VOLUME 119, NUMBER 36
SEPTEMBER 10, 1997

JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

Atomic Group "Mutagenesis" Reveals Major Groove Fine Interactions of a tRNA Synthetase with an RNA Helix

Penny J. Beuning, Miriam Gulotta,† and Karin Musier-Forsyth*

Contribution from the University of Minnesota, Department of Chemistry, Minneapolis, Minnesota 55455

Received March 31, 1997[⊗]

Abstract: RNA discrimination by aminoacyl-tRNA synthetases involves both major and minor groove interactions with the acceptor stem domain of tRNA substrates. In the case of class II *Escherichia coli* alanyl-tRNA synthetase (AlaRS), minor groove atomic groups in and around the unique G3:U70 base pair previously have been shown to be critical for recognition. In this work, we probe the role of the first (1:72) base pair in discrimination by AlaRS by incorporating 26 new base pair combinations at this site. We find that atomic groups in the wild-type G1:C72 base pair do not contribute as significantly to positive recognition by AlaRS as the minor groove elements in and around the G:U base pair. Our results, however, are consistent with the importance of major groove discrimination at this site. In particular, substrates with a major groove carbonyl oxygen presented by either a G or a U at position 72 are very poor alanine acceptors. Comparison of inactive N1:G72 duplex^{Ala} variants with active N1:2-aminopurine72 variants shows that deletion of the 6-keto oxygen and the N1-hydrogen of G72 results in a transition state stabilization of at least 3.0 kcal/mol. This work provides an example of a system that combines minor groove interactions at an internal position with the high selectivity of major groove interactions that are possible at the end of an RNA helix.

Introduction

In RNA A-form helices, the minor groove is much more accessible to protein side chains than the deep, narrow major groove. For this reason, it has been proposed that sequence-specific interactions between proteins and RNA are more likely to occur in the minor groove.[1] In accordance with this expectation are the well-documented interactions of class I *Escherichia coli* glutaminyl-tRNA synthetase and class II *E. coli* alanyl-tRNA synthetase (AlaRS) with minor groove functional groups in and around the 3:70 base pair of their cognate tRNA substrates.[2−5] In all the co-crystal structures of class II synthetases reported to date, it has been observed that these enzymes approach the top of the tRNA acceptor stem from the major groove side.[6−9] As seen clearly in the yeast aspartyl-tRNA synthetase (AspRS) system, this enzyme gains access to normally less-accessible major groove functional groups at the

* To whom correspondence should be addressed: Karin Musier-Forsyth, University of Minnesota, Department of Chemistry, 207 Pleasant Street S.E., Minneapolis, MN 55455. Telephone: 612-624-0286. Fax: 612-626-7541. E-mail: musier@chemsun.chem.umn.edu.

† Current address: Department of Physics, City College of the City University of New York, New York, New York 10031.

⊗ Abstract published in *Advance ACS Abstracts*, September 1, 1997.

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S0002-7863(97)01020-2 CCC: $14.00

end of the RNA helix.[7,10] Major groove interactions are advantageous in nucleic acid discrimination, since the patterns of hydrogen bond donors and acceptors in the major groove of base pairs are more diverse than those in the minor groove.[11] By analogy to class II AspRS, we wished to understand whether a class II synthetase where minor groove interactions are prominent at an internal position, such as AlaRS, can also gain access to the major groove at the end of the helix.

In the work reported here, we performed the complete set of 16 standard nucleotide base pair substitutions at the terminal acceptor stem position of chemically synthesized duplex^{Ala} substrates and tested the effect of these substitutions on aminoacylation by *E. coli* AlaRS. We also performed an atomic "mutagenesis" study by deleting specific major and minor groove functional groups in G1:C72- and C1:G72-duplex^{Ala} variants. Seventeen nonstandard base pair combinations were prepared at 1:72 in duplex^{Ala}, and we examined the effects of these changes on discrimination by AlaRS. We determined the thermal stability of active and inactive 1:72 duplex^{Ala} variants and also compared their circular dichroism (CD) spectra.

Our analysis suggests that the principle of major groove discrimination at the end of an RNA helix revealed in the AspRS system may be extended to other class II synthetases. In particular, AlaRS achieves an extraordinary degree of overall discrimination by combining minor groove interactions at an internal position with the richer set of interactions that are possible in the major groove at the end of an RNA helix.

Experimental Section

All chemicals were obtained from Aldrich or Fischer, unless noted otherwise. RNA synthesis chemicals, the 2′-deoxy-2-aminopurine phosphoramidite and the controlled pore glass supports were from Glen Research (Sterling, VA). All other RNA phosphoramidite monomers, the modified base 2′-deoxy-7-deazaguanosine, 4,4′-dimethoxytrityl chloride and 2-(cyanoethyl)-*N*,*N*,*N*′,*N*′-tetraisopropylphosphane were purchased from Chemgenes (Waltham, MA). Ultrahigh purity acetonitrile and dichloroethane were obtained from Baxter. NMR spectra were acquired on Bruker AC-200, AC-300, or Varian VXR 300 MHz spectrometers. Plasmid pQE-875, which harbors the gene for AlaRS with an amino-terminal six-histidine tag, was a gift from Prof. Paul Schimmel (M.I.T., Cambridge, MA). Reversed phase high performance liquid chromatography (RP-HPLC) was performed on a Beckman System using a Beckman 4 × 250 mm C_{18} column with diode array detection. All buffers were prepared using diethylpyrocarbonate-treated water to reduce RNase contamination.[12]

Synthesis of 2-Pyrimidinone-1-*β*-D-{2′-*O*-(*tert*-butyldimethylsilyl)-5′-*O*-(dimethoxytrityl)ribonucleoside 3′-*O*-[2-cyanoethyl (diisopropylamino)phosphoramidite]} (4HC). The modified base 4HC was synthesized essentially as described,[13−15] except for the final reaction.[16,17] In the final step, the 5′-(dimethoxytrityl)-2′-(*tert*-butyldimethylsilyl)-protected nucleoside was dissolved in a 10-fold volume of acetonitrile and diisopropylamine (1.2 equiv) followed by the addition of 2-(cyanoethyl)-*N*,*N*,*N*′,*N*′-tetraisopropylphosphane (1.5 equiv) and tetrazole (1.1 equiv from a 0.45 M solution in anhydrous acetonitrile). The reaction was stirred under N_2 at room temperature until complete by TLC (CH_3OH:CH_2Cl_2, 7:93, silica gel), 16 h, and then the mixture was dissolved in CH_2Cl_2, washed with 5% (aq) $NaHCO_3$ followed by brine, and finally passed through anhydrous Na_2SO_4. The product was purified by chromatography on silica gel (ethyl acetate:CH_2Cl_2:Et_3N, 24:76:0.5, v/v/v) (40% yield). The ^{1}H-NMR spectrum agreed with that previously published.[14] The final product was dried azeotropically from benzene and CH_3CN three times each and stored under Ar at −20 °C until use.

RNA Preparation. RNA oligonucleotides were synthesized using the phosphoramidite method on a Gene Assembler Special (Pharmacia), deprotected, gel purified on denaturing 16% polyacrylamide-TBE gels, eluted and desalted as previously described.[18,19] All oligonucleotides containing the modified base 4HC were gel purified and then further purified by RP-HPLC.[14] The presence of the modified base 4HC in the synthesized RNA was confirmed by monitoring the UV absorbance at 310 nm as well as at 260 nm.[14] Full-length tRNA^{Ala} was prepared by *in vitro* transcription with T7 RNA polymerase.[20] For the determination of RNA concentrations, the following extinction coefficients were used (units M^{-1} cm^{-1}): full-length tRNA, 60.4×10^4; 13-mer, 10.7×10^4; 9-mer, 8.9×10^4.[21]

Aminoacylation Assays. Histidine-tagged *E. coli* AlaRS was purified essentially as described previously for *E. coli* ProRS,[22] except for the following modifications. Induction with isopropyl thiogalactoside was performed overnight. An imidazole step gradient was used to elute the protein from the Ni^{2+}-nitrilotriacetic acid (Ni-NTA) resin (Qiagen). The column was washed with 5 mL of 0.1 M imidazole, 5 mL of 0.3 M imidazole, and finally 15 mL of 0.5 M imidazole. The fractions that eluted with 0.3 M imidazole contained AlaRS and were pooled. The enzyme was judged to be >95% pure by sodium dodecyl sulfate−polyacrylamide gel electrophoresis and was stored at −20 °C in a final concentration of 30 μM (11.6 mg/mL) in a buffer that contained 25 mM *N*-(2-hydroxyethyl)-1-piperazine-*N*′-2-ethanesulfonic acid (HEPES), pH 7.5, 0.15 M NaCl, 40% glycerol, and 1 mM dithiothreitol. For aminoacylation assays, the AlaRS concentration was based on the active-site titration.[23] Aminoacylation assays were performed using the published conditions.[21] Because the K_M for alanine is so high (∼200 μM),[24] these assays were routinely conducted at subsaturating concentrations of [^{3}H]-alanine (∼22 μM). The aminoacylation efficiency of duplex^{Ala} variants that we tested was independent of alanine concentration over the range 20−200 μM (P.J.B. and K.M.F., unpublished data), as was previously shown for full-length tRNA^{Ala}.[25] Prior to performing the assays, duplex^{Ala} substrates were annealed by heating in 50 mM HEPES, pH 8 at 80 °C for 2 min, then cooling to 60 °C for 2 min, and finally adding $MgCl_2$ to a final concentration of 10 mM before placing the samples on ice. The final concentration of duplex substrates in the assays was 10 μM. The reactions were initiated by adding purified AlaRS to 0.25 μM. Rates of aminoacylation, which are proportional to k_{cat}/K_M under the conditions used, were determined from the slope of a plot of picomoles of alanine charged versus time, and represent the average of at least two determinations. A 1000-fold decrease in aminoacylation represents the detection limit of the assay. In inhibition experiments, all reaction components were incubated for at least 5 min prior to initiating the reaction with full-length, *in vitro* transcribed tRNA^{Ala} to a final concentration of 1 μM. The conditions for inhibition assays were the same as for the charging assays, except that the concentration of AlaRS was 0.063 μM, and the concentrations of the inhibiting duplex were 10, 20, 30, 40, and 50 times that of tRNA^{Ala}.

Circular Dichroism and Melting Studies. CD spectra and CD melting curves were obtained on a Jasco J-710 spectropolarimeter fitted

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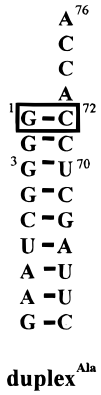

Figure 1. Sequence of chemically synthesized RNA duplex^{Ala} used in this work, which is based on the acceptor stem and part of the TΨC stem of *E. coli* tRNA^{Ala}. The 1:72 base pair investigated in this study is boxed.

with a Neslab variable-temperature circulating water bath. UV melting curves were obtained on a GBC UV/VIS 918 spectraphotometer equipped with a Peltier device. Spectra were obtained at 25 °C in 0.1 cm cells with approximately 15 μM RNA duplex that was freshly annealed in 50 mM NaCl, 10 mM $NaPO_4$ (pH 7), and 10 mM $MgCl_2$. Final CD spectra are the average of 10 scans.

Results

Probing Potential Recognition Elements in the G1:C72 Base Pair in Duplex^{Ala} Substrates. To determine whether functional groups in the major or minor grooves of the wild-type G1:C72 base pair contributed toward positive recognition by AlaRS, we used functional group mutagenesis to systematically delete possible hydrogen bond donors and acceptors from the terminal base pair of duplex^{Ala} substrates (Figure 1). Acceptor stem-derived duplexes are good substrates for *E. coli* AlaRS,[21] which facilitates the use of chemical RNA synthesisto probe the role of specific major and minor groove functional groups.[26] It was previously shown that when the minor groove 2-amino group of G1 was deleted by incorporating the modified base inosine (Figure 2A), aminoacylation activity was essentially unchanged.[5] We find that upon incorporation of the base pair A1:U72, which maintains the same minor groove functional groups as I:C but changes the arrangement of atoms in the major groove (Figure 2A), the activity is decreased 250-fold relative to the wild-type G1:C72 duplex^{Ala} (Table 1). This result suggests that if there are functional groups responsible for positive recognition, they are likely to reside in the major groove. We therefore determined the influence on aminoacylation of each hydrogen bond donor and acceptor in the major groove of the 1:72 base pair (Figure 2B). Incorporation of 7-deazaguanosine opposite C72 (7DAG1:C72) actually resulted in slightly improved aminoacylation of duplex^{Ala} (+2-fold), whereas substitutions of 2-aminopurine (2AP) at position 1 (2AP1:C72) and G1:4HC72 resulted in 6.4-fold and 3.0-fold decreases, respectively (Figure 2B and Table 1). The variant 2AP1:4HC72, which lacks the 6-keto oxygen, the 1-imino proton, and the 4-amino group, resulted in a slightly greater decrease in activity (17-fold, Figure 2B and Table 1). This decrease is approximately what we would have predicted on the basis of the effects of the individual deletions. We conclude that, although there is not a single functional group in the G1:C72 pair that has a major effect on positive recognition of duplex^{Ala} by AlaRS, modest decreases in aminoacylation are observed upon making changes at major groove positions.

Probing Potential Negative Elements in the C1:G72 Base Pair in Duplex^{Ala} Substrates. We previously showed that a C1:G72 duplex^{Ala} variant is inactive, and that activity could be partially restored by substitution of 2AP at position 72.[27] Relative to G, this modified base has a deletion of the 6-keto oxygen and the N1 proton. In the present work, we determine if additional functional groups in the major and minor grooves of the C1:G72 pair act as negative or blocking elements to prevent aminoacylation. Activity was indeed restored to varying levels when each of the functional groups of the C1:G72 base pair that we probed was deleted (Figure 3, Table 1). When the minor groove 2-amino group of G72 was deleted by incorporating inosine at position 72 (Figure 3A), the level of aminoacylation was now detectable, but still 420-fold decreased relative to the wild-type duplex (Table 1). Deletion of the major groove 4-amino group of C1 also resulted in a duplex that could be weakly aminoacylated; however, activity of the 4HC1:G72 duplex (Figure 3B) is still reduced 410-fold relative to the wild-type duplex (Table 1). If the potential hydrogen bond acceptor N7 of G72 is replaced by carbon (Figure 3B, C:7DAG), aminoacylation also improves and is now only 160-fold decreased relative to the wild-type duplex (Table 1). However, the greatest recovery in activity occurs upon substitution of 2AP at position 72. The C:2AP base pair has recently been shown to be in the wobble configuration in a DNA helix (Figure 3B).[28] Despite the probable conformational shift relative to a standard Watson-Crick base pair in an RNA helix, we observe only an 18-fold decrease in aminoacylation relative to the wild-type duplex (Table 1). This corresponds to a >50-fold increase in k_{cat}/K_M relative to the inactive C1:G72 duplex. Moreover, substitution of 2AP for G72 dramatically improves aminoacylation regardless of the identity of the base at position 1 (Table 1, Figure 4). The triple-deletion mutant 4HC1:2AP72 (Figure 3B) lacks the 4-amino group of C1 and the major groove carbonyl and N1 proton of G72. Our results with this variant (40-fold decrease in k_{cat}/K_M, Table 1), which contains only a single hydrogen bond, also support the role of major groove functional groups of the C1:G72 base pair in blocking aminoacylation by AlaRS.

We note that a carbonyl oxygen in the major groove appears to have a negative effect on aminoacylation by AlaRS whether presented in the context of a purine or a pyrimidine. Regardless of the identity of the base at position 1, U at position 72 always displayed lower activity than C or 4HC (Table 1, Figure 4). Comparison of the duplex containing 2AP1:U72 (−75-fold) with the 2AP:C variant (−6.4-fold) provides another example of this trend (Table 1). Aminoacylation of the duplex^{Ala} variants is generally highest with C72, whereas 4HC72 variants with the major groove 4-amino group deleted display intermediate efficiencies (Figure 4 and Table 1). On the other hand, a G at position 72 completely blocks aminoacylation in three cases (A:G, C:G, and G:G). We can conclude, therefore, that while a carbonyl oxygen in the major groove at position 72 appears to block aminoacylation, a major groove amino group presented by a pyrimidine (but not a purine) at this position has a positive influence on recognition by AlaRS.

To gain more insight into the effect of a G1:C72 → C1:G72 base pair transversion on aminoacylation by AlaRS, we determined whether the C1:G72 duplex could inhibit aminoacylation of full-length wild-type tRNA^{Ala}. A 50% inhibition

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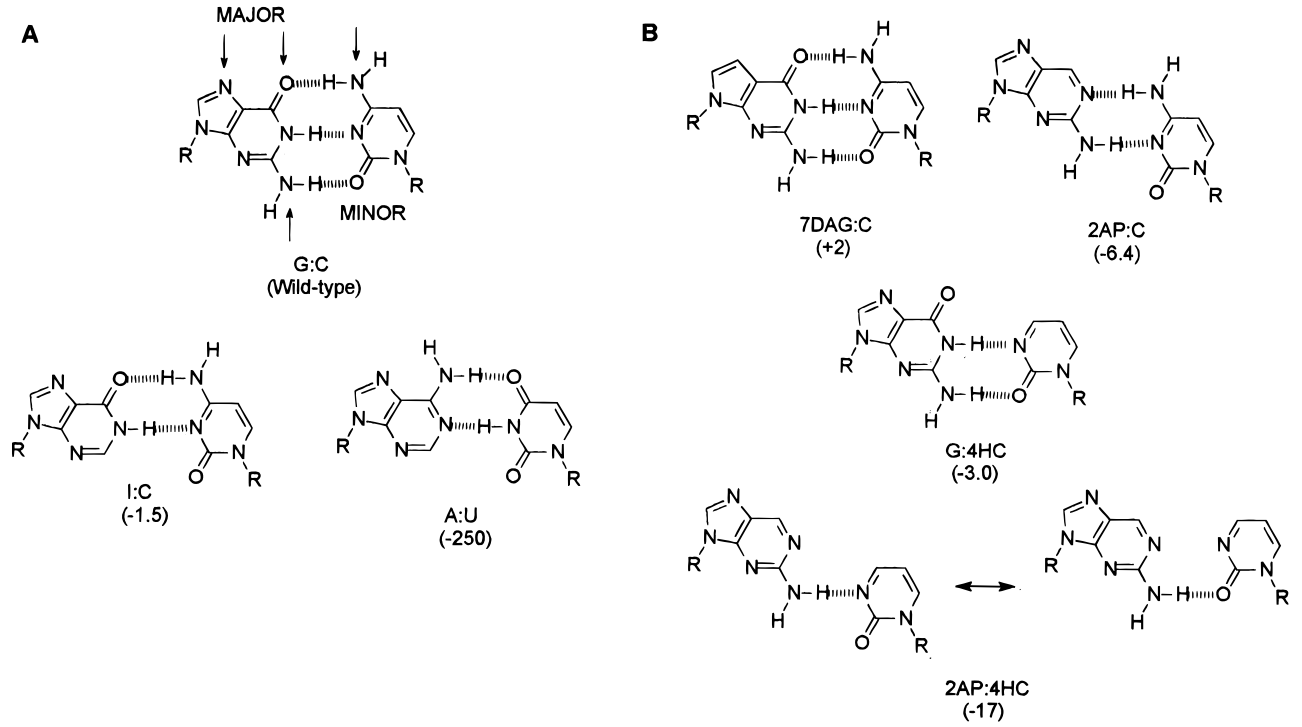

Figure 2. Proposed hydrogen bonding of base pairs incorporated at position 1:72 of duplex^{Ala} substrates designed to probe minor (panel A) and major (panel B) groove functional groups of the G1:C72 base pair for their role in recognition by *E. coli* AlaRS. The locations of the major and minor grooves are indicated for the wild-type base pair, along with the specific functional groups examined in this study (arrows). Fold changes in k_{cat}/K_M relative to the wild-type base pair are given in parentheses. I is inosine, 7DAG is 2′-deoxy-7-deazaguanosine, and 2AP is 2′-deoxy-2-aminopurine. Aminoacylation of duplex^{Ala} is not decreased significantly upon incorporation of a 2′-deoxy base at positions 1 or 72.[5]

in aminoacylation of tRNA^{Ala} was achieved upon incubation of AlaRS with a 16-fold excess concentration of the C1:G72 duplex relative to wild-type, full-length tRNA^{Ala}. A similar result was obtained using the C1:G72 duplex to inhibit charging of the wild-type duplex^{Ala} substrate. We conclude that AlaRS is able to bind the mutant duplex, but unable to charge it.

Biophysical Studies. The room-temperature CD spectra of the wild-type and C1:G72 duplexes are shown in Figure 5. These spectra are characteristic of A-form RNA, and it appears from these spectra, which were obtained at room temperature in the presence of 10 mM Mg^{2+}, that there are no major differences in the base stacking or helicity.[29,30] However, CD spectroscopy may not be able to discern subtle conformational differences between the duplexes. In particular, changes in the conformation of the single-stranded ACCA-3′ end of the RNAs may not have a significant effect on the spectra.

An RNA helix that closes with a 5′-G:C-3′ base pair is thermodynamically more stable than one closing in a 5′-C:G-3′ pair.[31,32] The effect of appending a single-stranded ACCA-3′ sequence on the relative stability of these RNA duplexes, however, is unknown. We wanted to determine whether the dramatic decrease in activity between the wild-type and C1:G72 variants was due to major differences in helix stability. The melting temperatures of the G1:C72 and the C1:G72 duplex^{Ala} were determined from circular dichroism melting curves to be 68 and 62 ± 1 °C, respectively. These differences indicate that the wild-type duplex is indeed slightly more stable than the C1:G72 variant, which is in agreement with previous findings on related systems.[31,32] However, CD results at low temperature (data not shown) showed no change relative to the spectra at 25 °C, indicating that the helix is fully annealed at the aminoacylation assay temperature of 25 °C used in these studies.

Discussion

It is well established that the acceptor stem domain of tRNA^{Ala} contains nucleotides responsible for specific recognition by class II *E. coli* AlaRS.[33−36] In particular, *in vitro* data showed that minor groove atomic groups in and near the unique G3:U70 base pair are functionally important.[3,5] The region of AlaRS that interacts with these minor groove functionalities has been localized to a fold-back peptide appended to the class-defining catalytic domain.[37] *In vivo* data support the notion that a "helical irregularity" caused by the G:U wobble pair also contributes to recognition by AlaRS.[38,39] On the basis of structural data obtained by X-ray crystallography, class II synthetases are all believed to approach the acceptor stem of tRNAs from the major groove side.[6−9] In this study, we investigate the extent of discrimination within this groove for AlaRS at the first base pair. Previous studies had already established that the 1:72 base pair was important for tRNA^{Ala} identity *in vivo*.[35] Furthermore, substitution of C72 → G in full-length tRNA^{Ala} results in a 62000-fold decrease in *in vitro* aminoacylation by AlaRS and a complete loss in aminoacylation

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Table 1. Aminoacylation Efficiency of Duplex^{Ala} Substrates Containing Base Pair Substitutions at the 1:72 Position

base pair	k_{cat}/K_M[a] (relative)	fold decrease[b]	$-\Delta\Delta G^{\ddagger}$[c] (kcal/mol)
G:C (wild-type)	1.0		0
	Positive Recognition		
I:C[d]	0.67	1.5	0.3
7DAG:C[e]	2.1	+2.0	−0.42
2AP:C[e]	0.16	6.4	1.1
2AP:U[e]	0.013	75	2.6
G:4HC	0.34	3.0	0.65
2AP:4HC[e]	0.059	17	1.7
	Blocking Elements		
C:I	0.0024	420	3.6
4HC:G	0.0024	410	3.6
C:2AP[e]	0.057	18	1.7
C:7DAG[e]	0.0063	160	3.0
4HC:2AP[e]	0.025	40	2.2
	A1 Variants		
A:A	0.0034	290	3.4
A:G	0	>1000	>4.9
A:2AP[e]	0.040	25	1.9
A:C	0.030	33	2.1
A:U	0.0040	250	3.3
A:4HC	0.012	80	2.6
	C1 Variants		
C:A	0	>1000	>4.9
C:G	0	>1000	>4.9
C:C	0.15	6.6	1.1
C:U	0.0087	120	2.8
C:4HC	0.024	41	2.2
	G1 Variants		
G:A	0	>1000	>4.9
G:G	0	>1000	>4.9
G:2AP[e]	0.076	13	1.5
G:U	0.0096	100	2.8
	U1 Variants		
U:A	0.0040	250	3.3
U:G	0.0016	630	3.8
U:2AP[e]	0.055	18	1.7
U:C	0.076	13	1.5
U:U	0.012	83	2.6
U:4HC	0.070	14	1.6

[a] Values reported are averages of two to four determinations with average standard deviations of ±30%. [b] Fold decrease in k_{cat}/K_M is given relative to wild-type duplex^{Ala}. In the case of 7DAG:C, an increase in k_{cat}/K_M was observed. [c] $-\Delta\Delta G^{\ddagger}$ is defined as $RT \ln[(k_{cat}/K_M)^{variant}/(k_{cat}/K_M)^{wild-type}]$, where $R = 1.98272$ cal/mol·K and $T = 298$ K. [d] Data was taken from ref 5. [e] It was previously established that single deoxynucleotide substitutions at positions 1 and 72 of duplex^{Ala} substrates have no negative effect on aminoacylation by AlaRS.[5] To test specific major groove functional groups, we therefore incorporated the commercially available deoxynucleotide version of the base analogs 7DAG and 2AP. Modified bases are abbreviated as in the legend to Figure 2.

of duplex^{Ala}.[40] In the duplex^{Ala} system, it was shown that the dramatic effect of a G1:C72 → C1:G72 transversion was at least in part caused by a blocking effect of major groove functional groups of G72.[27] The results of the 26 new substitutions at the 1:72 position presented here support and extend the results obtained previously and allow us to conclude that major groove discrimination does indeed play a significant role in acceptor stem recognition by AlaRS.

Comparison of inactive N1:G72 duplex^{Ala} variants with active N1:2AP72 variants shows that deletion of the 6-keto oxygen and the N1-hydrogen of G72 results in a transition state

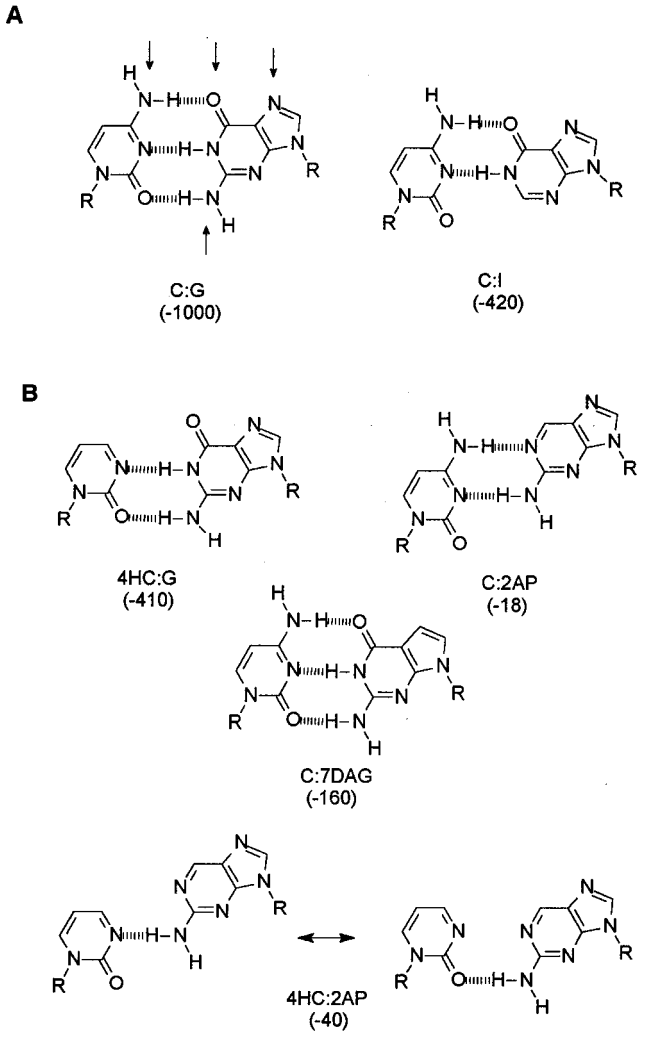

Figure 3. Proposed hydrogen bonding of base pairs incorporated at position 1:72 of duplex^{Ala} substrates designed to probe minor (panel A) and major (panel B) groove functional groups of the C1:G72 base pair for their role in blocking recognition by *E. coli* AlaRS. The specific functional groups examined in this study are indicated by the arrows. Fold changes in k_{cat}/K_M relative to the wild-type base pair are given in parentheses. Modified bases are abbreviated as in Figure 2.

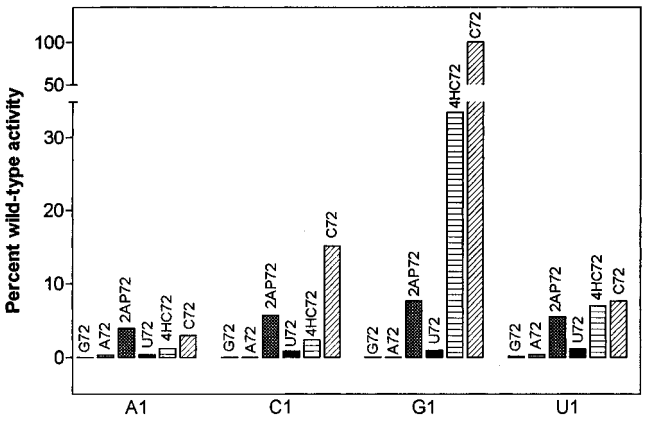

Figure 4. Histogram showing the aminoacylation efficiency of selected 1:72 variants relative to the wild-type G1:C72 duplex^{Ala} (defined as 100%). The nucleotide at position 1 is indicated on the *x*-axis, and the base at position 72 is indicated above the bars. Modified bases are abbreviated as in Figure 2.

stabilization of at least 3.0 kcal/mol (Table 1). This result is of particular interest because only one *E. coli* tRNA, tRNA^{Pro}, has a G at position 72,[41] and this is one mechanism by which

(40) Liu, H.; Kessler, J.; Peterson, R.; Musier-Forsyth, K. *Biochemistry* **1995**, *34*, 9795−9800.

(41) Steinberg, S.; Misch, A.; Sprinzl, M. *Nucleic Acids Res.* **1993**, *21*, 3011−3015.

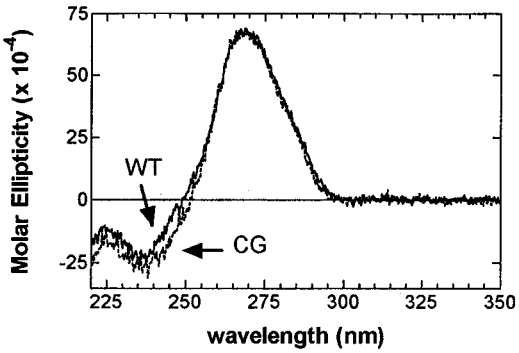

Figure 5. CD spectra of wild-type G1:C72 and mutant C1:G72 duplexes. The spectra were obtained using 15 μM duplex in 50 mM NaCl, 10 mM $NaPO_4$ (pH 7.0), and 10 mM $MgCl_2$ in 0.1 cm cells at room temperature and represent the average of 10 scans. Molar ellipticity is presented in units of $M^{-1}\cdot cm^{-1}$.

AlaRS is likely to discriminate against this noncognate tRNA. Furthermore, we show that deletion of major groove functional groups also affects positive recognition by AlaRS. A comparison of the wild-type duplex with the 4HC72 variant tested (Table 1) shows that the 4-amino group of C72 contributes 0.65 kcal/mol to transition state stabilization. The decrease in aminoacylation efficiency observed with the 2AP:C duplex (6.4-fold) shows that a modest contribution to transition state stabilization is also provided by the carbonyl group and N1 proton of G1 ($-\Delta\Delta G = 1.1$ kcal/mol). Many of the modified bases tested are likely to alter the base pair conformation to a non-Watson−Crick or wobble configuration. In these cases, it is difficult to assess the relative contribution of specific functional groups versus conformational differences. Nevertheless, our results agree with the hypothesis that class II synthetases approach their tRNA substrates from the major groove side of the acceptor stem, and therefore positive and negative recognition elements are expected to reside in this groove.

Since the C1:G72 mutant duplex can inhibit aminoacylation of wild-type substrates, the base pair transversion does not completely block binding to the synthetase. Discrimination by AlaRS against the C1:G72 duplex apparently occurs, at least in part, during the transition state of catalysis. We showed that the CD spectra of the C1:G72 mutant and wild-type duplexes at 25 °C are very similar and that the observed difference in melting temperatures was as expected.[31,32] Although CD is a useful probe of nucleic acid secondary structure, little tertiary structural information may be derived from analysis of these spectra, and therefore, our results cannot rule out structural differences in the conformation or stacking of the ACCA-3′ end onto the first base pair. Additional structural data, such as that which can be obtained using NMR spectroscopy, will be necessary to further explore these possibilities.

On the basis of the biochemical studies reported in this work, we conclude that while there is not a single functional group in the G1:C72 base pair that contributes as much toward positive recognition by AlaRS as the previously characterized minor groove elements in and around the G3:U70 base pair, the enzyme has a preference for a C at position 72 and discriminates strongly against substrates containing a major groove carbonyl group presented by either a G or a U residue at this position. Class II AlaRS is, therefore, an example of a system that achieves an extraordinary degree of overall discrimination by exploiting both the greater accessibility of minor groove functional groups in internal positions of an RNA helix with the high degree of specificity afforded by major groove interactions at the end of the helix.

Acknowledgment. We thank Dr. Hongjian Liu for the preparation of $tRNA^{Ala}$ and Professor Craig Forsyth, Mr. Feryan Ahmed, and Dr. Chris Adams for assistance and advice on the synthesis of the modified base 4HC. We are grateful to Professor Victor Bloomfield for his support of this work and for allowing us to use his instruments for CD and UV/Vis measurements. We also thank Professors Paul Schimmel and Christopher Francklyn for helpful comments on the manuscript. This work was funded by grants from the National Institutes of Health (GM 49928) and the Petroleum Research Fund, administered by the American Chemical Society. P.J.B. was supported by a National Institutes of Health Molecular Biophysics Training Grant (5T32 GM08277) and an Interdisciplinary Research Grant from the University of Minnesota.

JA971020C