Identification of Discriminator Base Atomic Groups That Modulate the Alanine Aminoacylation Reaction*

(Received for publication, September 13, 1999)

Abbey E. Fischer, Penny J. Beuning, and Karin Musier-Forsyth‡

From the Department of Chemistry, University of Minnesota, Minneapolis, Minnesota 55455

Specific aminoacylation of tRNAs involves activation of an amino acid with ATP followed by amino acid transfer to the tRNA. Previous work showed that the transfer of alanine from *Escherichia coli* alanyl-tRNA synthetase to a cognate RNA minihelix involves a transition state sensitive to changes in the tRNA acceptor stem. Specifically, the "discriminator" base at position 73 of minihelixAla is a critical determinant of the transfer step of aminoacylation. This single-stranded nucleotide has previously been shown by solution NMR to be stacked predominantly onto G1 of the first base pair of the alanine acceptor stem helix. In this work, RNA duplexAla variants were prepared to investigate the role of specific discriminator base atomic groups in aminoacylation catalytic efficiency. Results indicate that the purine structure appears to be important for stabilization of the transition state and that major groove elements are more critical than those located in the minor groove. This result is in accordance with the predicted orientation of a class II synthetase at the end of the acceptor helix. In particular, substitution of the exocyclic amino group of A73 with a keto-oxygen resulted in negative discrimination at this site. Taken together, these new results are consistent with the involvement of major groove atomic groups of the discriminator base in the formation of the transition state for the amino acid transfer step.

Aminoacylation, which refers to the covalent attachment of an amino acid to its cognate tRNA, occurs via a two-step enzyme-catalyzed reaction. In the first step, an amino acid reacts with ATP to form an aminoacyl-adenylate (aminoacyl-AMP) intermediate in the active site of the appropriate aminoacyl-tRNA synthetase. While still bound by the synthetase, the amino acid is then transferred to the 3′-end of the tRNA. The so-called "discriminator" base at position 73 and other acceptor stem elements proximal to the site of amino acid attachment are critical determinants of specificity and catalytic efficiency in most synthetase systems (1, 2).

In the period since the original discriminator site hypothesis (3), the fourth single-stranded nucleotide from the 3′-end of tRNAs (N73) has been the subject of extensive investigation. Indeed, although the details of the original hypothesis, which proposed a relationship between the nature of the amino acid and the identity of the base at position 73, are not entirely correct, biochemical and genetic experiments have shown that N73 is generally important for aminoacylation (1, 4, 5). Despite the importance of this position, to our knowledge, the energetic contribution of individual discriminator base functional groups to aminoacylation catalytic efficiency has not been examined in detail.

In the case of *Escherichia coli* tRNAAla, it has been shown that mutagenesis of wild-type A73 to other standard nucleotides does not completely eliminate aminoacylation in vitro as long as the substrate contains the critical G3:U70 base pair (6, 7), which is the major determinant in this system (8, 9). Although a detailed kinetic analysis was not reported, N73 substitution in minihelixAla reduced the extent of aminoacylation to ~6% of the wild-type minihelix. Thus, the nucleotide at position 73 modulates the efficiency of the aminoacylation reaction in the presence of the essential G3:U70 base pair (6, 7). This nucleotide has been shown by solution NMR spectroscopy to stack onto the end of the helix and to stabilize the G1:C72 base pair (10–12). Stacking of A73 with the first base pair occurs predominantly with G1 on the opposite strand (Fig. 1). In addition, NMR experiments indicate that A73, C74, and C75 all stack onto each other in a manner that resembles stacking within each strand of a double helix (12).1

In accordance with the relatively weak binding affinity between tRNAs and synthetases, presumably due to the need for rapid turnover during protein synthesis, the transition state of catalysis has often been found to be a more significant factor in discrimination than the binding step. To determine the mechanism by which N73 modulates aminoacylation with alanine, Shi and Schimmel (7) studied the single turnover charging of wild-type minihelixAla and three N73 variants (U73, C73, and G73). Using preformed, enzyme-bound aminoacyl-adenylate, they determined that modulation of the reaction upon N73 substitution does not arise from hydrolysis of the adenylate or a transiently charged RNA, but rather from a reduced rate of alanine transfer to the minihelix substrate. They hypothesized that this reduced efficiency is most likely due to a conformational change, which is sensitive to the discriminator base, and that this change occurs during the transition state of the transfer step. Although the transfer reaction was previously shown to proceed more slowly with N73 variants of minihelixAla (7), the specific atomic groups responsible for this defect were not identified. In the present work, we wished to further define the critical elements for the amino acid transfer reaction by examining the effect of atomic group substitutions at position 73 of duplexAla variants.

* This work was supported by Grant GM49928 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Dept. of Chemistry, University of Minnesota, 207 Pleasant St. S.E., Minneapolis, MN 55455. Tel.: 612-624-0286; Fax: 612-626-7541; E-mail: musier@chem.umn.edu.

‡ To whom correspondence should be addressed: Dept. of Chemistry, University of Minnesota, 207 Pleasant St. S.E., Minneapolis, MN 55455. Tel.: 612-624-0286; Fax: 612-626-7541; E-mail: musier@chem.umn.edu.

1 The structure of the single-stranded region of microhelixAla resembles that of a strand within a double helix (12). We will therefore refer to functional groups of A73 as located in "grooves." For example, N-6 and N-7 are in what would be the major groove of a double helical region, and position 2 is in the minor groove.
EXPERIMENTAL PROCEDURES

Chemicals—Inosine and all four standard RNA phosphoramidite monomers were purchased from Chemgenes (Waltham, MA). All other modified bases and RNA synthesis chemicals were from Glen Research Corp. (Sterling, VA).

RNA Preparation—Solid-phase synthesis of RNA oligonucleotides was accomplished using the phosphoramidite method on a Gene Assembler Special (Amersham Pharmacia Biotech) (13, 14). The oligonucleotides were gel-purified on 16% polyacrylamide gels, eluted, and desalted as described (13, 14).

Aminoacylation Assays—E. coli histidine-tagged alanyl-tRNA synthetase was purified as described previously (15). RNA duplex substrates were annealed immediately before use by heating at 80 °C for 2 min, cooling to 60 °C for 2 min, adding MgCl₂ to 10 mM, and placing on ice. Aminoacylation assays were performed at room temperature using published conditions (16). RNA duplexes (4.5 mM) and all other assay components were pre-equilibrated to room temperature prior to initiating the reaction with E. coli alanyl-tRNA synthetase (45 nM). Initial rates of aminoacylation are proportional to RNA concentration under the conditions used for these experiments. Thus, Vo/[S] is an accurate reflection of kcat/Km.

RESULTS

RNA duplexes that mimic the acceptor stem of tRNAAla are specific substrates for aminoacylation by E. coli alanyl-tRNA synthetase (16) (Fig. 2). In this work, we examined aminoacylation of eight duplexAla variants containing both standard and modified bases at position 73 (Fig. 3). As expected from previous reports (6, 7, 17, 18), substitution of A73 with the other three standard bases caused significant decreases in aminoacylation activity. The cytosine and uracil substitutions resulted in 34- and 67-fold decreases in aminoacylation efficiency, respectively (Table I). Whereas pyrimidine substitutions are not well tolerated, an even greater decrease occurred upon substitution of another purine base, guanine, which reduced the activity by 100-fold relative to the wild-type duplex (Table I). The guanine substitution replaced the 6-amino group of adenine with a keto-oxygen and also added an N-1 proton and an amino group at position 2.

Because standard nucleotide substitutions result in multiple atomic group changes relative to the wild-type base, they are not as useful in elucidating specific major groove versus minor groove effects. For this reason, atomic group mutagenesis was next performed using modified purine nucleotides. When 2'-deoxy-2-aminopurine (2AP) was substituted at position 73, activity was only reduced 2-fold relative to the wild-type duplex (Table I). The guanine substitution replaced the 6-amino group of adenine with a keto-oxygen and also added an N-1 proton and an amino group at position 2.

Because standard nucleotide substitutions result in multiple atomic group changes relative to the wild-type base, they are not as useful in elucidating specific major groove versus minor groove effects. For this reason, atomic group mutagenesis was next performed using modified purine nucleotides. When 2'-deoxy-2-aminopurine (2AP) was substituted at position 73, activity was only reduced 2-fold relative to the wild-type duplex (Table I). The guanine substitution replaced the 6-amino group of adenine with a keto-oxygen and also added an N-1 proton and an amino group at position 2.

The 14-fold decrease relative to the wild-type duplex corresponds to a 1.6 kcal/mol contribution to transition state formation.
Discriminator Base Recognition by Alanyl-tRNA Synthetase

TABLE I

| N73 variant | kcat/Km (relative)$^a$ | Fold change$^b$ | $-\Delta G^c$ kcal/mol |
|-------------|------------------------|-----------------|----------------------|
| Standard bases |                        |                 |                      |
| A (WT)$^e$ | 1                      | 1               | 0                    |
| C           | 0.029                  | 34              | 2.1                  |
| U           | 0.015                  | 67              | 2.5                  |
| G           | 0.0097                 | 100             | 2.7                  |
| Single atomic group mutants |                |                 |                      |
| Neb$^d$    | 0.48                   | -2.1            | 0.43                 |
| 7DAA$^e$   | 0.070                  | -14             | 1.6                  |
| 2AA$^e$    | 1.3                    | 1.3             | -0.17                |
| Double atomic group mutants |                |                 |                      |
| 2AP$^d$    | 0.67                   | -1.5            | 0.24                 |
| I$^d$       | 0.0049                 | -200            | 3.1                  |

$^a$ Values reported are averages of at least four determinations with an average S.D. of ±22%.

$^b$ Fold change is given relative to wild-type duplex$^{Ala}$. 

$^c$ $-\Delta G^c = RT \ln(k_{cat}/K_m)^{mutant}/k_{cat}/K_m^{wild-type}$, where $R = 1.98272$ kcal/mol·K and $T = 298$ K.

$^d$ Wild-type.

$^e$ It was previously determined that a single deoxy substitution at N73 has little effect on aminoacylation (20). We therefore incorporated the commercially available deoxynucleotide version of the base analogs Neb, 7DAA, 2AA, and 2AP.

To probe the minor groove, 2'-deoxy-2-aminoadenine (2AA) was substituted at position 73. This substitution results in the addition of an exocyclic 2-aminogroove to the wild-type base. Interestingly, this change resulted in a small (1.3-fold) but reproducible increase in activity relative to the wild-type substrate. A second substitution, that of 2'-deoxy-2-aminopurine (2AP), confirmed this result. 2AP lacks the 6-amino group of adenine, but contains a minor groove amino group; thus, when compared with Neb, it specifically tests the effect of placing an amino group in the minor groove. This substitution retained 67% of the wild-type activity and was 1.4-fold more active than the Neb$^{73}$ variant.

Finally, substitution of A73 with inosine, a guanine analog lacking the exocyclic 2-aminogroove group, reduced aminoacylation efficiency by 200-fold relative to the wild-type duplex. This decrease in activity is even greater than that observed upon A73 → G substitution and supports the hypothesis that introduction of a major groove 6-keto-oxygen results in a blocking effect.

**DISCUSSION**

Using atomic group mutagenesis, we previously showed that tRNA acceptor stem recognition by alanyl-tRNA synthetase involves minor groove recognition at an internal (3:70) position (19, 20) and major groove discrimination at the terminal base pair (1:72) of the acceptor stem helix (15). We now extend this analysis to the discriminator base position. This nucleotide is of particular interest because it is a well known determinant for recognition by most synthetases, including alanyl-tRNA synthetase (5). Moreover, standard base substitutions at this site in minihelix$^{Ala}$ are known to slow transition state formation for the amino acid transfer step of the alanine aminoacylation reaction (7). The 2'-hydroxyl group has previously been shown to be dispensable at this site (20); however, the function of specific nucleotide base atomic groups was not examined in this earlier work. In the present study, using duplex$^{Ala}$ substrates, both positive determinants and negative or "antideterminants" involved in the function of this key single-stranded acceptor stem nucleotide were elucidated. Similar to our previous results at the adjacent 1:72 base pair (15), we found that major groove substitutions at position 73 significantly affected aminoacylation activity, whereas minor groove substitutions had only small effects. The importance of major groove interactions at the end of the tRNA$^{Ala}$ acceptor stem helix is in accordance with the hypothesis, based primarily on known x-ray crystal structures of synthetase-tRNA complexes, that class II synthetases approach the top of the acceptor stem from the major groove side (21, 22).

The largest positive contribution by a single atomic group of A73 was observed upon removal of a hydrogen bond acceptor at position 7. By substituting N-7 of A73 with a carbon atom (A73 → 7DAA), we determined that this position contributes 1.6 kcal/mol to the free energy of transition state formation. Introducing a 6-keto-oxygen and an N-1 proton in the major groove (A73 → I) has a negative effect on aminoacylation. A similar effect was previously observed in a study of the 1:72 position (15). At both positions 72 (15) and 73 (Fig. 3 and Table I), the negative contribution of these functionalities is worth −3 kcal/mol. The large decrease in aminoacylation efficiency upon A73 → I substitution can be largely attributed to a blocking element rather than to the loss of the 6-amino group of A73 because Neb substitution, which removes all exocyclic functional groups (Fig. 3), is well tolerated ($-\Delta G = 0.43$ kcal/mol). Thus, the N-6-amino group is not a major contributor to transition state stabilization. The minor groove of A73 was also found to contribute very little to catalytic efficiency. Interestingly, the addition of an exocyclic 2-aminogroove to the minor groove had a small but reproducible positive impact on aminoacylation. Comparison of A73 → 2AA, Neb → 2AP, and I → G indicates that the presence of a 2-aminogroove group contributes 0.17–0.4 kcal/mol to transition state stabilization (Fig. 3 and Table I). It should be noted that for the purine substrations, atomic group changes are unlikely to significantly affect stacking interactions at the end of the helix (23, 24).

The atomic group mutagenesis data presented here allow us to begin to understand the strong preference for A73 over G73 in the alanine system. Although the 6-amino group of A73 is not a major positive recognition element, the presence of the 6-keto-oxygen of G results in a significant block to aminoacylation, presumably by slowing the amino acid transfer step (7). The preference for a purine base at N73 of the tRNA$^{Ala}$ acceptor stem is also evident from this work. At least part of the reduction in activity upon pyrimidine substitution can be explained by the lack of the positive N-7 determinant present in the purine bases. A recent high resolution NMR structure of microhelix$^{Ala}$ also sheds light on the preference for a purine discriminator base (12). The structure shows that there is substantial base stacking between the A at position 73 and the guanine located on the opposite strand of the first base pair (G1) (Fig. 1). This stacking interaction may contribute to proper orientation of the discriminator base during amino acid transfer. Moreover, according to nearest-neighbor free energy parameters (25, 26), an RNA duplex closed with a 5’-G-C-3’ pair containing a 3’-A single base overhang is predicted to have greater stability than the same duplex with a 3’-pyrimidine overhang (ΔG = 0.5–0.9 kcal/mol). Thus, the local conformation and stability of the RNA are likely to be altered upon pyrimidine substitution at position 73.

NMR studies of acceptor stem microhelices previously showed that the conformation of the CCA 3'-end and the stability of the first base pair are dependent on the identity of N73 (27, 28). In particular, in the context of an E. coli microhelix$^{Met}$ variant containing a terminal G1:C72 base pair, the presence of an A at position 73 results in an extended CCA 3'-end (27), similar to the conformation observed in the high resolution structure of microhelix$^{Ala}$ (12). In contrast, a U73 substitution results in a fold-back conformation with the terminal A73 in close proximity to G7 (27). The conformation of the CCA 3'-end in duplex$^{Ala}$ variants containing subtle atomic group changes,
such as 7DAA$^{A73}$, is unlikely to be altered in such a dramatic way. However, even small changes in stability and stacking can have large effects on aminoaacylation. For example, a discriminator base change from A$^{73}$ to G in human tRNA$_{Leu}^{AA}$ switches acceptor specificity from leucine to serine (29). Moreover, structural studies of human microhelix$_{Leu}$ variants support a close correlation between the identity of the nucleotide at position 73 and terminal base pair stability (28). In particular, an A$^{73}$ → G change resulted in a destabilization of the G$^{1}$C$^{72}$ pair also present in this system.

A previous study of discriminator base recognition in the human tRNA$_{Ser}$ system used atomic group mutagenesis to incorporate modified bases into position 73 of semi-synthetic tRNAs (30). Although a detailed kinetic analysis was not carried out, when wild-type G$^{73}$ was changed to 2AP, tRNA$_{Ser}$ could be aminoaacylated, albeit very weakly. An I$^{73}$ substitution completely eliminated charging (30). Thus, in contrast to the alanine case, a minor groove atomic group is critical in the human serine system. Since seryl-tRNA synthetase also belongs to class II, major versus minor groove effects at the discriminator base do not appear to be a class-specific feature of tRNA aminoaacylation.

In summary, in E. coli tRNA$_{Ala}$, an A$^{73}$ discriminator base is required for optimal formation of the transition state for the amino acid transfer step in the aminoaacylation reaction. Insights into the strong preference for an adenine over the other three natural bases are gained from the atomic group mutagenesis results presented here. The positive contribution of a purine N-7 and a strong negative effect upon introduction of a 6-keto-oxygen substitution indicate that major groove determinants are critical at this site. Whether these functional groups exert their effect via a direct or an indirect mechanism remains to be determined.

Acknowledgments—We thank Professor Paul Schimmel for helpful comments on the manuscript and Maria Nagan for assistance in the preparation of Fig. 1.

REFERENCES

1. Giegé, R., Sissler, M., and Florentz, C. (1998) Nucleic Acids Res. 26, 5017–5035
2. Musier-Forsyth, K., and Schimmel, P. (1999) Acc. Chem. Res. 32, 368–375
3. Crothers, D. M., Seno, T., and Soll, D. G. (1972) Proc. Natl. Acad. Sci. U. S. A. 69, 3063–3067
4. Shimizu, M., Asahara, H., Tamura, K., Haseqawa, T., and Himeno, H. (1992) J. Mol. Evol. 35, 436–443
5. Hou, Y.-M. (1997) Chem. Biol. 4, 93–96
6. Shi, J.-P., Francklyn, C., Hill, K., and Schimmel, P. (1990) Biochemistry 29, 3621–3626
7. Shi, J.-P., and Schimmel, P. (1991) J. Biol. Chem. 266, 2705–2708
8. Hou, Y.-M., and Schimmel, P. (1988) Nature 333, 140–145
9. McClain, W. H., and Foss, K. (1988) Science 240, 783–786
10. Limmner, S., Hofmann, H.-P., Ott, G., and Sprinzl, M. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 6199–6202
11. Limmer, S., Reif, B., Ott, G., Lubos, A., and Sprinzl, M. (1996) FEBS Lett. 385, 15–20
12. Ramos, A., and Varani, G. (1997) Nucleic Acids Res. 25, 2083–2090
13. Scaringe, S. A., Francklyn, C., and Usman, N. (1990) Nucleic Acids Res. 18, 5433–5441
14. Sproat, B., Colonna, F., Mullah, B., Tsou, D., Andrus, A., Hampel, A., and Vinayak, R. (1995) Nucleosides Nucleotides 14, 255–273
15. Beuning, P. J., Gulotta, M., and Musier-Forsyth, K. (1997) J. Am. Chem. Soc. 119, 8397–8402
16. Musier-Forsyth, K., Scaringe, S., Usman, N., and Schimmel, P. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 209–213
17. McClain, W. H., Foss, K., Jenkins, R. A., and Schneider, J. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 9272–9276
18. Buechter, D. D., and Schimmel, P. (1995) Biochemistry 34, 6014–6019
19. Musier-Forsyth, K., Usman, N., Scaringe, S., Doudna, J., Green, R., and Schimmel, P. (1991) Science 253, 784–786
20. Musier-Forsyth, K., and Schimmel, P. (1992) Nature 357, 513–515
21. Ruff, M., Krishnaswamy, S., Boeglin, M., Peterszian, A., Mitschler, A., Poddarjany, A., Rees, B., Thierry, J. C., and Moras, D. (1991) Science 252, 1682–1689
22. Cusack, S., Yaremchuk, A., and Tukalo, M. (1996) EMBO J. 15, 2834–2842
23. Turner, D. H., Sugimoto, N., Kierzek, R., and Dreike, S. D. (1987) J. Am. Chem. Soc. 110, 3783–3785
24. Sciascia-Lucia, J. Jr., Kierzek, R., and Turner, D. H. (1991) J. Am. Chem. Soc. 113, 4313–4322
25. Sugimoto, N., Kierzek, R., and Turner, D. H. (1987) Biochemistry 26, 4554–4558
26. Turner, D. H., Sugimoto, N., and Freier, S. M. (1988) Annu. Rev. Biophys. Biophys. Chem. 17, 167–192
27. Puglisi, E. V., Puglisi, J. D., Williamson, J. B., and RajBhandary, U. L. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 11467–11471
28. Metzger, A. U., Heckl, M., Willbold, D., Breitschopf, K., RajBhandary, U. L., Rosch, P., and Gross, H. J. (1997) Nucleic Acids Res. 25, 4551–4556
29. Breitschopf, K., and Gross, H. J. (1994) EMBO J. 13, 3166–3169
30. Breitschopf, K., and Gross, H. J. (1996) Nucleic Acids Res. 24, 405–410