

COMMUNICATION

## Amino Acid Activation of a Dual-Specificity tRNA Synthetase is Independent of tRNA

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Transfer RNA can play a role in amino acid activation by aminoacyl-tRNA synthetases. For the prolyl-tRNA synthetase (ProRS) of *Methanococcus jannaschii*, which activates both proline and cysteine, the role of tRNA in amino acid selection and activation is of interest in the effort to understand the mechanism of the dual-specificity. While activation of proline does not require tRNA, whether or not tRNA is required in the activation of cysteine has been a matter of debate. Here, investigation of a series of buffer conditions shows that activation of cysteine occurs without tRNA in a wide-range of buffers. However, the extent of cysteine activation is strongly buffer-dependent, varying over a 180-fold range. In contrast, the extent of proline activation is much less sensitive to buffer conditions, varying over only a 36-fold range. We also find that addition of tRNA has a small threefold stimulatory effect on cysteine activation. The lack of a major role of tRNA in activation of cysteine suggests that the dual-specificity enzyme must distinguish cysteine from proline directly, without the assistance of each cognate tRNA, to achieve the necessary specificity required for protein synthesis.

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Transfer RNAs decode genetic information by carrying amino acids that match the anticodon triplets of the genetic code. For each tRNA, aminoacylation is catalyzed by its cognate aminoacyl-tRNA synthetase (aaRS) in a two-step reaction. The first step involves activation of an amino acid with ATP to form an enzyme-bound aminoacyl-adenylate intermediate, while the second step is transfer of the activated amino acid to the tRNA.<sup>1,2</sup> Due to the close chemical or structural similarity of some amino acids, synthetases have developed specific mechanisms to activate the cognate amino acid and discriminate against sterically smaller or isosteric non-cognate substrates.<sup>3,4</sup> Although tRNA is a substrate for the second step of aminoacylation, it may also play an active role in the first step. For example, three of the 20 synthetases, ArgRS,

GluRS, and GlnRS, carry out tRNA-dependent amino acid activation,<sup>1</sup> where interactions between tRNA and synthetase are required for synthesis of the cognate aminoacyl-adenylate.<sup>5</sup> Another example is the tRNA-dependent pre-transfer editing reaction that hydrolyzes and removes mis-activated amino acids prior to transfer to tRNA. This mechanism is used by IleRS which mis-activates valine,<sup>6–11</sup> by ValRS which mis-activates threonine,<sup>12–14</sup> and by LeuRS which mis-activates homocysteine and several non-natural amino acids.<sup>15–18</sup> The participation of tRNA in amino acid activation and editing for some synthetases suggests the role of tRNA as a co-factor throughout the two-step process of aminoacylation.

The selection and specific activation of amino acids is a particularly challenging problem for a recently identified dual-functional tRNA synthetase. The ProRS of *Methanococcus jannaschii* not only recognizes and activates proline but also cysteine.<sup>19,20</sup> This enzyme possesses all the signature sequences of ProRS,<sup>21</sup> but lacks those of CysRS,<sup>22</sup> and thus its ability to activate cysteine is unexpected. The dual-specificity ProRS is also found in several other archaeal methanogens,<sup>19</sup>

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Abbreviations used: RS, tRNA synthetase.

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such as *Methanococcus maripaludis* and *Methanobacterium thermoautotrophicum*, and in the deep rooted eucarya *Giardia lamblia*.<sup>23</sup> The presence of the dual-specificity ProRS in *M. jannaschii* and *M. thermoautotrophicum* is biologically significant, as these organisms lack the gene for the traditional CysRS. Although the genome of *M. jannaschii* also encodes the gene for a non-canonical CysRS that does not contain sequence motifs of known synthetases,<sup>24</sup> this novel gene is absent from *M. thermoautotrophicum*. Thus, at least in *M. thermoautotrophicum*, the dual-specificity enzyme appears to be responsible for activation of cysteine and synthesis of cysteinyl-tRNA<sup>Cys</sup>.

An interesting feature of the dual-specificity ProRS from *M. jannaschii* is that the cysteine binding site appears to be extensively overlapped with the proline binding site. Analogs of proline (such as thiaproline and prolinamide) inhibit synthesis of not only prolyl-adenylate but also cysteinyl-adenylate,<sup>19,20</sup> and excess of proline or cysteine (unlabeled) inhibits transfer of labeled cysteine to tRNA<sup>Cys</sup> or labeled proline to tRNA<sup>Pro</sup>.<sup>19</sup> Mutational studies have identified enzyme residues that selectively affect activation of proline or cysteine, and these residues are located proximal to one another.<sup>25</sup> One solution to the problem of using an overlapping site to properly activate proline and cysteine might be to use tRNA<sup>Pro</sup> or tRNA<sup>Cys</sup> as a co-factor to help selection of the respective amino acid. Thus, the recent reports that activation of cysteine is dependent on tRNA<sup>Cys</sup> by the dual-specificity *M. jannaschii* ProRS are of interest,<sup>19,25</sup> as activation of proline is not tRNA-dependent.<sup>19,20</sup> However, we have found that activation of cysteine by this enzyme occurs in the absence of tRNA<sup>Cys</sup>.<sup>20,26</sup> For example, we have observed activation of cysteine and transfer of the activated amino acid to an unmodified transcript of tRNA<sup>Pro</sup>.<sup>27</sup>

The question of whether tRNA<sup>Cys</sup> is a necessary co-factor for activation of cysteine is important for the general understanding of the dual-specificity, which changes the traditional view of one synthetase for one amino acid. The mechanism by which this enzyme functions may also shed light on how a primordial synthetase of multiple-specificity evolved to exact amino acid-specificity.<sup>28,29</sup> We are particularly interested in the question of whether *M. jannaschii* ProRS uses or requires tRNA to achieve specificity, which has been the subject of debate. We noted that the various published studies reporting activation of cysteine by *M. jannaschii* ProRS were carried out using different buffer conditions. Those reporting tRNA-dependent activation were performed in a Hepes buffer system,<sup>19,25</sup> while those reporting tRNA-independent activation were carried out in Tris buffer.<sup>20,26,27</sup> To understand the discrepancy between these reports, we investigated the buffer-dependence of amino acid activation by *M. jannaschii* ProRS. The studies presented here help to

clarify the role of tRNA in activation by the dual-specificity enzyme.

### Activation of cysteine does not require tRNA

A recombinant clone of *M. jannaschii* ProRS was purified to homogeneity by taking advantage of a His-tag.<sup>20</sup> The purified enzyme, when assayed for aminoacylation with cysteine in the absence of exogenous tRNA<sup>Cys</sup>, yielded less than 0.01% of charged tRNA per mole of enzyme. This indicated that the enzyme was free of tRNA contamination. The capacity of the enzyme to activate cysteine and proline in the absence of tRNA was evaluated under a variety of reaction conditions (Table 1). The activation was measured at 65°C by the [<sup>32</sup>P]PPi-ATP exchange reaction, which monitors the enzyme-catalyzed conversion of PPi to ATP, the reverse direction of aminoacyl-adenylate synthesis.<sup>30</sup> This assay was used to report both the tRNA-dependent and tRNA-independent reactions of activation of cysteine.<sup>19,20,25,26</sup> The previously used standard Hepes buffer (condition 6<sup>25</sup>) and Tris buffer (condition 1<sup>20</sup>) were included in the test. Components present in the standard buffers such as KF, KCl, BSA, β-mercaptoethanol, DTT, and PPi were varied (Table 1). In addition, the imidazole buffer (condition 12) previously used to assay amino acid activation by *Escherichia coli* MetRS was tested,<sup>31,32</sup> as well as Pipes (condition 13) and triethanolamine (condition 14) buffers. Amino acid activation was monitored by the initial rate of the exchange reaction ( $V_o/[S]/[E]$ ), and the activity was expressed in units of M<sup>-1</sup> s<sup>-1</sup>. As shown in Table 1, all buffer conditions supported activation of both proline and cysteine in the absence of tRNA. In particular, cysteine activation was observed (0.027 unit) in the standard Hepes buffer (condition 6<sup>25</sup>) previously reported to require tRNA for activation. The previously reported tRNA-independent cysteine activation activity (0.084 unit) was also observed in the standard Tris buffer (condition 1).

The extent of activation of cysteine without tRNA was highly variable, depending on buffer conditions. The highest activity (0.23 unit) was achieved in condition 7, which was created by replacing Hepes (100 mM, pH 7.2) with Tris (144 mM, pH 7.5) in the standard Hepes buffer (condition 6). Thus, by simply substituting Tris for Hepes, the activity was increased from 0.027 to 0.23 unit, an improvement of almost tenfold. Conversely, substitution of Tris (144 mM, pH 7.5, condition 1) with Hepes (100 mM, pH 7.5, condition 3) in the standard Tris buffer condition decreased the cysteine activity by nearly tenfold from 0.084 to 0.011 unit. Other components that also had an effect on activation of cysteine were PPi and reducing agents. A twofold increase in the PPi concentration (from 1 to 2 mM) decreased the cysteine activity by fourfold (conditions 2 and 5). Similarly, replacement of 10 mM β-mercaptoethanol with 40 mM DTT decreased the activity by fourfold

**Table 1.** Activation of cysteine and proline by *M. jannaschii* ProRS in the absence of added tRNA

Condition	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Cys ( $10^{-2}$ unit)	8.4	1.6	1.1	0.43	0.43	2.7	23	0.76	0.13	1.2	2.2	1.7	0.25	0.32	17
Pro (units)	3.7	4.1	3.6	3.8	1.1	22	7.6	2.2	1.1	1.1	2.6	7.2	1.7	1.6	40
Pro/Cys	44	260	330	880	260	810	33	290	850	92	120	420	680	500	240
Tris (7.5)	144	-	-	-	-	-	144	0.1	1	5	10	-	-	-	100
Hepes (7.2)	-	100	-	-	-	100	-	-	-	-	-	-	-	-	-
Hepes (7.5)	-	-	100	100	100	-	-	100	100	100	100	-	-	-	-
IMDZ (7.0)	-	-	-	-	-	-	-	-	-	-	-	20	-	-	-
Pipes (7.2)	-	-	-	-	-	-	-	-	-	-	-	-	100	-	-
TEA (7.3)	-	-	-	-	-	-	-	-	-	-	-	-	-	100	-
KF	10	10	10	10	10	2	2	10	10	10	10	10	10	10	2
KCl	-	-	-	-	-	50	50	-	-	-	-	-	-	-	50
BSA	0.2	0.2	-	0.2	0.2	-	-	0.2	0.2	0.2	0.2	0.2	0.2	0.2	-
$\beta$ -ME	10	10	10	-	10	-	-	10	10	10	10	10	10	10	-
DTT	-	-	-	40	-	40	40	-	-	-	-	-	-	-	40
PPi	1	1	1	1	2	2	2	1	1	1	1	1	1	1	2

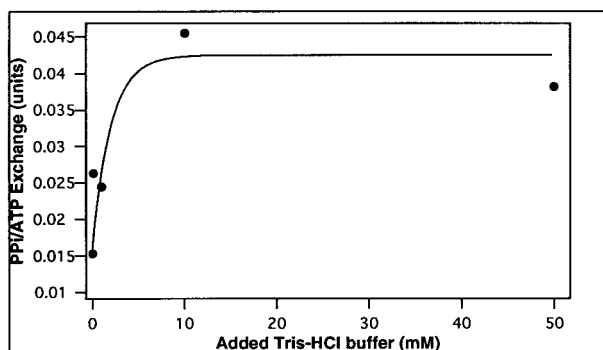
Units of enzyme activities for cysteine and proline are shown on the top, while components of reaction conditions are shown at the bottom part of the Table. Abbreviations: units in  $M^{-1} s^{-1}$ ; Tris, Tris-HCl; IMDZ, imidazole; TEA, triethanolamine; BSA, bovine serum albumin;  $\beta$ -ME,  $\beta$ -mercaptoethanol; DTT, dithiothreitol. Numbers in parentheses are pH values. All concentrations are given in mM, except for BSA, which is in mg/ml. In addition to the listed components, all buffer conditions included 2 mM ATP and 10 mM  $MgCl_2$ . Proline concentrations were 2 mM in assays in conditions 6, 7, and 15, and 0.05 mM in all others. Cysteine concentration was 2 mM in all cases. These concentrations were well above the  $K_M$  for proline (12  $\mu M$ )<sup>26</sup> and for cysteine (50  $\mu M$ ).<sup>35</sup> The enzyme was purified from a recombinant clone constructed by Dr K. Shiba (Department of Cell Biology, Cancer Institute, Japan).<sup>36</sup> The entire coding sequence has been verified by three independent facilities: the Nucleic Acid facility of Thomas Jefferson University, the Microchemical facility of University of Minnesota, and the Japanese Foundation for Cancer Research (Kami-Ikebukuro, Toshima-Ku). The concentration of the purified enzyme was determined by the active-site titration<sup>37</sup> and was 1 nM in all assays.

(conditions 2 and 4). The lowest detectable activities were observed in Pipes (100 mM, pH 7.2, condition 13) and in triethanolamine (100 mM, pH 7.3, condition 14), which gave 0.0025 and 0.0032 unit, respectively. Overall, variations among buffer conditions resulted in a range of cysteine activities that differed by as much as 180-fold.

Several conditions in Table 1 indicate that Tris buffer had a moderate stimulatory effect on activation of cysteine. For example, analysis of conditions 8-11 showed that increasing concentrations of Tris (0.1, 1, 5, and 10 mM) added to a Hepes buffer (100 mM, pH 7.5) improved the activity (from 0.008 to 0.02 unit). This was confirmed by analysis of a broader range of concentrations of Tris (0.1, 1, 10, and 50 mM) added to the same Hepes buffer. As shown in Figure 1, an approximately threefold stimulation of cysteine activation was observed relative to the activity in the absence of Tris. The maximum effect occurred at 10 mM Tris without further increase in activity at concentrations of Tris up to 50 mM.

The sensitivity of the cysteine activity to buffer conditions offers at least a partial explanation for the discrepancy between published reports. It should be noted that the accuracy of the gene encoding the enzyme used here has been verified by three independent facilities. Thus, the discrepancy with previous reports of tRNA-dependent activation is not due to errors in the enzyme sequence. However, other laboratory-specific differences, such as enzyme preparation, active site concentration determination, and the amount of enzyme used in the assays, may also affect the experimental outcome. These issues are not addressed here.

In the absence of tRNA, the activity of cysteinyl-AMP formation was consistently lower than that of prolyl-AMP synthesis. This was observed in all buffers tested (Table 1), although the degree of



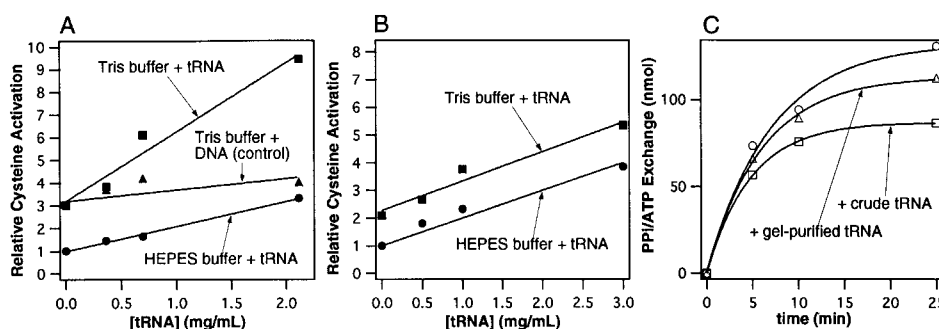
**Figure 1.** Stimulatory effect of added Tris on activation of cysteine (2 mM) by *M. jannaschii* ProRS (10 nM) in the absence of added tRNA. Initial rate of the cysteine-dependent ATP-PP<sub>i</sub> exchange reaction was measured in the Hepes buffer (condition 5) of Table 1 in the presence of increasing concentrations of Tris at 0, 0.1, 1.0, 10, and 50 mM (pH 7.5). The curve was fit to a simple exponential equation.

difference between the activity with cysteine and that with proline varied significantly. For example, the largest difference was observed in a Hepes buffer (condition 4), where the activity with proline was 3.8 units and that with cysteine was 0.0043 unit. This yielded a ratio of the activity for proline over that for cysteine of 880. The smallest difference was observed in a Tris buffer (condition 7), where the activity with proline was 7.6 units and that with cysteine was 0.23 unit, yielding a ratio of 33. In these two conditions, the proline activity varied only twofold, whereas the cysteine activity varied 53-fold. In Table 1, the proline concentration was 2 mM in conditions 6, 7, and 15, but was 0.05 mM in all other conditions. Comparison of the proline activity in conditions of 2 mM proline indicates a range of activity of fivefold (7.6 to 40 units). At proline concentration of 0.05 mM, the range of activity for proline was fourfold (1.1 to 4.1 units). Thus, compared to the much larger range of the cysteine activity (180-fold), the proline activity was less sensitive to variation of buffer conditions.

#### Activation of cysteine is slightly stimulated by tRNA

Although tRNA was not required for activation of cysteine in Hepes or Tris buffer, the effect of tRNA on the activity was examined in these two buffers. The Hepes buffer (condition 6) was as previously described,<sup>25</sup> while the Tris buffer (condition 15) was created by replacing Hepes (100 mM) with Tris (100 mM) under otherwise identical conditions. Total *M. jannaschii* tRNA was prepared by two methods, both of which yielded tRNA<sup>Cys</sup> and tRNA<sup>Pro</sup> in similar quantities. In the first approach, the tRNA was isolated from cells by phenol-extraction, ethanol-precipitation, and deacylation in a Tris buffer, followed by ethanol-precipitation.<sup>25</sup> This tRNA was added to the two buffers in increasing concentrations and its effect on activation of cysteine was measured (Figure 2(a)). The basal activities (in the absence of the tRNA) in the Hepes and Tris buffers were 0.040 and 0.12 unit, respectively, which confirmed that the activity was generally higher in Tris than in Hepes buffers. In both buffers, addition of the tRNA stimulated the basal activity in a nearly linear manner. At the highest tRNA concentration tested (2.1 mg/ml), the activities in the Hepes and Tris buffer were 0.11 and 0.40 unit, respectively, approximately threefold above that of the basal activity in both cases. To determine if the Tris buffer used during the deacylation step of the tRNA was responsible for the increase, salmon DNA treated in the same way as the tRNA, beginning with the deacylation step, was also tested. As the control DNA did not significantly affect cysteine activation, the observed stimulation of activity appears to be tRNA-dependent (Figure 2(a)).

In the second method of tRNA preparation, total tRNA was further purified by fractionation through a denaturing polyacrylamide gel



**Figure 2.** (a) Effect of crude *M. jannaschii* tRNA on activation of cysteine by the *M. jannaschii* ProRS (10 nM) in a HEPES buffer (condition 6, but pH 7.5) or a Tris buffer (condition 15). (b) Effect of gel-purified *M. jannaschii* total tRNA on activation of cysteine as described in (a). (c) Effect of crude (2.1 mg/ml) or gel-purified (3.0 mg/ml) tRNA on activation of proline by the *M. jannaschii* ProRS (1 nM) in a Tris buffer (condition 15). In (a) and (b), activities relative to that of the control in the HEPES buffer without tRNA are shown, whereas in (c), the rate of synthesis of prolyl-AMP is shown as a function of time. Lines were fit to a simple linear regression equation while curves were fit to an exponential equation. The crude tRNA was prepared as described,<sup>25</sup> resuspended in 200 mM Tris-HCl (pH 8.0), and was deacylated at 37 °C for 30 minutes. The tRNA was recovered by ethanol-precipitation, and resuspended in water for use. The amounts of tRNA<sup>Pro</sup> (0.027%) and tRNA<sup>Cys</sup> (0.25%) were determined by plateau-levels of aminoacylation by *M. jannaschii* ProRS. The gel-purified tRNA was prepared as described,<sup>20</sup> and was fractionated by denaturing 12% (w/v) PAGE. Various fractions were identified by UV shadow, extracted from the gel, and ethanol-precipitated. The amounts of tRNA<sup>Pro</sup> (0.2%) and tRNA<sup>Cys</sup> (1.21%) were determined by plateau-levels of aminoacylation.<sup>20,36</sup> Both tRNA preparations were derived from *M. jannaschii* cells that were grown under optimal fermentation condition,<sup>20</sup> thus were believed to contain natural modifications. However, specific modifications in tRNA<sup>Pro</sup> or tRNA<sup>Cys</sup> have not been determined.

(pH 8.3).<sup>20</sup> Deacylation was achieved during extraction of the tRNA from the gel (pH 8.3, 12 hours). The gel-purified tRNA, when added to the two buffer systems, also stimulated activation of cysteine in a concentration-dependent manner (Figure 2(b)). The basal activities in the HEPES (condition 6) and Tris (condition 15) buffers were 0.072 and 0.16 unit, respectively, and increased to 0.29 and 0.51 unit in the presence of 3 mg/ml of the tRNA. Thus, the stimulatory effect of tRNA on activation of cysteine was the same (~threefold) in each of the two buffers, and this effect was independent of the method used to prepare the tRNA (Figure 2(a) and (b)). In contrast, addition of crude or gel-purified tRNA had no effect on activation of proline (Figure 2(c)). Because the observed tRNA-dependent stimulation of cysteine activation is small, and because the activation is highly sensitive to buffer conditions, these two factors together may explain the lack of a tRNA effect under our previous assay conditions.<sup>20</sup> Additionally, higher levels of enzyme were used previously relative to the amounts used in the present study and this might mask the stimulatory effect of tRNA.

Thus, tRNA is not a necessary component for activation of cysteine, but it has the ability to moderately enhance the activity. For example, under near-optimal conditions for cysteine activation (condition 15), the rate of synthesis of cysteinyl-AMP (0.17 unit) is now below that of prolyl-AMP (40 units) by 240-fold. Addition of tRNA up to 3 mg/ml can improve the rate of synthesis of

cysteinyl-AMP by threefold (to 0.51 unit) such that this rate is below that of synthesis of prolyl-AMP by only 80-fold. Although the stimulatory effect is small, it nonetheless provides an incremental improvement to cysteinyl-AMP synthesis, which is the rate-limiting step in the overall process of aminoacylation with cysteine.<sup>25</sup>

### Activation of cysteine independent of tRNA

Recent studies show that the dual-specificity *M. jannaschii* ProRS has an editing activity against alanine. Although capable of hydrolyzing the mis-activated alanyl-AMP, the enzyme does not hydrolyze prolyl or cysteinyl-AMP,<sup>26,27</sup> indicating that it recognizes proline and cysteine as correct substrates. Because activation of cysteine does not require tRNA<sup>Cys</sup>, this suggests that cysteine must directly compete with proline for the overlapping amino acid binding site. The direct competition, without the assistance of tRNA, is consistent with the lack of an effect of tRNA on the ability of *M. jannaschii* ProRS to maintain prolyl-AMP and cysteinyl-AMP. If tRNA<sup>Cys</sup> had a major role in activation of cysteine, one might envision it to have an effect on the stability of prolyl-AMP or cysteinyl-AMP. However, even the addition of total tRNA (which includes fully modified tRNA<sup>Cys</sup>) does not stimulate hydrolysis of either adenylate.<sup>26</sup>

Insights into the ability of the dual-specificity ProRS to activate proline and synthesize prolyl-tRNA<sup>Pro</sup> are obtained from analysis of crystal

structures of *Thermus thermophilus* ProRS,<sup>33,34</sup> which shares strong homology with the ProRS of *M. jannaschii*.<sup>21,33</sup> Comparison of the crystal structure in the absence and presence of bound proline shows that the binding site is not pre-formed, as observed for many other synthetases, but is created upon binding of the amino acid.<sup>34</sup> This binding-site consists of a network of extensive H-bonding interactions between enzyme side-chains and proline-specific functional groups. The "induced-fit" mechanism of binding of proline is also observed with binding of ATP and tRNA<sup>Pro</sup>.<sup>33,34</sup> Most importantly, the synthesis of prolyl-AMP, which induces a further conformational change, appears to be a pre-requisite for the enzyme to properly place the 3' end of tRNA<sup>Pro</sup> into the active site for aminoacylation.<sup>34</sup> These successive steps of induced-fit and synthesis of the adenylate intermediate help to ensure the specificity with proline.

In the absence of a major role of tRNA<sup>Cys</sup>, activation of cysteine may also use an induced-fit mechanism to ensure the specificity for aminoacylation with cysteine. In this mechanism, the binding of cysteine precludes the binding of proline and the synthesis of cysteinyl-AMP would allow only tRNA<sup>Cys</sup> to position its 3' end into the active site for aminoacylation. The ordered binding of cysteine and the 3' end of tRNA<sup>Cys</sup> would be necessary to prevent transfer of the activated cysteinyl-AMP to tRNA<sup>Pro</sup>. Indeed, recent studies suggest that the *M. jannaschii* ProRS cannot remove cysteine from mis-acylated cysteinyl-tRNA<sup>Pro</sup>. Specifically, when incubated with cysteinyl-tRNA<sup>Pro</sup>, transcribed and mis-acylated *in vitro*, the enzyme failed to catalyze hydrolysis of the cysteinyl group.<sup>26,27</sup> Thus, at least with the unmodified transcript of tRNA<sup>Pro</sup>, the enzyme does not possess a post-transfer editing mechanism against cysteine. This lack of editing is consistent with the induced-fit mechanism, where synthesis of cysteinyl-AMP would only lead to synthesis of cysteinyl-tRNA<sup>Cys</sup>, but not cysteinyl-tRNA<sup>Pro</sup>, thus eliminating the need for an editing reaction. Further studies are necessary, however, to determine if the enzyme remains incapable of hydrolyzing mis-acylated cysteine or proline from native tRNA.

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