Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 2755–2758, April 1996
Biochemistry

Protein synthesis editing by a DNA aptamer

(accuracy of protein synthesis/amino acid discrimination/aminoacyl-tRNA synthetase/genetic code)

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Contributed by Paul Schimmel, December 22, 1995

ABSTRACT **Potential errors in decoding genetic information are corrected by tRNA-dependent amino acid recognition processes manifested through editing reactions. One example is the rejection of difficult-to-discriminate misactivated amino acids by tRNA synthetases through hydrolytic reactions. Although several crystal structures of tRNA synthetases and synthetase-tRNA complexes exist, none of them have provided insight into the editing reactions. Other work suggested that editing required active amino acid acceptor hydroxyl groups at the 3′ end of a tRNA effector. We describe here the isolation of a DNA aptamer that specifically induced hydrolysis of a misactivated amino acid bound to a tRNA synthetase. The aptamer had no effect on the stability of the correctly activated amino acid and was almost as efficient as the tRNA for inducing editing activity. The aptamer has no sequence similarity to that of the tRNA effector and cannot be folded into a tRNA-like structure. These and additional data show that active acceptor hydroxyl groups in a tRNA effector and a tRNA-like structure are not essential for editing. Thus, specific bases in a nucleic acid effector trigger the editing response.**

Several aminoacyl-tRNA synthetases catalyze editing reactions that greatly improve the accuracy of protein synthesis (1–4). These reactions hydrolyze misactivated aminoacyl adenylate and misacylated aminoacyl-tRNA linkages.

Isoleucyl-tRNA synthetase is a class I enzyme (5) that also activates valine (to give valyl-AMP) with an efficiency of ≈0.5% compared to that of isoleucine (6). This high error rate is reduced by $tRNA^{Ile}$-induced hydrolysis of the activated valyl-AMP. Like other class I enzymes, the initial site of amino acid attachment for isoleucyl-tRNA synthetase is the 2′-OH of an adenosine at the 3′ end of the molecule (5, 7, 8). [After being attached, the aminoacyl group rapidly migrates back and forth between 2′ and 3′ positions (9).] These hydroxyl groups and the capacity for aminoacylation have been thought to be integral to the editing sites and mechanisms. For example, oxidation of the vicinal 2′-, 3′-hydroxyl groups simultaneously destroys the acceptor function and the ability to induce the hydrolytic editing activity without destroying the capacity to bind to isoleucyl-tRNA synthetase (10). Additional experiments have pointed to the need for both hydroxyl groups for the editing activity (11).

With these observations in mind, we thought that editing might use the 2′-OH as a "tentative" acceptor or as an attacking nucleophile for the hydrolytic reaction. Alternatively and seemingly less likely, the acceptor function of the terminal adenosine might not play a direct role in the editing reaction. For the purpose of exploring further this question, we designed a strategy to select for DNA aptamers (cf. refs. 12–14), which might inhibit or induce the editing activities of isoleucyl-tRNA synthetase. An aptamer of this sort would presumably be directed to a site on the enzyme where it could affect the editing activity without being aminoacylated by virtue of its lacking a 2′-hydroxyl group acceptor.

METHODS AND MATERIALS

Isoleucine tRNA Synthetase. Wild-type isoleucyl-tRNA synthetase was isolated as described (15) from *Escherichia coli* cells harboring a multicopy plasmid containing the gene for *E. coli* isoleucyl-tRNA synthetase. To facilitate aptamer selection, isoleucyl-tRNA synthetase was coupled to CNBr-activated Sepharose (Pharmacia Biotech); the immobilized enzyme possessed full synthetic and editing activities (unpublished results).

Aptamer Selection. The selection reaction mixtures contained 20 mM Hepes, pH 7.5/150 mM NH_4Cl/1 mM $MgCl_2$/10 μg of bovine serum albumin per ml/1 mM 2-mercaptoethanol. The immobilized enzyme was saturated with a tightly bound valyl-adenylate (cf. ref. 16) and bound DNA was eluted from the enzyme with the major $tRNA^{Ile}$ isoacceptor at saturating concentrations. DNAs were amplified by methods of the PCR containing 10 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM dNTPs, and 50 pmol each of the primers 5′-CACGCGTG-GAATTCTACG-3′ and 5′-CATCGCAAGCTTCCAGAG-3′. The amount of bound versus unbound DNA after each selection cycle was determined by visual examination (after agarose/ethidium bromide gel electrophoresis) of the PCR-amplified DNA present in the flow-through compared to the eluted fractions.

Aptamer Characterization. DNA collected from the final selection cycle (cycle 7) was digested with *Eco*RI and *Hin*dIII (New England Biolabs) and cloned into the appropriate sites in the phagemid pTZ19R (Pharmacia Biotech). After transformation into the *E. coli* strain MVII84 (17), colonies were chosen randomly for sequence analysis (United States Biochemical). Melting temperatures were obtained using a Cary 3E UV-visible spectrophotometer equipped with a Cary temperature controller under the selection conditions.

Nitrocellulose Binding Assay. The nitrocellulose binding assay was performed as described (18) under the conditions used for aptamer selection.

Aminoacylation. Aminoacylation reactions were performed at 1–10 μM DNA as described (15).

Effector-Induced Activity. The effector-induced rate of hydrolysis of the enzyme-bound valyl adenylate was determined by following ATP consumption using a modification of a method previously described (17). The reaction mixture contained 150 mM Tris·HCl (pH 7.5), 10 mM $MgCl_2$, 5 mM valine or 50 μM isoleucine, 3 mM [γ-^{32}P]ATP, 2 units of pyrophosphatase per ml (Boehringer Mannheim), 1.5 μM isoleucyl-tRNA synthetase, and 20 μM $tRNA^{Ile}$ or 10 μM DNA. The amino acid concentrations chosen were 10-fold above the respective K_m values (17). Reactions were performed at 25°C with time points being quenched in 25 vol of 7% $HClO_4$ containing 10 mM sodium pyrophosphate. Activated charcoal was added to bind ATP and was removed by centrifugation; [^{32}P]phosphate levels in the supernatant were determined by scintillation counting (19).

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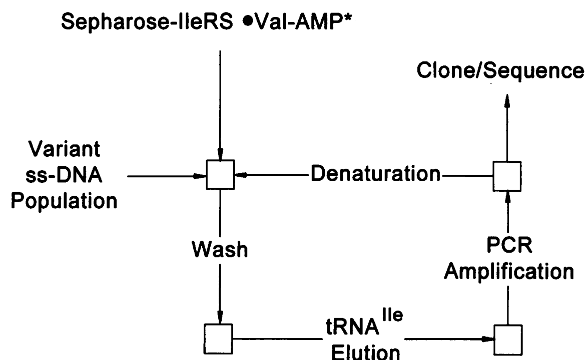

FIG. 1. Schematic diagram of the *in vitro* selection/amplification scheme targeting the tRNA^{Ile}-dependent editing activity of IleRS.

RESULTS

We constructed a library of DNA aptamers made up of 61 nucleotides, which consisted of a central sequence of 25 randomized nucleotides flanked on either side by a fixed 18-nucleotide sequence. The latter served as binding sites for primers of the PCR that was used to amplify specific sequences that emerged from our selection process. Equimolar amounts of each of the four deoxynucleotides were introduced at each position of the randomized segment to give in principal a library of 10^{15} aptamers. In practice, because of physical limitations on the mass of DNA that could be synthesized and used in an experiment, the sequence complexity of our initial library was estimated as 10^{14}. That the initial library was randomized was established by DNA sequencing after PCR amplification and subsequent cloning of the library into a phagemid vector.

To select for aptamers that might affect the editing reaction, isoleucyl-tRNA synthetase was coupled to CNBr-activated Sepharose and subsequently saturated with a tight binding (nonreactive) valyl-AMP analog (cf. ref. 16). The randomized DNA library was passed through a column of the immobilized complex and the bound molecules were eluted with tRNA^{Ile} (Fig. 1). The eluted molecules were then subjected to another round of selection. We observed that, as the number of rounds of selection increased, the amounts of bound (which could be eluted with tRNA^{Ile}) versus unbound DNA progressively

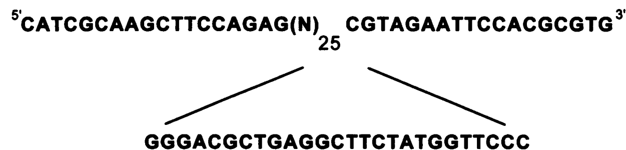

FIG. 2. Overall design of the initial 61-nucleotide DNA molecule with a 25-nucleotide random region being flanked by 18-nucleotide constant regions. After seven cycles of *in vitro* selection and amplification, the major species identified (80% of all selected sequences) is of the inserted sequence presented as DNA^{A}.

increased. By the seventh round, virtually all of the DNA applied to the column was adsorbed and subsequently eluted by tRNA^{Ile}.

Twenty-five randomly chosen clones containing the DNA eluted from the seventh round of selection were subjected to sequence analysis. Of these clones, 20 encoded the identical sequence (designated DNA^{A}; Fig. 2) while each of the others had a similar but unique sequence. We investigated DNA^{A} further and found that it displayed a cooperative melting transition (T_m = 54°C) that was independent of concentration over the range 0.2–2.0 μM. This observation suggested that DNA^{A} had a secondary structure that was formed by unimolecular folding. None of the theoretically possible secondary structures remotely resemble that of a tRNA cloverleaf (data not shown).

We tested this aptamer to see whether it had any effect on the tRNA^{Ile}-dependent selective hydrolysis of valyl-AMP. Although valyl- and isoleucyl-AMP have the same stability to hydrolysis when bound to isoleucyl-tRNA synthetase (19), we found that, remarkably, DNA^{A} specifically induced hydrolysis of the enzyme-bound valyl adenylate (Fig. 3). The rate of hydrolysis induced by saturating amounts of the aptamer was 1.0 s^{-1}, which compares with a rate of 2.1 s^{-1} achieved by induction with saturating amounts of tRNA^{Ile}.

We used the nitrocellulose filter binding assay (18) to measure directly the binding of DNA^{A} to isoleucyl-tRNA synthetase. We determined an apparent K_d of 1.5 μM for the synthetase–aptamer complex under the same conditions used to study the editing activity induced by the aptamer. The dissociation constant for a DNA sample based on the randomized pool was too high to be measured (estimated to be at

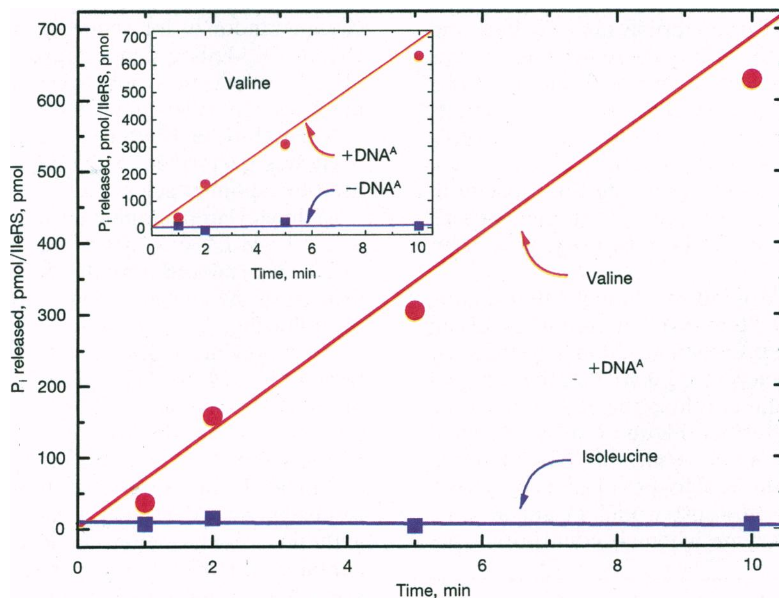

FIG. 3. DNA^{A}-induced ATP hydrolysis in the presence of valine (●) or isoleucine (■). (*Inset*) Comparison of ATP consumption in the presence of valine for reactions with DNA^{A} (●) or without DNA^{A} (■).

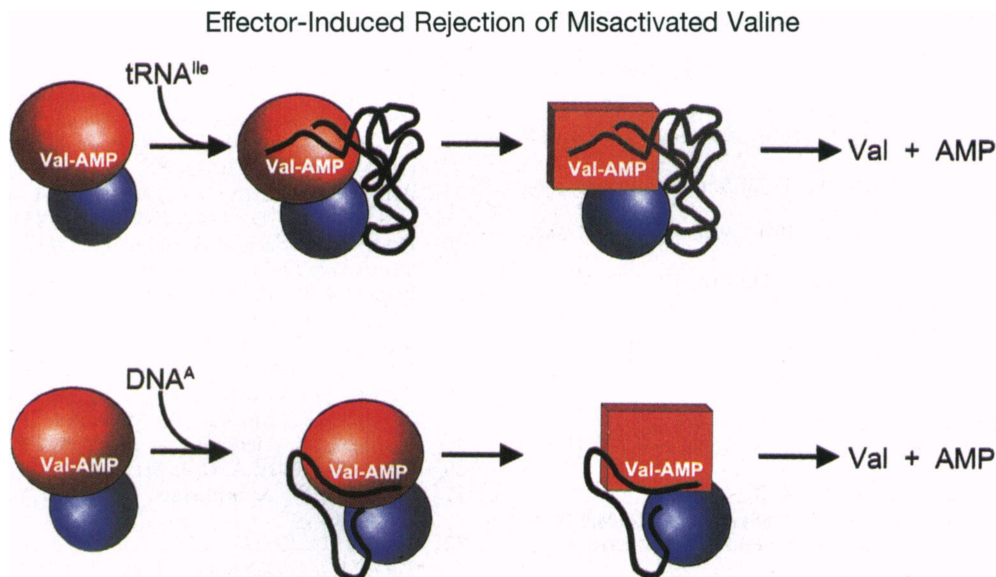

FIG. 4. Proposed effector-induced conformational change of IleRS that triggers discrimination between the correct and incorrectly activated amino acid resulting in rejection of misactivated valine. Isoleucyl-tRNA synthetase is a single polypeptide that is divided into two domains which are represented by red (active site domain) and blue (anticodon binding domain). tRNA and DNA are represented in black.

least 2 orders of magnitude greater than that for DNA^A), suggesting that binding of DNA^A to the synthetase was specific.

Because earlier work had concluded that the acceptor function of $tRNA^{Ile}$ was required for the pretransfer editing activity (10), we determined whether DNA^A was a serendipitous substrate for aminoacylation, even though it lacked the 2′-OH required by all class I enzymes as the initial site of the amino acid molecule (5). However, we could detect no aminoacylation of DNA^A. Thus, an acceptor function is not a necessary part of the site or mechanism for this particular editing reaction.

DISCUSSION

Although x-ray structures of 11 different synthetases are known (20–30), the site(s) and mechanisms for editing have not been clarified by structural analysis. This circumstance occurs either because the synthetases whose structures are solved happen to lack an editing activity or because they have been crystallized without a mischarged or misactivated amino acid. Our results are consistent with a recent genetic analysis showing that the site(s) for editing is operationally independent of that for initial amino acid binding (17). Our data also show a surprising lack of requirement for an acceptor hydroxyl group in the tRNA substrate. Thus, editing is triggered by an effector-induced conformational change that discriminates the correct from the incorrect misactivated amino acid (Fig. 4). Effectors such as $tRNA^{Ile}$ and DNA^A can trigger the same conformational change, suggesting that specific bases in each case are the effectors.

Although the lack of a requirement for an acceptor hydroxyl group is clear from these experiments, we do not know whether the DNA aptamer acts directly, indirectly, or by some combination of both in effecting the discrimination of valine from isoleucine when each is bound to isoleucyl-tRNA synthetase in the form of the respective aminoacyl adenylate. For example, one or more bases in the aptamer could make direct contact with the bound aminoacyl adenylate; alternatively, the aptamer may bind at a site removed from the adenylate and effect a conformational change that results in the hydrolytic response. Current experiments are attempting to crosslink the aptamer to the enzyme as a way to estimate the position of the aptamer relative to the bound adenylate.

Editing reactions catalyzed by tRNA synthetases generally have a "pre-" and a "posttransfer" component (2, 3). In the pretransfer reaction, the addition of the tRNA cognate to the enzyme stimulates hydrolysis of the misacylated adenylate. In the posttransfer reaction, the tRNA is transiently misacylated (for example, to give Val-$tRNA^{Ile}$) and the misattached amino acid is then hydrolytically removed (31). The results reported here show that an aptamer can mimic the tRNA in the pretransfer reaction. In preliminary experiments, we obtained no evidence that the aptamer effects the hydrolysis of Val-$tRNA^{Ile}$ catalyzed by isoleucyl-tRNA synthetase.

Our results demonstrate that aptamers have useful applications for answering questions in mechanistic enzymology, such as the role of an acceptor hydroxyl group in the editing reaction. Although DNA aptamers have been selected to bind small molecule ligands and specific proteins such as thrombin (32), the experiments reported here are the first to show that a DNA aptamer can work in concert with an enzyme to produce a catalytic (turnover) event with high substrate specificity. Because DNA oligonucleotides are relatively easy to synthesize, specific nucleotides needed for the induction of catalytic function can be identified and manipulated with some efficiency. Identification of nucleotides critical for the editing response in the DNA aptamer studied here and in $tRNA^{Ile}$ are currently being investigated.

We thank Professor Jack W. Szostak for comments on this work and manuscript. The work was supported by National Institutes of Health Grant GM 15539. S.P.H. is a National Institutes of Health postdoctoral fellow.

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