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Active Site of an Aminoacyl–tRNA Synthetase Dissected by Energy-Transfer-Dependent Fluorescence

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Abstract—Aminoacyl–tRNA synthetases establish the rules of the genetic code by catalyzing attachment of amino acids to specific transfer RNAs (tRNAs) that bear the anticodon triplets of the code. Each of the 20 amino acids has its own distinct aminoacyl–tRNA synthetase. Here we use energy-transfer-dependent fluorescence from the nucleotide probe *N*-methylanthraniloyl dATP (mdATP) to investigate the active site of a specific aminoacyl–tRNA synthetase. Interaction of the enzyme with the cognate amino acid and formation of the aminoacyl adenylate intermediate were detected. In addition to providing a convenient tool to characterize enzymatic parameters, the probe allowed investigation of the role of conserved residues within the active site. Specifically, a residue that is critical for binding could be distinguished from one that is important for the transition state of adenylate formation. Amino acid binding and adenylate synthesis by two other aminoacyl–tRNA synthetases was also investigated with mdATP. Thus, a key step in the synthesis of aminoacyl–tRNA can in general be dissected with this probe.

Aminoacyl–tRNA synthetases arose early during the evolution of the genetic code.[1] The proteins catalyze the esterification reaction between an amino acid and its cognate tRNA, thereby linking the anticodon triplet genetic code to a specific amino acid.[2] This esterification reaction occurs in two steps:

$$\text{aaRS} + \text{aa} + \text{ATP} \rightleftharpoons \text{aaRS}\cdot(\text{aa-AMP}) + \text{PP}_\text{i}$$
$$\text{aaRS}\cdot(\text{aa-AMP}) + \text{tRNA} \rightleftharpoons \text{aaRS} + \text{aa-tRNA} + \text{AMP}$$

In the first step (termed amino acid activation), the aminoacyl–tRNA synthetase (aaRS) activates the amino acid (aa) through a condensation reaction with ATP to generate aminoacyl adenylate (aa-AMP). In the second step, the activated amino acid is attached to the 3′ end of the cognate tRNA to yield the aminoacylated tRNA (aa-tRNA).

Aminoacyl–tRNA synthetases are organized into two classes of 10 synthetases each, designated as class I and class II.[3–7] This classification is based on the sequences and structures of their active site domains. Isoleucyl–tRNA synthetase (IleRS) is a typical class I aminoacyl–tRNA synthetase whose active site consists of a Rossmann nucleotide-binding fold of alternating β-strands and α-helices.[7–10] *Escherichia coli* IleRS is a monomer consisting of 939 amino acids. The gene encoding for the protein is essential for cell viability. This then raises the possibility of exploiting the enzyme as a target for novel antibiotics, particularly because inhibitors can be obtained that block microbial enzyme but not the human counterpart.[11] In addition, point mutations in IleRS that result in reduced amino acid activation exhibit a dominant negative effect in vivo. For example, expression of a plasmid-encoded mutant enzyme defective in amino acid activation in a cell containing the wild-type chromosomal allele of IleRS resulted in cell death.[12] Lethality occurs because the defective enzyme sequesters cellular tRNA without being able to achieve aminoacylation. This observation raises the possibility of using engineered genes for defective synthetases of pathogens in specific therapeutic applications.

Analysis of the catalytic mechanism of the active site is a key step towards utilizing any enzyme as a target for drug development or for gene-based therapy. The

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crystal structure of *Thermus thermophilus* IleRS bound to isoleucine provides an initial snapshot of the mechanism of isoleucine activation.[10] The bound amino acid in this structure is in close proximity to several highly conserved residues. Substitutions of some of these conserved residues have been made in the *E. coli* enzyme, and the effects of these substitutions were initially examined by an ATP–pyrophosphate exchange assay. In this assay, the activity of the enzyme is measured by following the reverse reaction of adenylate formation; [^{32}P]-PP_i is added to the enzyme in the presence of amino acid and ATP, and the incorporation of [^{32}P]-PP_i into ATP is determined.[13] Using this approach, amino acid activation was observed to be completely abolished in two mutant proteins that have substitutions in the amino acid binding site—D96A and G56P IleRS.[12,14] On the other hand, a G56A substitution resulted in an enzyme that retained activity, but had a significantly higher K_m (7 mM) for isoleucine activation than did the wild-type enzyme (4 μM).[13]

To extend these studies, the fluorescent nucleotide mdATP was used to investigate both the binding of amino acid and synthesis of aminoacyl adenylates in wild-type and mutant IleRS.[15] Using methods described here and elsewhere,[15–19] this analysis enabled us to distinguish between mutations that blocked binding from those that severely attenuated the synthesis of Ile-AMP (k_{cat} defect). The results of these studies provided a clearer understanding towards the adenylate synthesis mechanism. In addition, the versatility of this approach was explored by examination of amino acid interactions of two other aminoacyl–tRNA synthetases.

Rationale for Monitoring Active Site with a Probe

The fluorescent nucleotide mdATP (Fig. 1a) competitively inhibits binding of aminoacyl adenylates to the active site of IleRS.[15] Figure 1b shows the excitation and emission spectra of mdATP (solid lines), together with the tryptophan emission spectrum of IleRS (dashed line, 295 nm excitation). The excitation spectrum of mdATP has a peak at 360 nm that overlaps well with the tryptophan emission spectrum. In addition, the emission spectrum of tryptophan (peak at 330 nm) is well separated from that of mdATP (peak at 440 nm). These circumstances raised the possibility that the interaction between IleRS and mdATP can be visualized by energy transfer between endogenous tryptophan residues of IleRS and mdATP.

The rationale for the fluorescence assay that monitors the occupation of the synthetase active site with mdATP versus with aminoacyl adenylates is illustrated in Figure 1c. When bound to IleRS, mdATP is close to one or more tryptophans in the active site region, so that excitation at 295 nm leads to energy-transfer-dependent emission at 440 nm. The mdATP binds with a K_d of approximately 1 μM compared to a K_d for the adenylate of <10 nM. Hence, the displacement of mdATP from the active site by the more tightly bound aminoacyl adenylates leads to a loss of energy transfer that results in a decrease in emission at 440 nm. As a consequence, energy-transfer-dependent emission at 440 nm measures the fractional occupation of the active site with mdATP versus with aminoacyl adenylates.

When mdATP is complexed to IleRS, the subsequent addition of isoleucine and ATP results in formation of Ile-AMP, leading to the displacement of mdATP from the active site (pyrophosphatase is included to ensure that formation of Ile-AMP goes to completion) (Fig. 2a). This, in turn, results in a decrease in emission at 440 nm. In this particular assay, the concentration of ATP used is always less than the concentration of mdATP, so that ATP will not displace mdATP by itself. Instead, in the presence of amino acid, it is the enzymatic formation of the tightly bound adenylate that allows mdATP to be displaced from the active site. Hence, the rate of fluorescence decrease directly corresponds to the rate of adenylate formation, and this can be estimated by a single exponential fit (Fig. 2b) (Because the concentration of ATP ($\sim$1.2 μM) is significantly less that the K_m for ATP (440 μM),[14] the velocity of the reaction is well below V_{max}.) Increasing the concentration of isoleucine results in an increase in the rate of the fluorescence decrease. The reciprocals of the observed rate constants at each Ile concentration were plotted versus the reciprocals of the Ile concentrations (Fig. 2c). The points were fit to a line, and the x-intercept of this line corresponds to the negative reciprocal of the K_m for amino acid activation.

Using this fluorescence assay, a K_m value of 3.2 μM for isoleucine activation was determined. This K_m is in good agreement with the previously determined K_m for isoleucine activation (4 μM), which was measured by the isoleucine dependent ATP–PP_i exchange assay.[13] At the ATP concentration used here (1.2 μM), the observed $k_{cat}-k_{cat,obs}$ is 0.15 s^{-1}. Assuming the usual linear dependence between k_{cat} and ATP concentration (at ATP concentrations below the K_m (440 μM)), the true k_{cat} for isoleucine activation (at saturating ATP) was estimated to be 55 s^{-1} at pH 7.5, 20 °C, in good agreement with the measured k_{cat} of 28 s^{-1} at pH 8.0, 37 °C[13] (the discrepancy may be due to the different conditions used in the two assays).

This approach is general and can, therefore, be used to determine the K_m's for other amino acids activated by IleRS. In particular, certain amino acids are activated provided that they are smaller than Ile and fit into the binding pocket. For example, in the case of valine, whose isopropyl side chain lacks one methylene group compared to the isobutyl group of isoleucine, a K_m of 390 μM was determined (Fig. 2d). This value is in good agreement with the previously determined K_m of 450 μM.[13]

Application to Enzymes with Point Mutations in Active Site

The aforementioned fluorescence assay provides a convenient tool for establishing the roles played by conserved residues in the active site. For example, G56

of *E. coli* IleRS is highly conserved. In the crystal structure of *T. thermophilus* IleRS, the equivalent glycine (G45) is in close proximity to the bound Ile side chain, suggesting that the glycine is conserved to ensure that the active site is able to accommodate the isobutyl side chain of Ile.[10] Substituting the glycine for alanine (G56A IleRS) results in the introduction of an additional methyl group within the active site, leading to the partial steric occlusion of Ile. Still, activation can be detected,[13] thereby allowing determination of the K_m's for both isoleucine and valine (using the fluorescence assay). As expected, the K_m for the activation of Ile by G56A IleRS is increased ~3000-fold (9.5 mM vs 3.2 μM for wild-type IleRS). In contrast, the increase in the K_m for Val activation is only ~4-fold (1.5 mM vs 390 μM for wild-type IleRS). Because valine is smaller than isoleucine by a single methylene group, the additional methyl group in the active site resulting from the G56A substitution has a much smaller effect on Val binding.

On the other hand, when the glycine was substituted with proline (G56P IleRS), amino acid activation was no longer detectable in the ATP–PP_i exchange assay.[14] In the fluorescence assay, no significant energy transfer between G56P IleRS and mdATP was detected (data not shown). Thus, G56P IleRS appears unable to bind mdATP. This result suggests that, as a result of the G56P substitution, the structure of the active site is disrupted in a way that affects mdATP (and probably amino acid) binding.

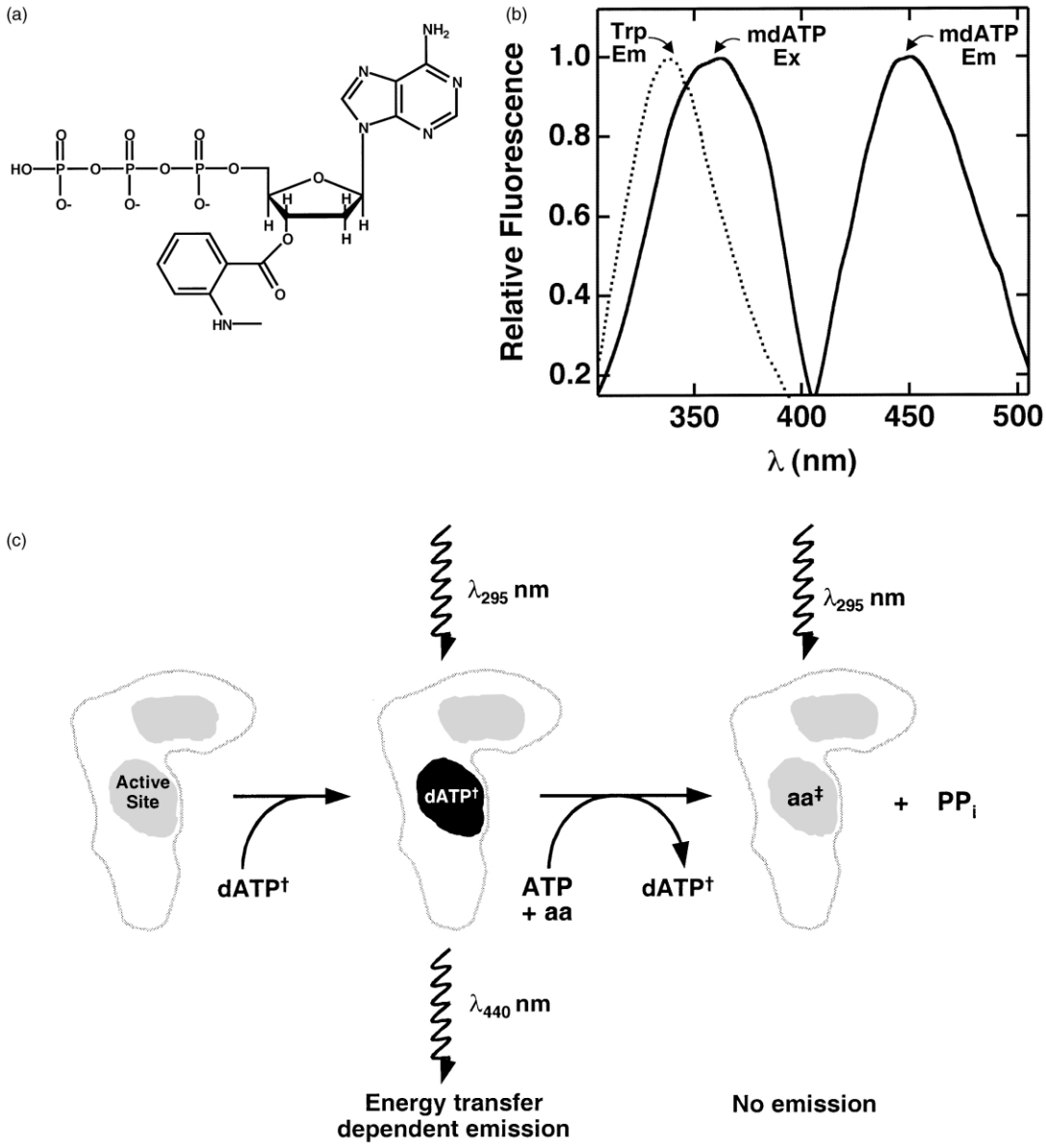

Figure 1. Fluorescence energy transfer between *N*-methylanthraniloyl dATP (mdATP) and tryptophan residues of IleRS. (a) Structure of mdATP with the anthraniloyl group attached to the 3′-OH of ATP. (b) Excitation and emission spectra of mdATP (solid lines) and the tryptophan emission spectrum of IleRS (dashed line). (c) The binding of mdATP to the enzyme's active site results in energy-transfer-dependent emission at 440 nm. The mdATP nucleotide can be displaced from the active site by an aminoacyl adenylate (aa‡), resulting in the loss of energy-transfer-dependent emission at 440 nm.

D96 of *E. coli* IleRS is highly conserved throughout class I tRNAs synthetases.[20] The equivalent residue in *E. coli* MetRS is D52. This aspartate was substituted with alanine to give D52A MetRS. Although it exhibited no significant defect in binding either ATP or methionine, the k_{cat} for adenylate formation by D52A MetRS was found to be reduced by 10^{-4}.[21] This result showed that the aspartate is primarily involved in the transition state of amino acid activation.

As was observed for the G56P substitution, D96A IleRS showed no detectable activity in the ATP–PP_i exchange assay.[12] However, in contrast to G56P IleRS, significant energy transfer between the enzyme and bound mdATP was observed. Thus, the active site was not disrupted by the G56P mutation. Due to the inability of the enzyme to synthesize Ile-AMP, the addition of isoleucine and ATP (in the presence of pyrophosphatase) did not result in any decrease in mdATP fluorescence (Fig. 3a). To verify that the active site of D96A IleRS retained its ability to interact both with ATP and amino acid, the mdATP nucleotide was displaced with chemically synthesized Ile-AMP. For both wild-type IleRS and the D96A point mutant, the concentrations of Ile-AMP required to displace mdATP were almost identical (concentrations of Ile-AMP at which displacement of mdATP was half-maximal were 407 and 330 nM, respectively (Fig. 3b and 3c). This result supports the

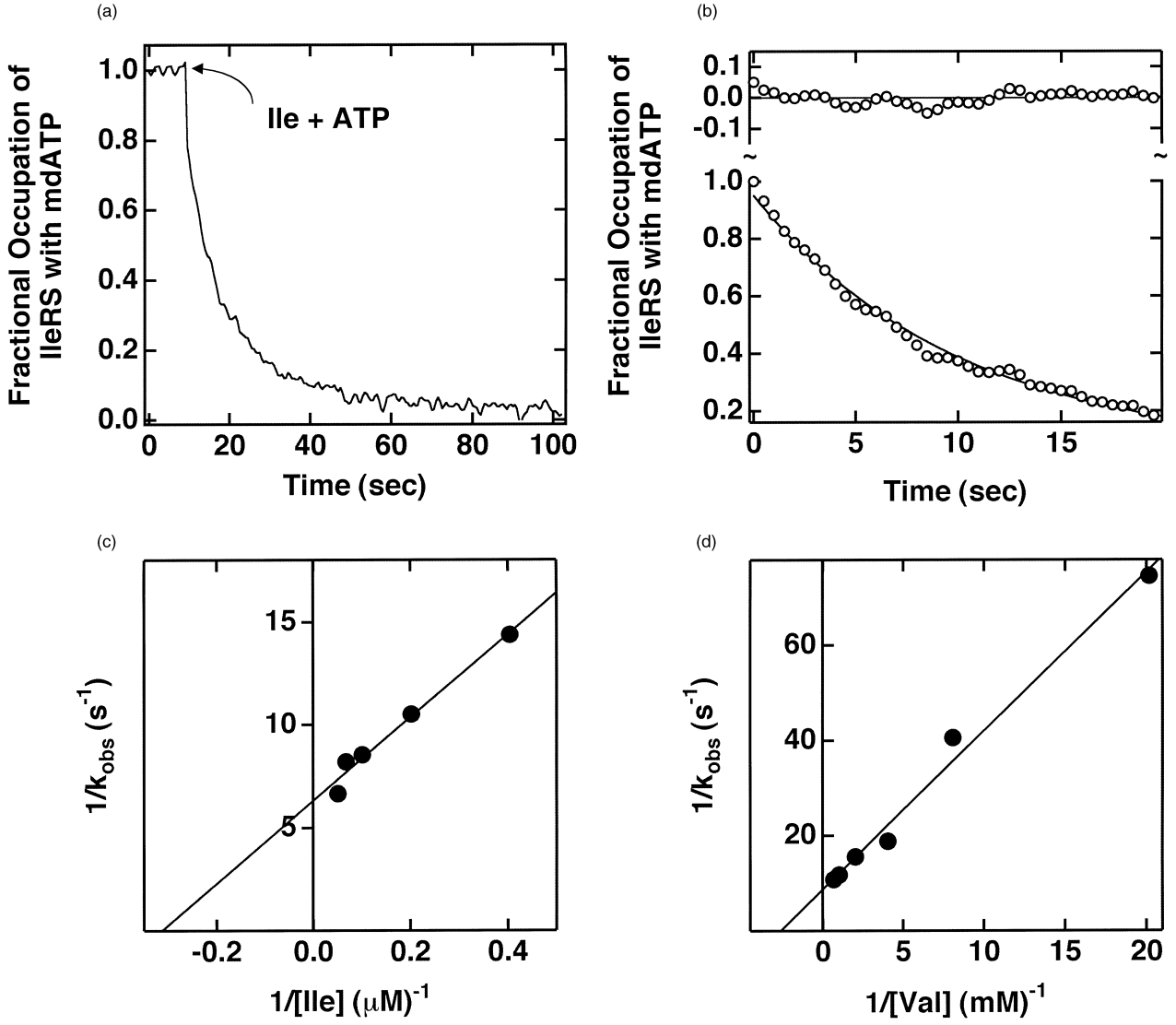

Figure 2. Time-dependent displacement of mdATP by the enzymatic formation of aminoacyl adenylate. (a) IleRS (700 nM) was incubated with mdATP (8 μM) and pyrophosphatase (2 nM), and energy-transfer-dependent emission was monitored (excitation = 295 nm, emission = 440 nm). Isoleucine (6.2 μM) and ATP (1.2 μM) were then added (arrow) to generate isoleucyl adenylate. (b) The observed rate constant for the displacement of mdATP by the formation of isoleucyl adenylate was estimated by a single exponential fit (lower panel). The residual plot (showing the difference between the experimental data and the fit for each observed point) is shown in the upper panel. (c) The observed rate constants for the displacement of mdATP by isoleucyl adenylate were determined over a range of isoleucine concentrations. The reciprocals of the observed rate constants were plotted versus the reciprocals of the isoleucine concentration, and the points were fit to a line so as to determine the K_m for the activation of isoleucine by the enzyme. (d) Identical to (c) except that observed rate constants were measured for the displacement of mdATP by the enzymatic formation of valyl adenylate over a range of valine concentrations.

notion that the defect caused by the D96A substitution does not alter the ability of the enzyme to interact with the amino acid and ATP. Instead, the catalytic event per se is eliminated.

Application to Other Aminoacyl tRNA Synthetases

Having demonstrated that mdATP can be used to measure the occupancy of the IleRS active site with aminoacyl adenylates versus mdATP, we examined whether the assay could be applied to other aminoacyl–tRNA synthetases. For this purpose, we chose two other class I synthetases that are closely related to IleRS: valyl– and methionyl–tRNA synthetases (ValRS and MetRS, respectively). We observed energy transfer between mdATP and tryptophans of each enzyme. Here again, for both enzymes, adenylate formation could be followed by the displacement of mdATP from the active site (Fig. 4a and 4b, respectively). However, the success with which the assay was applied to these different aminoacyl–tRNA synthetases varied considerably. In the case of IleRS, the fluorescence increase upon binding mdATP was about 30%, while for ValRS, this fluorescence increase was about 50%. In contrast, the corresponding fluorescence increase for MetRS was only 8% (hence the increase in noise in Fig. 4b).

Discussion

An understanding of the specific tryptophans that contribute to the energy transfer would be an important step towards identifying aminoacyl–tRNA synthetases for which this fluorescence assay is applicable.

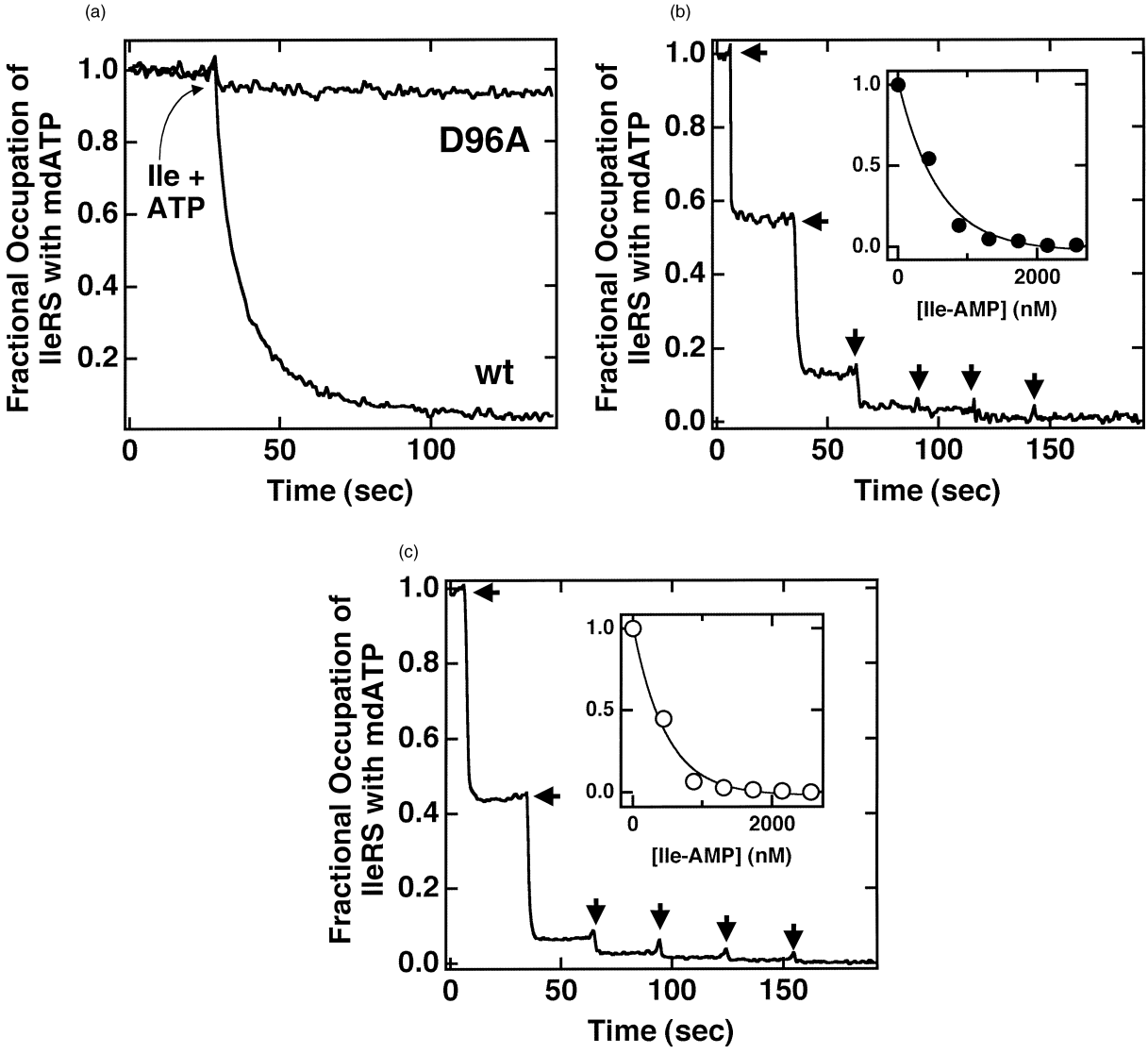

Figure 3. Comparison of wt IleRS with D96A IleRS. (a) Either wild-type IleRS (700 nM) or D96A IleRS (700 nM) was incubated with mdATP (8 μM) and pyrophosphatase (2 nM), and energy-transfer-dependent emission was monitored (excitation = 295 nm, emission = 440 nm). Isoleucine (15 μM) and ATP (2.5 μM) were then added to generate isoleucyl adenylate. (b) Wild-type IleRS (700 nM) was incubated with mdATP (5 μM), and energy-transfer-dependent emission was monitored (excitation = 295 nm, emission = 440 nm). Aliquots (~440 nM) of chemically synthesized Ile-AMP were added at different times (arrows) to displace mdATP from the active site. The displacement of mdATP upon addition of each aliquot of Ile-AMP was plotted versus the Ile-AMP concentration (inset). (c) Identical to (b), except that D96A IleRS (700 nM) was used instead of the wild-type enzyme.

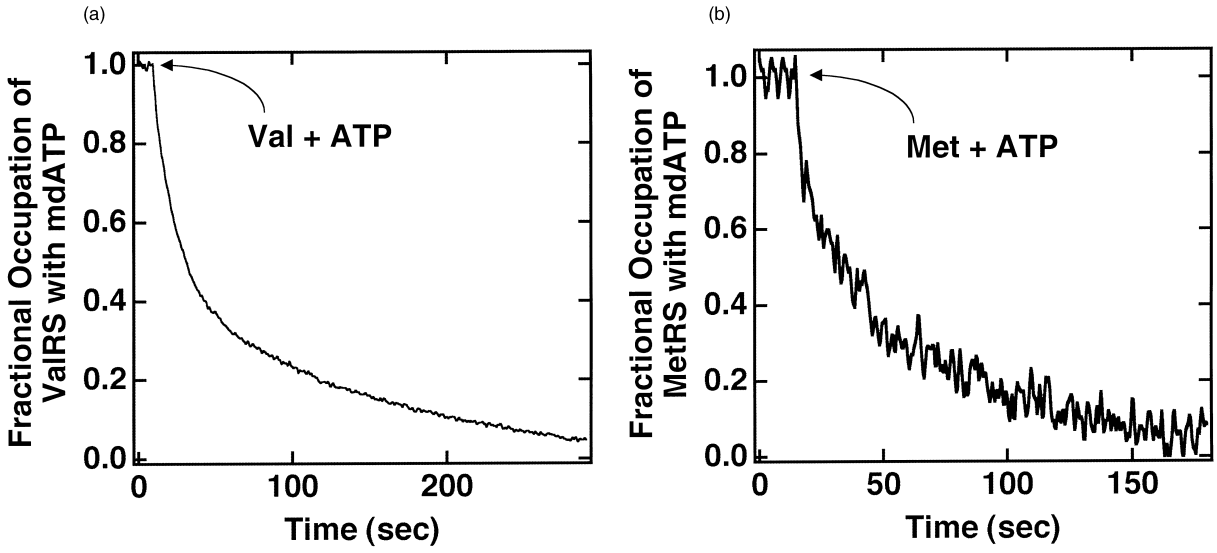

Figure 4. Fluorescence energy transfer between mdATP and the tryptophans of ValRS and MetRS. (a) ValRS (830 nM) was incubated with mdATP (8 μM) and pyrophosphatase (2 nM), and energy-transfer-dependent emission was monitored (excitation = 295 nm, emission = 440 nm). Valine (15 μM) and ATP (1.2 μM) were then added to generate valyl adenylate. (b) Identical to (a) except that MetRS (700 nM) was used instead of ValRS, and methionine (15 μM) was used instead of valine.

Alternatively, specific tryptophans could be introduced into a synthetase to improve its energy-transfer efficiency with mdATP. The Förster distance (R_0) (i.e., the distance between the donor and acceptor probes at which the energy transfer efficiency is 50%[22]) for a tryptophan–mdATP donor/acceptor pair is about 21 Å.[23] The structure of *Staphylococcus aureus* IleRS complexed with $tRNA^{Ile}$ and mupirocin was examined to identify tryptophans close to mdATP.[24] Using mupirocin to approximate the position of mdATP, the distances from mupirocin to the equivalent tryptophans in *E. coli* IleRS were determined. Five tryptophans were identified within 15 Å from mupirocin (efficiency of energy transfer ~90%). Amongst these tryptophans, three are conserved in ValRS, while only one is conserved in MetRS.[25] Hence, the energy transfer efficiency between the enzyme and mdATP is significantly better in ValRS than in MetRS. Introducing additional tryptophans at these positions in MetRS could improve energy transfer between the enzyme and mdATP.

In the case of class II synthetases, *E. coli* alanyl–tRNA synthetase (AlaRS) and *E. coli* threonyl–tRNA synthetase (ThrRS) were examined. In neither case was energy transfer observed between the enzyme and mdATP. The structure of *E. coli* ThrRS bound to a threonyl adenylate analogue has been determined[26] (a structure is not available for the alanine enzyme). Only a single tryptophan was identified within 15 Å of the active site. Hence, the absence of energy transfer between ThrRS and mdATP may be due to the lack of tryptophans close to mdATP. However, we cannot rule out the possibility that structural differences between the active sites of class I and class II aminoacyl–tRNA synthetases may sterically occlude the anthraniloyl moiety from the class II active site.

Compared to the conventional ATP–PP_i exchange assay, a limitation of the mdATP fluorescence assay is that the amount of ATP added to generate adenylate is significantly below the K_m for ATP; hence, this assay cannot directly measure the k_{cat} for amino acid activation, although reasonable estimates can be made (see above). The mdATP probe has also been useful for investigation of tRNA binding and of editing of misactivated amino acids.[15] However, studies with tRNA are somewhat limited because of the inner-filter effect from the UV-absorbing tRNA nucleotides that causes a quenching of mdATP fluorescence, leading to poor signal to noise ratios. Nevertheless, the fluorescence assay has provided information beyond that obtained by ATP–PP_i exchange assay. Specifically the assay allows distinguishing mutations that disrupt binding of amino acid or adenylate from those that affect k_{cat}. For example, the D96A and G56P IleRS mutants appeared to have identical defects in the ATP–PP_i exchange assay. However, in the fluorescence assay, D96A is seen to be specifically defective in the transition state of amino acid activation, while the G56P mutant appears to result in a more global disruption of the active site of the enzyme. It is also worth noting that, because binding of adenylates to the active site displaces mdATP, the fluorescence assay could also be exploited to screen directly for active site inhibitors of aminoacyl–tRNA synthetases.

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