Noncovalent Assembly of Microhelix Recognition by a Class II tRNA Synthetase

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We report here an experimental demonstration of the noncovalent assembly of a tRNA synthetase whose major determinant for aminoacylation specificity is in the tRNA acceptor stem. This assembly may resemble a step in the overall development of the genetic code. The code is established through aminoacylation reactions, whereby amino acids are attached specifically to tRNAs that bear cognate anticodons.[1] As the genetic code was established, aminoacyl tRNA synthetases are thought to be among the first proteins to appear. One model for the assembly of synthetases in evolution is that idiosyncratic RNA binding domains noncovalently associated with primitive catalytic domains which carried out amino acid activation.[2]

Alanyl-tRNA synthetase makes no contact with the RNA anticodon, thus demonstrating that the genetic code relationship between the nucleotide triplets for alanine and the amino acid are indirect.[3] Instead, the major determinant for aminoacylation of $tRNA^{Ala}$ is a G3:U70 base pair in the acceptor stem.[4] As a consequence, alanyl-tRNA synthetase aminoacylates a microhelix substrate that reconstructs the $tRNA^{Ala}$ acceptor stem.[5] Other synthetases also charge their cognate acceptor stem oligonucleotide substrates with high specificity.[6] The relationship between the sequences/structures of these oligonucleotides and specific amino acid constitutes an operational RNA code for amino acids which historically may have preceded the genetic code.[2] Thus, the assembly of synthetases that charged acceptor stem substrates may have been a step in the evolution of the full synthetase−tRNA interaction and of the genetic code. We sought to test the feasibility of this step by investigating the alanine system.

The N-terminal 461-residue fragment of *Escherichia coli* AlaRS (461N) is active *in vivo* and can aminoacylate RNA microhelix or duplex substrates with the same efficiency as the wild-type AlaRS (875 amino acids).[7] Residues 1−249 contain the three conserved motifs that make up the active sites of all class II synthetases.[8] We used a split gene approach[9] to engineer a translational stop−restart sequence after residue 249 (end of the conserved domain) in protein 461N to create two fragments: 249N and 212C (Figure 1a). This approach results in the biosynthesis of two separate but translationally coupled polypeptides. The fragments, when coexpressed in *E. coli*, can be copurified indicating that the two pieces form a stable complex.[10] Furthermore, 461N-split (249N + 212C) is active for adenylate synthesis and G3:U70-dependent aminoacylation of $microhelix^{Ala}$ in vitro (Figure 1b). Thus, specific acceptor helix interactions have been maintained in the split construct.

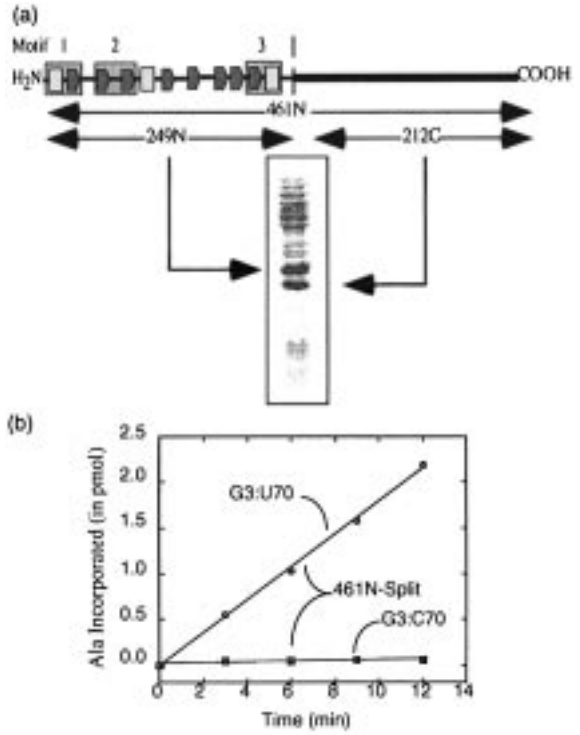

Figure 1. (a) Schematic representation of 461N showing the active site domain (residues 1−249) containing the three conserved motifs and a C-terminal 212-residue fragment (rectangle, α-helix; pentagon, β-sheet). The gene for 461N-split was constructed by introducing a translational stop−restart sequence between residues 249 and 250. A 14% SDS-PAGE gel stained with Coomassie Blue dye shows the expression in *E. coli* of the two independent polypeptide chains. (b) Plot showing alanine incorporation in an aminoacylation assay using 461N-split (1 μM) and $microhelix^{Ala}$ substrates (5 μM) under standard conditions.[7,11] The activity on a microhelix substrate of 461N-split is about 1% of that of 461N or the native full length protein (875N). Thus, the split raises the apparent free energy of activation by about 2.8 kcal/mol.

Next, we synthesized RNA microhelix and duplex substrates containing an azidophenacyl moiety appended to a phosphorothioate linkage between residues C69 and U70 (AP-RNA)-(Figure 2). Figure 3 shows the AP-RNA-protein cross-link generated upon irradiation of 461N or 461N-split in the presence of $AP\text{-}microhelix^{Ala}$. Significantly, the site of cross-linking in

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(10) Proteins 461N, 461N-split, and 212C were expressed as C-terminal 6-His-tagged proteins and purified by Nickel affinity chromatography (Quiagen Resin).

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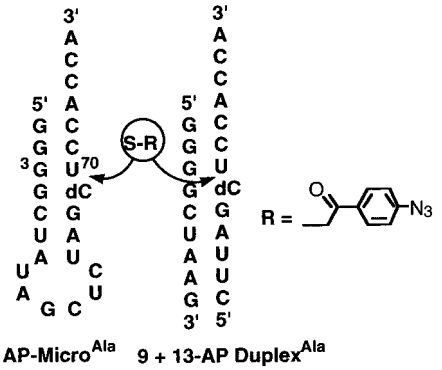

Figure 2. Azidophenacyl RNA substrates synthesized for cross-linking studies.[12,15]

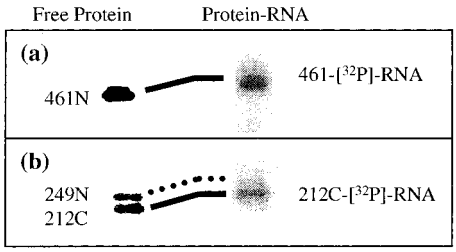

Figure 3. Formation of a protein-RNA cross-link between (a) 461N or (b) 461N-split and AP-microhelix^{Ala}. Samples containing protein (4 μM) and AP-microhelix^{Ala} (20 μM, 100 000 cpm) were prepared in reaction buffer (50 mM HEPES, pH 7.5; 10 mM $MgCl_2$; 20 mM KCl; 20 mM β-mercaptoethanol; 20 μM alanine), incubated at room temperature for 5 min, and irradiated at 300 nm for 2 min. The samples were then mixed with 2 μL 10× SDS loading buffer and denatured at 37 °C for 10 min before electrophoresis on a 14% SDS-polyacrylamide gel for 1 h. The proteins were transferred to a PVDF membrane by electro-blotting and visualized by amido black staining (free protein, shown on the left) or phosphorimagery ([^{32}P]RNA−protein, shown on the right). The mass added by the cross-linked RNA shifts the bands by an amount equal to the added molecular mass of 7.8 kDa, as seen in both panels a and b.

461N-split is the C-terminal fragment (212C) that is noncovalently associated with the active-site-containing domain. We established that the cross-links were active site directed and reflected true substrate binding in two ways (data not shown). First, we were able to compete out the AP-RNA cross-links in the presence of unmodified microhelix. Second, we observed a decrease in the initial rates of aminoacylation by 461N when irradiated at 300 nm in the presence of AP-microhelix^{Ala}, but no decrease in the absence of irradiation.

Figure 4 shows discrimination in cross-linking of G3:U70- versus I3:U70- and G3:C70- containing duplex^{Ala} by 461N.[17] We also showed that 212C alone could cross-link to duplex^{Ala} and that this cross-link was preferential to the G3:U70 substrate. (The 249N fragment is highly insoluble, and thus, we could not determine if it alone bound RNA.) The results show that the 250−461 region is an RNA binding domain and includes elements that distinguish between subtle variations of the 2-amino group in the RNA minor groove. This RNA binding domain may cooperate with a region that is within the N-terminal catalytic fragment.[13]

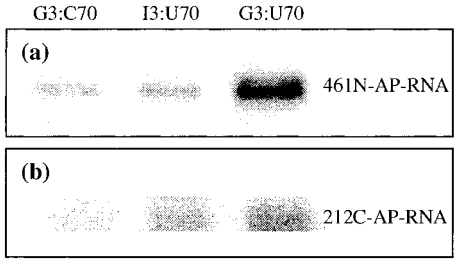

Figure 4. Autoradiograms showing the discrimination in cross-linking of the 9+13-AP duplex^{Ala} substrates by proteins (a) 461N and (b) 212C.[17] Samples containing protein (5 μM) and 9+13-AP duplex^{Ala} (5 μM, 100 000 cpm) were treated as in Figure 3 except that 50 mM sodium acetate, pH 6.0 was used in the reaction buffer.

Some tRNA synthetases bind to the anticodon of the cognate tRNA in addition to the acceptor stem. Previous work showed that the noncovalent assembly of an anticodon binding domain with a catalytic domain (containing a presumptive acceptor helix binding element) resulted in an enzyme that was active on the full tRNA.[9a] In contrast, the data in Figures 1, 3, and 4 show that an acceptor helix binding domain alone can be noncovalently assembled with an active site to yield a synthetase that has microhelix binding and aminoacylation specificity. Thus, primitive enzymes that made adenylates may have had a significant capacity for noncovalently associating with RNA binding elements that, in turn, enabled assembly of the microhelix-based operational RNA code for amino acids. These primitive enzymes could then assemble with additional RNA binding elements such as the anticodon binding domain.

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(12) RNA oligonucleotides were synthesized on a Pharmacia Gene Assembler Special using standard phosphoramidite chemistry. A unique phosphorothioate linkage was introduced between dC69 and U70 by using tetra ethyl thiuram disulfide instead of iodine at the oxidation step during synthesis. The single deoxyribo nucleotide was introduced immediately 3′ to the phosphorothioate to prevent probe elimination.[13] Azidophenacyl bromide (Aldrich) was reacted with the phosphorothioate containing RNA oligomers following a protocol similar to that reported previously.[14] The reaction mixture was extracted thrice with 2-butanol to remove excess reagent (organic layer), and the RNA was ethanol precipitated from the aquaeous layer. The AP-RNA pellet was resuspended in 0.1 M triethylammonium acetate (TEAA) and further purified to homogeniety by reverse phase HPLC using a C-18 column and 0.1 M TEAA/acetonitrile gradient. The two diastereomers of AP-RNA were collected together and used without further diastereomeric resolution.

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(15) AP-microhelix or AP-13 (200 pmol) was 5′-end labeled with [γ-^{32}P]-ATP and T4 polynucleotide kinase and purified by 16% 8 M urea-PAGE.[15] The [^{32}P]AP-RNA was recovered from the gel by by electroelution followed by ethanol precipitation.

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(17) We chose to look for discrimination in binding using duplex substrates because of the ease of mixing and matching G3- and I3-containing 9-mer oligonucleotides with U70- and C70-containing 13-mer AP-oligonucleotides. Duplexes (9+13) are active substrates for aminoacylation by 461N.[18] Furthermore, AP-microhelix^{Ala} and 9+13-AP duplex^{Ala} are also efficient substrates for alanine charging by 461N (data not shown).

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