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Inhibition by L-aspartol adenylate of a nondiscriminating aspartyl-tRNA synthetase reveals differences between the interactions of its active site with $tRNA^{Asp}$ and $tRNA^{Asn}$.

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Abstract

Asparaginyl-tRNA formation in *Pseudomonas aeruginosa* PAO1 involves a nondiscriminating aspartyl-tRNA synthetase (ND-AspRS) which forms Asp-$tRNA^{Asp}$ and Asp-$tRNA^{Asn}$, and a tRNA-dependent amidotransferase which transamidates the latter into Asn-$tRNA^{Asn}$. We report here that the inhibition of this ND-AspRS by L-aspartol adenylate (Asp-ol-AMP), a stable analog of the natural reaction intermediate L-aspartyl adenylate, is biphasic because the aspartylation of the two tRNA substrates of ND-AspRS, $tRNA^{Asp}$ and $tRNA^{Asn}$, are inhibited with different K_i values (41 μM and 215 μM, respectively). These results reveal that the two tRNA substrates of ND-AspRS interact differently with its active site. Yeast $tRNA^{Asp}$ transcripts with some identity elements replaced by those of $tRNA^{Asn}$ have their aspartylation inhibited with K_i values different from that for the wild-type transcript. Therefore, aminoacyl adenylate analogs, which are competitive inhibitors of their cognate aminoacyl-tRNA synthetase, can be used to probe rapidly the role of various structural elements in positioning the tRNA acceptor end in the active site.

Keywords: *Aspartyl-tRNA synthetase, nondiscriminating, $tRNA^{Asn}$, competitive inhibitor, aspartol-AMP,* Pseudomonas aeruginosa

Introduction

Transfer RNA (tRNA) aminoacylation is the first step of protein biosynthesis. In extant organisms, this step is carried out by a set of aminoacyl-tRNA synthetases (aaRS) whose specific interactions with their cognate tRNAs are crucial for the faithful translation of the genetic information. Most tRNAs are directly aminoacylated correctly by a cognate aaRS, but in some organisms, some tRNAs are first misacylated by a nondiscriminating aaRS (ND-aaRS) [1–2], as in *Pseudomonas aeruginosa* PAO1 where $tRNA^{Asn}$ is aspartylated by an ND-AspRS [3]. The incorrect aminoacyl group is then modified on the tRNA to generate a correctly-acylated tRNA; in the case mentioned, a tRNA-dependent amidotransferase (AdT) [4] converts aspartyl-$tRNA^{Asn}$ into asparaginyl-$tRNA^{Asn}$ [3].

Phylogenetic and structural studies indicate that asparaginyl-tRNA synthetases (AsnRS) emerged from the duplication and divergent evolution of such an ancestral ND-AspRS [1,5–6]. The main identity elements of $tRNA^{Asp}$ for discriminating AspRSs (D-AspRS, such as that of *Escherichia coli*), and those of $tRNA^{Asn}$ for AsnRS are the anticodon and the discriminator base, and are conserved in evolution [7–8]. They share the same discriminator base (G73) and the two first anticodon bases (G34 and U35), but

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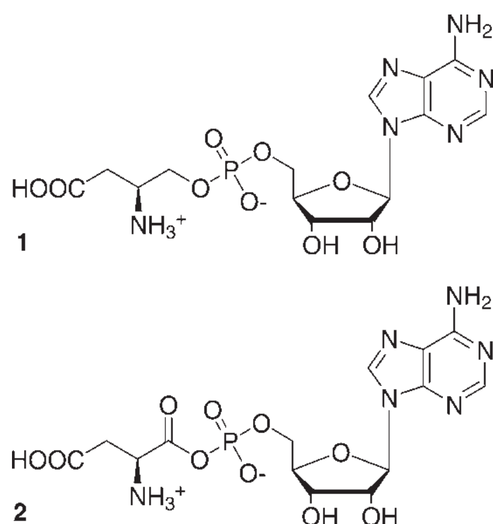

Figure 1. Structure of L-aspartol adenylate (Asp-ol-AMP)(**1**), a stable analog of the natural intermediate L-aspartyl adenylate (**2**) of the aminoacylation reaction catalyzed by AspRS.

differ at position 36. Other minor identity elements, such as C38 present in *Thermus thermophilus* and yeast, are species-specific. As *P. aeruginosa* ND-AspRS aspartylates both $tRNA^{Asp}$ and $tRNA^{Asn}$, one could expect a high degree of similarity between their identity elements.

Asp-ol-AMP (**1**, in Figure 1), a stable analog of aspartyl adenylate (**2**), is a competitive inhibitor of *E. coli* D-AspRS with respect to aspartate, with an inhibition constant (K_i) of about 45 μM [9]. Stable inhibitors of aaRSs have been helpful to produce high-quality crystals of these enzymes, and to study their reaction mechanism [10–11]. Since no structures of bacterial ND-AspRS have been resolved yet, we tested the inhibitory properties of Asp-ol-AMP on the aspartylation of unfractionated *P. aeruginosa* tRNA by *P. aeruginosa* ND-AspRS.

Materials and methods

General

L-aspartol adenylate (Asp-ol-AMP) was synthesized as previously reported [9]. The pUC18-derived plasmids expressing the wild-type, C36U and C38A variants of yeast $tRNA^{Asp}$ [17] were provided by Richard Giegé and Catherine Florentz (IBMC-CNRS and Université Louis Pasteur, Strasbourg, France). [^{14}C]aspartic acid was purchased from Amersham Biosciences, Ni-NTA resin from Qiagen Inc., *Bst*NI and T_7 RNA polymerase from New England Biolabs (NEB), RNase-free DNaseI from Ambion, and RNasin from Promega.

Purification of P. aeruginosa *AspRS,* $tRNA^{Asp}$ *and* $tRNA^{Asn}$

The overproduction of the C-terminal histidine-tagged *P. aeruginosa* AspRS was done using a strain of *P. aeruginosa* ADD1976, harboring a plasmid expressing the *aspS* gene from *P. aeruginosa* PAO1, as previously described [3], and purified to homogeneity using a nickel-containing resin (Ni-NTA). The $tRNA^{Asn}$ and the two $tRNA^{Asp}$ species were purified from unfractionated *P. aeruginosa* tRNA as hybrids with the 24-mer oligodeoxyribonucleotide probes 5′-GACCTGGACTCGAACCAGGGACCC-3′ and 5′-GGACGGGACTCGAACCCGCGACCC-3′ respectively, complementary to their respective T-arms, by polyacrylamide gel electrophoresis under non-denaturing conditions (the detailed experimental procedure will be published elsewhere). After removing the probe by denaturing PAGE, the $tRNA^{Asp}$ and $tRNA^{Asn}$ were recovered by electroelution.

Preparation of wild-type and variant $tRNA^{Asp}$ *from yeast*

Plasmids harboring wild-type or variant yeast $tRNA^{Asp}$ genes were digested with *Bst*NI, and then used for the *in vitro* transcription of the tRNAs. The run-off transcription was made in a reaction mixture containing 40 mM Tris-HCl (pH 7.9), 10 mM DTT, 20 mM $MgCl_2$, 4 mM ATP, CTP, UTP and GTP, 16 mM GMP, 2 mM spermidine, 400 units/mL of RNasin (Promega), 5 units/mL recombinant inorganic pyrophosphatase (Sigma) and 100 μg/mL plasmid digested with *Bst*NI, using 2000 units/mL of T_7 RNA polymerase. Following 5 h of incubation at 37°C, 160 units/mL of RNase-free DNaseI were added to the mixture, and the incubation was maintained for 1 h. Then, the tRNA was ethanol precipitated, and the pellet was resuspended in 30 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$ and 1 mM DTT. The sample was loaded on a MicroSpin G-25 column (Amersham Biosciences) to remove contaminating nucleotides before its use in aminoacylation reactions.

Aminoacylation reactions

Aminoacylation activity of *P. aeruginosa* ND-AspRS on unfractionated tRNA was determined as described [3]. The reaction was done in 100 mM Na-HEPES (pH 7.5), 10 mM $MgCl_2$, 30 mM KCl, 1 mM DTT, 2 mM ATP, 100 μM (~ K_M) [^{14}C]aspartic acid (25 mCi/mmol), 67 μM unfractionated tRNA from *P. aeruginosa*, and 14 μg/mL pure AspRS. For the aminoacylation of *P. aeruginosa* purified $tRNA^{Asp}$ or $tRNA^{Asn}$, the same conditions were used, except for the final concentration of $tRNA^{Asp}$ (0.34 μM) and $tRNA^{Asn}$ (0.14 μM). For the aminoacylation of yeast tRNA transcripts (wild-type, C36U and C38A), final concentrations of tRNA of 0.30 μM, 1.2 μM and 0.30 μM were used, respectively. Incorporation of [^{14}C]Asp on tRNA was detected at various times, and the rates of reaction were used to determine the relative activity (V_i/V) of the AspRS in the presence of Asp-ol-AMP (V_i) versus in the absence of this inhibitor (V).

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Determination of the inhibition constant (K_i)

Assuming that Asp-ol-AMP is a competitive inhibitor of AspRS with respect to aspartate, we could estimate the K_i from the rate of reaction with different concentrations of inhibitor (V_i) over the rate of reaction without inhibitor (V) as follows: $V = V_{max} S/(S + K_M)$, $V_i = V_{max}S/(S + K_M[1 + I/K_i])$, where S and I are the substrate and inhibitor concentrations, respectively. Hence $V_i/V = (S + K_M)/(S + K_M[1 + I/K_i])$. When $S = K_M$ and $I = K_i$, then $V_i/V = 0.66$.

Results

The inhibition by aspartol-AMP of total P. aeruginosa *tRNA aspartylation by AspRS is biphasic*

Unexpectedly, our inhibition data could not be fitted adequately with the competitive inhibition curve $V_i/V = 2/((I/K_i) + 2)$ (results not shown), contrarily to what has been observed for *E. coli* D-AspRS ([9], and see above). On the other hand, these data are fitted correctly by the sum of two competitive inhibition curves. We thus considered the possibility that the K_i for Asp-ol-AMP could be different for the aspartylation of the two very different tRNA substrates of this nondiscriminating AspRS, $tRNA^{Asp}$ and $tRNA^{Asn}$. This possibility implies that the tRNA substrate can influence the interaction of the active site with at least one of the two other substrates, aspartate and ATP. Such an influence was observed for several aaRSs, where the cognate tRNA increases the affinity of the enzyme for its amino acid and/or ATP substrates, and/or is required for the proper positioning of these substrates to allow the amino acid activation reaction to take place. For instance, glutamyl-tRNA synthetase (GluRS), glutaminyl-tRNA synthetase (GlnRS), arginyl-tRNA synthetase and class I lysyl-tRNA synthetase [12–14] cannot activate their amino acid substrate in the absence of tRNA. The molecular mechanism for this tRNA requirement has been elucidated for GluRS, which binds ATP in its active site in the absence of $tRNA^{Glu}$, but does so in such a way that the two reacting groups in the glutamate activation reaction, the α-phosphate of ATP and the α-COOH of glutamate, are too far apart for glutamate activation to occur [10]; in the presence of $tRNA^{Glu}$, ATP binds in another orientation which allows glutamate activation. In the case of GlnRS, and even in the case of tryptophanyl-tRNA synthetase (TrpRS) which can activate its amino acid substrate in the absence of tRNA, the tRNA identity elements influence the binding of the amino acid substrate [15], and therefore alter directly or indirectly the structure of the active site. Moreover, the structure of an inactive complex between yeast $tRNA^{Asp}$ and *E. coli* AspRS suggests that the acceptor stem controls the proper positioning of the amino acid substrate [16]. For these reasons, and considering that the reported nucleotide sequences of $tRNA^{Asp}$ and $tRNA^{Asn}$ (The Genomic tRNA Database (http://lowelab.ucsc.edu/GtRNAdb/Pseu_aeru/Pseu_aeru-summary.html)) differ in regions involved in their specific interactions with *P. aeruginosa* ND-AspRS, such as the acceptor stem, the anticodon stem-loop and the D-stem [17], we tested the possibility that the nature of the tRNA substrate could influence the interaction of Asp-ol-AMP with the active site of *P. aeruginosa* ND-AspRS.

Differential inhibition by aspartol-AMP of $tRNA^{Asp}$ and $tRNA^{Asn}$ aspartylation by AspRS

We thus measured the inhibition by Asp-ol-AMP of pure $tRNA^{Asp}$ and $tRNA^{Asn}$ aspartylation catalyzed by *P. aeruginosa* ND-AspRS (Figure 2). For both $tRNA^{Asp}$ and $tRNA^{Asn}$, the decrease in the rate of tRNA aspartylation as a function of inhibitor concentration is fitted correctly using the competitive inhibition equation $V_i/V = 2/((I/K_i) + 2)$, and yields K_i values of 41 μM and 215 μM Asp-ol-AMP for $tRNA^{Asp}$ and $tRNA^{Asn}$ aspartylation, respectively. The reason for this difference must lie within the nucleotide sequences of these tRNAs (Figure 3). A comparison of these sequences reveal that the main known identity elements of *P. aeruginosa* $tRNA^{Asp}$ are conserved in *P. aeruginosa* $tRNA^{Asn}$, except for two nucleotides located in the anticodon loop of $tRNA^{Asp}$, C36 and C38. The ND-AspRS could recognize $tRNA^{Asp}$ via its complete set of identity elements, and $tRNA^{Asn}$ via the same elements, excluding positions 36 and 38 where U36 and A38 are present in $tRNA^{Asn}$. To test if these identity elements can influence the K_i for Asp-ol-AMP, we used $tRNA^{Asp}$ variants altered in these elements. Transcripts of genes encoding wild-type yeast $tRNA^{Asp}$ and two variants (C36U and C38A) [17] where an identity element in the anticodon loop had been replaced by the corresponding one of $tRNA^{Asn}$, were aspartylated by *P. aeruginosa* AspRS in the presence of Asp-ol-AMP. The inhibition of their aspartylation reveals different K_i values for Asp-ol-AMP (Figure 4): 550 ± 30 μM for wild-type yeast $tRNA^{Asp}$, 280 ± 26 μM for the C38A variant, and 1100 ± 260 μM for the C36U variant.

Discussion

The inhibition patterns by Asp-ol-AMP of tRNA aspartylation by *P. aeruginosa* ND-AspRS, reported here, reveal that this aspartyl-AMP analog inhibits more efficiently the aspartylation of $tRNA^{Asp}$ ($K_i = 41$ μM Asp-ol-AMP) than that of $tRNA^{Asn}$ ($K_i = 215$ μM), the other natural tRNA substrate of this enzyme (Figure 2). The replacement of identity elements of the anticodon loop of $tRNA^{Asp}$ by those of $tRNA^{Asn}$ also alters the K_i (Figure 4). These results show that tRNA substrates of

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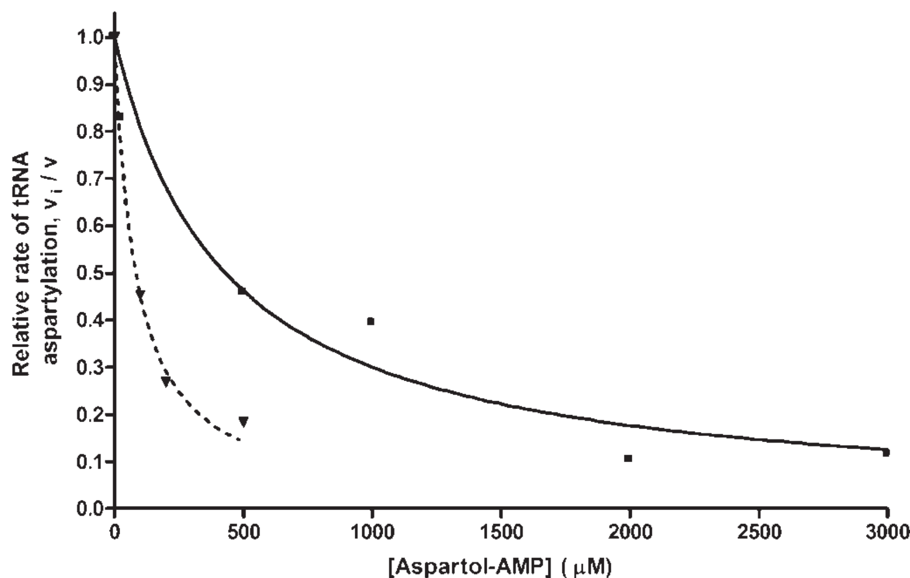

Figure 2. Inhibition by Asp-ol-AMP of pure $tRNA^{Asp}$ and $tRNA^{Asn}$ aspartylation catalyzed by the ND-AspRS of *P. aeruginosa*. ▼, *P. aeruginosa* $tRNA^{Asp}$ aspartylation (K_i = 41 μM; r^2 = 0.9943); ■, *P. aeruginosa* $tRNA^{Asn}$ aspartylation (K_i = 215 μM; r^2 = 0.9625). The straight and dashed lines represent the competitive inhibition curves plotted using the above mentioned inhibition constants, for $tRNA^{Asn}$ and $tRNA^{Asp}$ aspartylation, respectively.

this ND-AspRS, having different identity elements, position their acceptor ends differently into the active site where Asp-ol-AMP is presumed to bind. For this difference in the anticodon loop recognition to influence the K_i values for Asp-ol-AMP, tRNA binding must trigger intramolecular long-range interactions between the anticodon-binding domain in this ND-AspRS and its active site located about 40 Å away. Such interactions have been characterized before in GlnRS [18]. The large difference between the K_i of Asp-ol-AMP for aspartylation of *P. aeruginosa* $tRNA^{Asp}$ (41 μM, Figure 2) versus that for previously characterized yeast $tRNA^{Asp}$ transcripts [17] (553 μM, Figure 4) could be explained by the fact that the *P. aeruginosa* $tRNA^{Asp}$ used has modified nucleotides, whereas the yeast transcripts has none. Moreover, yeast $tRNA^{Asp}$ and *P. aeruginosa* $tRNA^{Asp}$

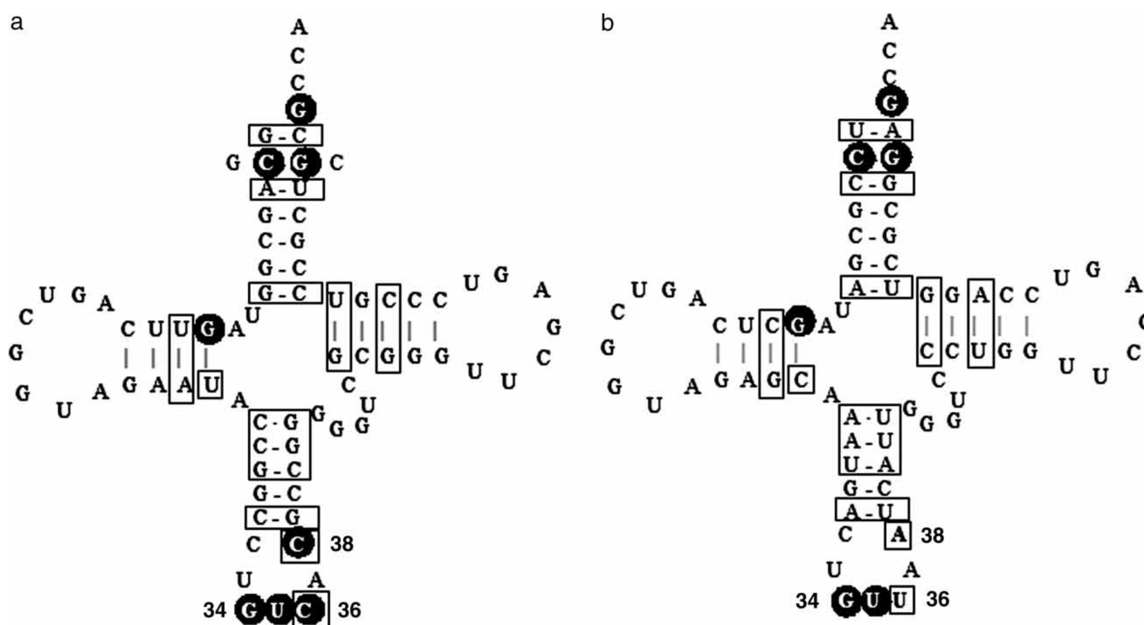

Figure 3. Secondary structures of (a) $tRNA^{Asp}$ and (b) $tRNA^{Asn}$ from *P. aeruginosa* PAO1. The sequences are derived from the genes, and thus do not show the nucleotide modifications. Two species of $tRNA^{Asp}$ exist in *P. aeruginosa*, and are only different at base pair number two (C:G or G:C), as shown in (a). The white letters in black circles represent the identity elements of $tRNA^{Asp}$ [16]. The differences between these two tRNAs are boxed. Some nucleotides in the anticodon loop are identified by their position number in the standard tRNA structure.

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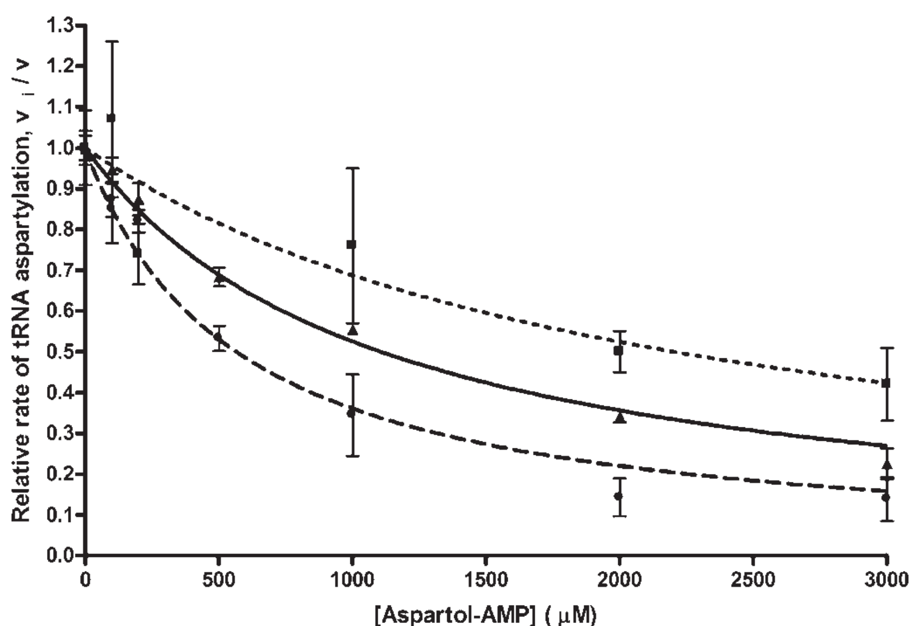

Figure 4. Inhibition by Asp-ol-AMP of yeast – wild-type, and C36U and C38A variants, $tRNA^{Asp}$ transcript aspartylation catalyzed by the ND-AspRS of *P. aeruginosa*. ●, C38A $tRNA^{Asp}$ transcript aspartylation ($K_i = 280\,\mu M$); ▲, wild-type $tRNA^{Asp}$ transcript aspartylation ($K_i = 550\,\mu M$); ■, C36U $tRNA^{Asp}$ transcript aspartylation ($K_i = 1100\,\mu M$). Lines represent the competitive inhibition curves plotted using the above mentioned inhibition constants. The mean ± spread is shown from duplicate (C36U) and standard error from triplicate (wild-type and C38A) experiments.

differ at 27 positions over 76 nucleotides, even if all the $tRNA^{Asp}$ identity elements are present in both molecules (The Genomic tRNA Database (http://lowelab.ucsc.edu/GtRNAdb/)).

Such comparisons of K_i values for an inhibitor bound to the active site provides structural information on the configuration of this site complexed with substrates. This structural information is complementary to that provided by the kinetic parameters K_M, k_{cat} and the specificity constant k_{cat}/K_M [19]. The position of the substrates in the active site has a direct influence on the maximal rate of the reaction, expressed by k_{cat}. This latter parameter plays a major role in defining the specificity of aaRSs for their tRNA substrates [20]. For instance, the identity elements of *E. coli* $tRNA^{Trp}$ influence its ability to bind the reaction intermediate TrpRS/Trp-AMP, affecting predominantly the rate at which the activated tryptophan is transferred to tRNA [21]. It will be interesting to see how the changes in K_i are correlated with changes in k_{cat} for the aspartylation of the tRNA substrates of the *Deinococcus radiodurans* ND-AspRS, which has a 3-fold higher k_{cat} for the aspartylation of $tRNA^{Asn}$ than for that of $tRNA^{Asp}$ [22].

Conclusion

The results reported here indicate that K_i values of the *P. aeruginosa* ND-AspRS for Asp-ol-AMP reflect the position of the acceptor end of the tRNA substrate in the active site. More generally, they reveal that the characterization of the inhibition of an aaRS activity by a stable analog of its aminoacyl adenylate intermediate can be used as a rapid tool to test the influence of any nucleotide of the tRNA substrate or of any residue of the synthetase on this positioning. For comparing the positions of a substrate in the active sites of a set of enzyme variants, K_i values of an inhibitor competitive with respect to this substrate can be obtained at a lower cost in time and material than k_{cat} values, since a precise measurement of the latter may require a titration of the active sites of the variants. Indeed, the altered activity observed for some variants may be due in part to the presence of a certain proportion of inactive enzyme molecules, which may be calculated by active site titration. This method, while used successfully for several aaRSs [23–24], requires a large amount of pure enzyme and is only possible in the case of a two-step reaction where the first step is much faster than the second [23,25]. In contrast, K_i measurements require neither a large amount of enzyme nor active site titration as they are not affected by the presence of inactive variant molecules in the samples tested.

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