rate of 1 °C/min. Melting curves were analyzed by fitting to a two-state model with sloping base lines with a nonlinear least-squares program (Petersheim & Turner, 1983a). Thermodynamic parameters were obtained by two methods. In one method, enthalpy and entropy changes obtained from fitted curves were averaged. In the second method, plots of the concentration dependence of the inverse melting temperature were fit to a straight line (Borer et al., 1974):

$$T_M^{-1} = (2.3R/\Delta H^\circ)\log(C_T/4) + \Delta S^\circ/\Delta H^\circ \quad (1)$$

RESULTS

Thermodynamic parameters for different coaxial stacking interfaces are determined by using a model system where a short oligomer binds to a four-nucleotide overhang at the 5′ end of a hairpin stem as shown in Figure 1. This forms a short helix that is adjacent to the preexisting hairpin stem helix, thus allowing coaxial stacking. The hairpin is designed to be stable below 80 °C, and the parameters are derived from the

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* Author to whom correspondence should be addressed.

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1) 5'GGACCAGUGGC^{G}C + 5'GUCC3' ⇌ 5'GGAC-CAGUGGC^{G}C
3'GUCACCG$_{A}$A 3'CCUG/GUCACCG$_{A}$A

2) 5'GGAGGAGUGGC^{G}C + 5'CUCC3' ⇌ 5'GGAG-GAGUGGC^{G}C
3'CUCACCG$_{A}$A 3'CCUC/CUCACCG$_{A}$A

3) 5'GGUGUGAGUGGC^{G}C + 5'CACC3' ⇌ 5'GGUG-UGAGUGGC^{G}C
3'ACUCACCG$_{A}$A 3'CCAC/ACUCACCG$_{A}$A

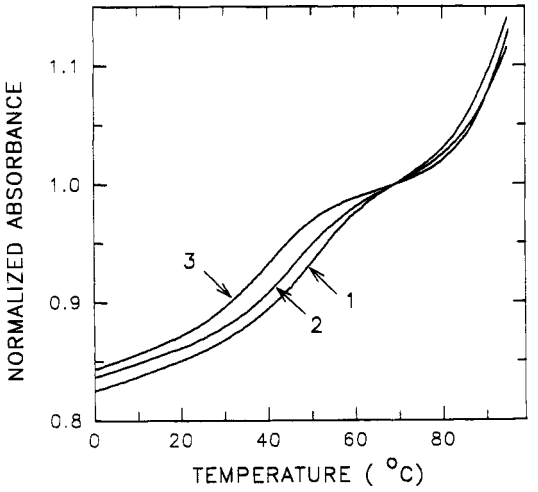

FIGURE 1: Typical melting curves. Absorbances were normalized by dividing by the absorbance at 69 °C. Sequences are shown above the curves. The first transition of each curve represents melting of the short oligomer from the hairpin. This transition is fit to obtain the parameters of coaxial stacking. The second transition is the melting of the hairpin, which has a T_M greater than 100 °C. The concentrations and T_M values for the curves are as follows: 1.11×10^{-4} M and 46.7 °C for curve 1, 9.96×10^{-5} M and 42.5 °C for curve 2, and 1.05×10^{-4} M and 38.7 °C for curve 3.

first transition in the melting curve which relects binding of the short oligomer to the 5′ overhang of the hairpin (Figure 1). In three cases, the short oligomer is a pentamer instead of a tetramer to increase the T_M of the first transition. The thermodynamic parameters from T_M^{-1} versus log (C_T/4) plots and from fitted melting curves are recorded in Table 1. T_M^{-1} versus log(C_T/4) plots are shown in Figure 2. The enthalpy changes obtained from these plots and curve fitting agree within 15%, consistent with the two-state model (Freier et al., 1983; Petersheim & Turner, 1983a). Thermodynamic increments for coaxial stacking are calculated from equations equivalent to $\Delta\Delta G°_{37} = \Delta G°_{37}$(hairpin + short oligomer) − $\Delta G°_{37}$(short oligomer duplex without the adjacent helix).

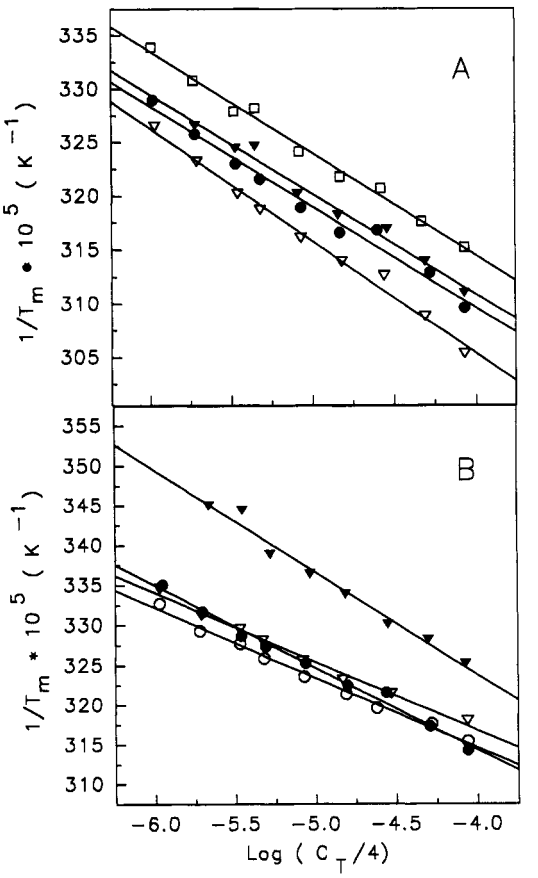

FIGURE 2: Plots of inverse melting temperature versus log concentration for (A) 5'GGAG-G-CCUC/C- (●), 5'GGAC-C-CCUG/G- (△), 5'GGAG-A-CCUC/U- (▲), and 5'GGUG-U-CCAC/A- (□) and for (B) 5'GGUA-G-ACCAU/C- (○), 5'GGAU-G-ACCUA/C- (●), 5'GGAU-A-ACCUA/U- (△), and 5'GGAC-G-CCUG/C- (▲).

These are listed in Table 2. For these calculations, the $\Delta H°$, $\Delta S°$, and $\Delta G°_{37}$ values for formation of the short oligomer duplex are calculated from nearest neighbor parameters (Freier et al., 1986a), since the T_M values of these oligomers are too low to measure accurately.

DISCUSSION

Coaxial stacking of helixes is thought to be a common motif in RNA structure (Gutell et al., 1994; Kim & Cech, 1987; Michel & Westhof, 1990). A recent study showed that

Table 1: Thermodynamic Parameters for Binding to Hairpins[a]

RNA duplex	T_M^{-1} vs log(C_T/4) $-\Delta H°$ (kcal/mol)	$-\Delta S°$ (cal/molK)	$-\Delta G°_{37}$ (kcal/mol)	T_M @ 10^{-4} M (°C)	curve fit $-\Delta H°$ (kcal/mol)	$-\Delta S°$ (cal/molK)	$-\Delta G°_{37}$ (kcal/mol)	T_M @ 10^{-4} M (°C)
5'GGAG-C-*b* CCUC/G-	43.27 ± 1	114.36 ± 3	7.80 ± 0.02	46.4	46.23 ± 2	123.73 ± 6	7.86 ± 0.10	46.2
5'GGAG-G-CCUC/C-	48.49 ± 2	131.82 ± 8	7.61 ± 0.05	44.0	44.23 ± 3	118.21 ± 9	7.57 ± 0.18	44.4
5'GGAC-C-CCUG/G-	43.64 ± 2	114.96 ± 5	7.99 ± 0.04	47.7	40.99 ± 2	106.59 ± 5	7.93 ± 0.11	48.0
5'GGAG-A-CCUC/U-	49.12 ± 3	134.41 ± 9	7.44 ± 0.05	42.8	50.66 ± 4	139.34 ± 11	7.44 ± 0.12	42.7
5'GGUG-U-CCAC/A-	48.03 ± 2	132.73 ± 6	6.86 ± 0.03	39.2	46.00 ± 3	126.08 ± 10	6.89 ± 0.08	39.5
5'GGAC-G-*b* CCUG/C-	42.70 ± 1	115.34 ± 4	6.92 ± 0.03	39.9	38.16 ± 2	100.47 ± 7	7.00 ± 0.16	40.9
5'GGUA-G-ACCAU/C-	52.01 ± 2	145.32 ± 5	6.94 ± 0.03	39.5	44.44 ± 2	120.85 ± 6	6.96 ± 0.17	40.0
5'GGAU-G-ACCUA/C-	44.54 ± 2	121.72 ± 6	6.79 ± 0.04	38.8	43.07 ± 3	117.04 ± 9	6.77 ± 0.09	38.7
5'GGAU-A-ACCUA/U-	52.99 ± 2	149.55 ± 7	6.61 ± 0.04	37.4	44.97 ± 2	123.36 ± 5	6.71 ± 0.16	38.2
5'GGAC/G-CCUG-C-	35.74 ± 2	97.43 ± 7	5.52 ± 0.09	28.5	32.53 ± 3	86.50 ± 11	5.70 ± 0.19	29.3

[a] Melting buffer is 1 M NaCl, 10 mM sodium cacodylate, and 0.5 mM EDTA, pH 7. Significant figures are given beyond error estimates to allow accurate calculation of T_M and other parameters. [b] Walter et al. (1994).

Table 2: Thermodynamic Parameters of Coaxial Stacking[a]

RNA duplex	coaxial stack $\Delta\Delta H°$ (kcal/mol)	coaxial stack $\Delta\Delta S°$ (cal/(mol K))	coaxial stack $\Delta\Delta G°_{37}$ (kcal/mol)
5'GGAG-C CCUC/G-	−10.2 ± 1.2	−19.2 ± 3.9	−4.3 ± 0.2
5'GGAG-G CCUC/C-	−15.4 ± 2.6	−36.6 ± 8.3	−4.1 ± 0.2
5'GGAC-C CCUG/G-	−7.9 ± 1.8	−12.8 ± 5.8	−4.1 ± 0.2
5'GGAG-A CCUC/U-	−16.0 ± 2.8	−39.2 ± 9.0	−3.9 ± 0.2
5'GGUG-U CCAC/A-	−15.1 ± 2.0	−38.2 ± 6.5	−3.5 ± 0.2
5'GGAC-G CCUG/C-	−7.0 ± 1.5	−13.1 ± 4.9	−3.0 ± 0.2
5'GGUA-G ACCAU/C-	−12.5 ± 2.1	−32.6 ± 6.8	−2.5 ± 0.2
5'GGAU-G ACCUA/C-	−4.3 ± 2.1	−6.8 ± 7.0	−2.4 ± 0.2
5'GGAU-A ACCUA/U-	−12.8 ± 2.5	−34.6 ± 8.2	−2.2 ± 0.2
5'GGAC/G CCUG-C-	0.0 ± 2.3	+4.8 ± 7.6	−1.6 ± 0.2 (−3.3)[b]

[a] Calculations were done by using equations equivalent to $\Delta\Delta G°_{37} = \Delta G°_{37}$(hairpin + short oligomer) − $\Delta G°_{37}$(short oligomer duplex without adjacent helix) and are based on results from T_M^{-1} versus $\log(C_T/4)$ plots. Predicted $\Delta H°$, $\Delta S°$, and $\Delta G°_{37}$ values were used for short oligomer duplexes (Freier et al., 1986a). [b] The value in parentheses is a corrected $\Delta\Delta G°_{37}$ where the value −1.7 kcal/mol for $^{G}_{3'GC}$ (Turner et al., 1988) is added to correct for the 3' G stacking on the hairpin stem.

oligomers bind to a 5' overhang at the base of a hairpin stem much more tightly than expected from the number of base pairs formed (Walter et al., 1994), presumably due to coaxial stacking. This provides a model system for studying the sequence dependence of coaxial stacking. In the previous study, the effect of changing the interface from $^{5'C\text{-}G}_{G/C}$ to $^{5'G\text{-}C}_{C/G}$ and of adding nucleotides adjacent to the interface, i.e.,

5'C-G
G/C
X Z

was measured (Walter et al., 1994). Changing the interface from $^{5'C\text{-}G}_{G/C}$ to $^{5'G\text{-}C}_{C/G}$ provided the largest sequence-dependent effect, suggesting that the base pairs at the interface are a major determinant of the thermodynamic stability of a coaxial stack. Here we report thermodynamic parameters for seven additional sequences at the interface.

Thermodynamic increments for the interfaces studied are listed in Table 2. The effect of the position of the break was tested by comparing $^{5'G\text{-}G}_{C/G}$ with $^{5'C\text{-}C}_{G/G}$. Both give the same $\Delta\Delta G°_{37}$, suggesting that the interaction of nearest neighbor base pairs is the primary determinant of the free energy increment. In Figure 3, the increments for coaxial stacking

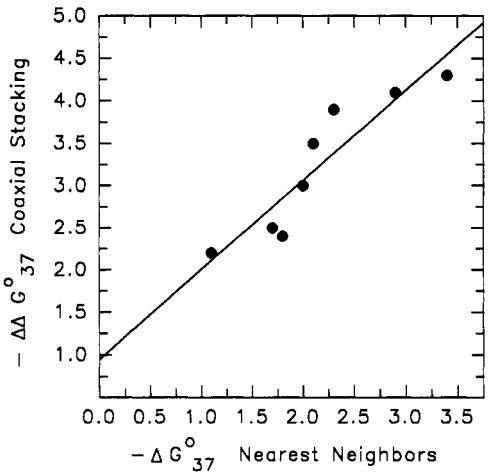

FIGURE 3: Plot of free energy increment for coaxial stacking versus free energy increment for nearest neighbor interactions in a continuous helix (Freier et al., 1986a). Each filled circle represents one Watson–Crick base paired interface.

are plotted versus the free energy increments for the equivalent nearest neighbor base pairs in an intact helix (Freier et al., 1986a). A monotonic increase is observed, except for $^{5'A\text{-}G}_{U/C}$ and $^{5'U\text{-}G}_{A/C}$ interfaces, which differ by only 0.1 kcal/mol. Thus coaxial stacking follows essentially the same sequence dependence as Watson–Crick base pairing in a continuous helix. The free energy increments for the interfaces range from 0.6 to 1.6 kcal/mol more favorable than for the equivalent nearest neighbor base pairs in a regular helix. On average, this difference in stability is 1.1 kcal/mol. Previous results suggest that extending both RNA chains after the interface

5'C-G
G/C
X Z

will make the interface less favorable by about 1 kcal/mol (Walter et al., 1994). Thus the data suggest that the nearest neighbor free energy increment for a regular helix is a reasonable approximation for the free energy increment for a coaxially stacked interface in a long RNA chain. A similar approximation has been shown to improve predictions of RNA secondary structure (Walter et al., 1994). The results in Table 2 provide an experimental foundation for this approximation.

For one sequence, $^{5'GGAC/G\text{-}}_{CCUG\text{-}C\text{-}}$, the overhang is at the 3' end of the hairpin stem. The $\Delta\Delta G°_{37}$ for this interface is −1.6 kcal/mol, compared with −3.0 kcal/mol for the same interface

Table 3: Occurrences of Coaxial Stacking Interfaces Composed of Watson–Crick Pairs in Phylogenetic Structures of 36 Large Subunit[a] and 21 Small Subunit[b] Ribosomal RNAs, 455 tRNAs,[c] and 87 group I introns[d]

interfaces	5'C-C G/G	5'U-G A/C	5'C-A G/U	5'C-G G/C	5'G-U C/A	5'U-A A/U	5'C-U G/A	5'G-C C/G
occurrences								
large subunit rRNA	22	79	54	62	36	26	8	27
small subunit rRNA	19	1	3	1	6	3	2	6
tRNA (acceptor/T stem)	73	1	30	8	21	34	41	14
group I intron (P4/P6)	0	7	0	0	0	0	0	0
total	114	88	87	71	63	63	51	47

interfaces	5'A-G U/C	5'U-C A/G	5'A-C U/G	5'G-G C/C	5'U-U A/A	5'G-A C/U	5'A-A U/U	5'A-U U/A
occurrences								
large subunit rRNA	5	14	34	15	4	14	8	2
small subunit rRNA	19	3	5	24	0	2	1	5
tRNA (acceptor/T stem)	0	22	4	2	21	5	5	4
group I intron (P4/P6)	21	5	0	0	2	0	3	6
total	45	44	43	41	27	21	17	17

[a] Gutell et al. (1992). [b] Gutell et al. (1985). [c] The adjacent base pairs at the interface of the amino acid acceptor and T stems are tabulated (Sprinzl et al., 1989). [d] The adjacent base pairs at the interface of the P4 and P5 stems are tabulated (Michel & Westhof, 1990).

formed with a 5′ overhang (Table 2). It is known that 5′ dangling ends contribute little to the stability of a double helix but that 3′ dangling ends can contibute almost as much stability as a base pair (Turner et al., 1988). This is thought to be because 5′ dangling ends do not interact with the opposite strand, whereas stabilizing 3′ dangling ends stack on the opposite strand (Petersheim & Turner, 1983a,b; Freier et al., 1985, 1986b; Turner et al., 1988). Thus the first nucleotide of the 3′ overhang is expected to stack on the adjacent base pair of the hairpin stem. If the 3′ overhang stacked in the appropriate conformation to form a helix with an oligomer and to coaxially stack with the adjacent hairpin stem, then binding to the 3′ overhang should be at least as favorable as binding to the 5′ overhang, if not more favorable. The opposite is observed. This suggests that the 3′ overhang is not preorganized in the conformation required for coaxial stacking of the helixes at the interface. Interestingly, the free energy increment for stacking of the first nucleotide of the 3′ overhang on the adjacent base pair, $_{3'\frac{G}{GC}}$, is −1.7 kcal/mol (Turner et al., 1988). If binding of the oligomer required breaking this stack, then the measured $\Delta\Delta G°_{37}$ would be the sum of the favorable free energy of forming the coaxial stack and the unfavorable free energy for breaking the 3′ dangling end stack. Thus the intrinsic free energy increment for the coaxial stack would be −1.6 + (−1.7) = −3.3 kcal/mol, close to the measured value for the 5′ overhang. The results suggest that stacking conformations are different for the free and base-paired 3′ overhangs.

Zhong and Kallenbach (1993) studied the effect on stability of introducing a nick in one strand of a DNA oligomer duplex. They report that the total free energy change for forming the three-strand complex at 20 °C is 8 kcal/mol less favorable than that for forming the two-strand complex. Presumably, some of this unfavorable free energy results from the fact that a three-strand complex requires two helix initiations. The $\Delta G°$ for a helix initiation at 20 °C can be estimated as 3.2 kcal/mol for RNA (Freier et al., 1986a) and 4.9 kcal/mol for DNA (Breslauer et al., 1986). This suggests that coaxial stacking in DNA may be unfavorable. This is opposite our result for RNA.

Phylogenetic structures from 36 large subunit (Gutell et al., 1992) and 21 small subunit (Gutell et al., 1985) ribosomal RNAs, 455 tRNAs (Sprinzl et al., 1989), and 87 group I introns (Michel & Westhof, 1990) were studied to determine the possibilities of coaxial stacking interfaces in nature. In tRNA the acceptor stem and the T stem are known to be coaxially stacked in the crystal structure (Kim et al., 1974; Robertus et al., 1974; Westhof et al., 1985; Biou et al., 1994). In group I introns the P4 and P6 helixes are thought to be coaxially stacked (Michel & Westhof, 1990; Kim & Cech, 1987). The occurrences of the 16 possible combinations of Watson–Crick base pairs at helix–helix interfaces are listed in Table 3. Comparison with Table 2 shows that the interfaces studied in this paper are representative of those found in nature.

Often in phylogenetic secondary structures, three helixes are adjacent, thus providing two possibilities for coaxial stacking. It is possible that thermodynamics will determine which of the two possibilities will be chosen. If so, then the results in Table 2 will also aid prediction of three-dimensional structure. For example, one RNA where three helixes converge with no intervening unpaired nucleotides is *Thermus thermophilus* serine tRNA. The crystal structure of this tRNA bound to its synthetase has recently been reported (Biou et al., 1994). The two possible interfaces for coaxial stacking are $^{5'G\text{-}A}_{C/U}$ and $^{5'U\text{-}A}_{A/U}$. In the crystal structure, the coaxial stacking occurs at the $^{5'G\text{-}A}_{C/U}$ interface. On the basis of the results in Table 2, the $^{5'G\text{-}A}_{C/U}$ interface is 1.7 kcal/mol more favorable than the $^{5'U\text{-}A}_{A/U}$ interface. Thus the three-dimensional structure of this tRNA is consistent with the suggestion that the thermodynamics of coaxial stacking determines the choice of helix stacking. This case of three helixes converging with adjacent Watson–Crick pairs is not found in the 455 other phylogenetic structures of tRNA (Sprinzl et al., 1989) examined, however. Thus definitive tests of this hypothesis must await determination of three-dimensional structures for other RNAs. It is quite possible that the thermodynamics of base triples (Michel & Westhof, 1990) and other tertiary interactions will be as important as coaxial stacking of base pairs for determining choice of helix stacking, if not more important.

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