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Use of β³-methionine as an amino acid substrate of Escherichia coli methionyl-tRNA synthetase

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β³-methionine is an attractive tool for the design of novel proteins having unique properties of medical or industrial interest. Incorporation of β-amino acids in vivo requires the development of efficient aminoacyl-tRNA synthetases specific for these non-canonical amino acids. Here, we have performed a detailed structural and biochemical study of the recognition and use of β³-Met by Escherichia coli methionyl-tRNA synthetase (MetRS). We show that MetRS binds β³-Met with a 24-fold lower affinity but catalyzes the esterification of the non-canonical amino acid onto tRNA with a rate lowered by three orders of magnitude. Accurate measurements of the catalytic parameters required careful consideration of the presence of contaminating α-Met in β³-Met commercial samples. The 1.45 Å crystal structure of the MetRS: β³-Met complex shows that β³-Met binds the enzyme essentially like α-Met, but the carboxylate moiety is mobile and not adequately positioned to react with ATP for aminoacyl adenylate formation. This study provides structural and biochemical bases for engineering MetRS with improved β³-Met aminoacylation capabilities.

1. Introduction

The design of polypeptides having enhanced or novel properties is a major challenge for synthetic biology. Indeed, although natural proteins mostly rely on the set of 20 canonical amino acids to perform their functions, additional groups are required for some of the biological activities. These groups are brought by rare amino acids such as selenocysteine or pyrrolysine, by post-translational modifications or by cofactors. Considerable work has been made for expanding the genetic code in order to direct the incorporation of non-standard amino acids at desired positions in polypeptides. Two types of approaches were used, either living cells or in vitro reconstituted ribosomal translation systems.

Using living cells to incorporate an artificial amino acid in vivo requires the development of aminoacyl-tRNA synthetases (aaRS) able to aminoacylate a wild-type tRNA. For instance, E. coli methionyl-tRNA synthetase mutants were selected for aminoacylation of tRNA⁷Met with the methionine surrogate azidonorleucine, resulting in efficient protein labelling in vivo in either bacterial or eukaryotic cells (Mahdavi et al., 2016; Ngo et al., 2009; Tanrikulu et al., 2009). The early studies led to the development of more general residue-specific methods that allow partial to quantitative replacement of canonical amino acids by non-canonical analogues (Johnson et al., 2010). For site-specific incorporation, the non-standard amino acid is generally encoded by a non-sense or a frameshift codon (Chin, 2017; Liu and Schultz, 2010). In this case, an orthogonal tRNA, not recognized by any endogeneous aaRS, is used together with an orthogonal aaRS, unable to aminoacylate any endogeneous tRNA (Wang et al., 2006). In vitro approaches generally use cell-free systems reconstituted from purified components (Shimizu et al., 2001). One important advantage of such systems is that aminoacyl-tRNAs can if necessary be prepared using methods that do not require aminoacylation by aaRSs. Indeed, highly flexible methods for in vitro aminoacylation of tRNAs with a wide variety of amino acids are available. These methods are based on RNA catalysts named flexizymes (Murakami et al., 2003, 2006; Ohuchi et al., 2007) and have virtually no limitation of acid donor substrates.

Most non-standard amino acids successfully used for in vivo incorporation were L-α-amino acids, resulting in the same polypeptide backbone as the canonical amino acids. β-amino acids bear an additional methylene group either between the nitrogen atom and the α-carbon (β¹ type) or between the α-carbon and the carboxylate carbon...
that results, when incorporated in a polypeptide, in a non-standard main chain. These non-canonical amino acids have deserved increasing interest because they give polypeptides unique properties, such as increased resistance to enzymatic hydrolysis (Heck et al., 2006; Webb et al., 2005) or the capacity to form peculiar helical structures (Pettersson et al., 2007; Pettersson and Schepartz, 2008; Seebach et al., 2006). Actually, β-amino acids were used to create peptides with interesting biological activities (Cheloha et al., 2015; Seebach and Gardiner, 2008).

Ribosomes could be modified to allow incorporation of β-amino acids in cell free systems (Dedkova et al., 2011; Maini et al., 2013). It was also shown that various β-amino acids were compatible with ribosomal translation (Fujino et al., 2016). Moreover, elongation of peptides with successive β-amino acids was more recently rendered possible thanks to the use of engineered tRNAs having enhanced affinities for elongation factors EF-Tu and EF-P (Katoz and Suga, 2018). These studies in cell-free systems required the supply of β-aminoacyl-tRNAs synthesized using flexizymes or chemical tools. Overall, the availability of aaRSs able to specifically aminoacylate tRNAs with β3-amino acids in cell free systems is limited by the use of engineered tRNAs having enhanced affinities for elongation factors EF-Tu and EF-P (Katoz and Suga, 2018).

2. Results and discussion

2.1. Evidence for the presence of contaminating α-Met in β3-Met samples

It is well established that interpretation of [32P]ATP-PPi exchange experiments can be obscured by the presence of methionine traces in commercial amino acids samples (Fersht and Dingwall, 1979). Prior to studying activation of β3-Met by MetRS, we used LC-HRMS experiments to search for the presence of α-Met in the β3-Met sample. A protonated molecule at m/z 164.074 (corresponding to β3-MeH+) retention time of 2.4 min) was mainly observed on the ESI-P mass spectrum for the β3-Met sample (Fig. 1). However, the results showed the presence of a compound having the exact molecular mass of α-MeH+ (m/z 150.058) at 2.2 min in β3-Met suggesting the occurrence of contaminating α-Met. Accordingly, the peak of controlling α-Met corresponded to the major peak observed in an α-Met sample analyzed as a reference (data not shown). From the relative responses of the β3-Met and α-Met samples, we evaluated the contamination of α-Met in the β3-Met sample of about 0.1%. Actually, BOC-L-methionine was used as a reactant during the synthesis of β3-Met, as communicated by the company from which the compound was purchased. As an attempt to remove contamination, we treated β3-Met samples with methionine gamma lyase (MGL), an enzyme that degrades L-methionine into methanethiol, ammonia and 2-oxobutanate. This enzyme was likely to be specific of α-Met as compared to β3-Met since it binds both the amino and carboxylate groups of methionine (Fukumoto et al., 2014). The treated sample was then analyzed by mass spectrometry (Fig. 1). As expected, the molecular ion at m/z 150.058 [α-MeH]+ became undetectable in the MGL sample (reduced by a factor of at least ten), whereas the peak corresponding to β3-Met remained present.

2.2. MetRS binds β3-Met

Because of this contamination, we investigated binding of β3-Met using enzyme intrinsic fluorescence titration experiments. This method is much less sensitive than ATP-PPi exchange to trace contaminations. Indeed, enzyme fluorescence varies measurably only if a significant proportion of the enzyme is bound to the ligand whereas even a very small fraction of enzyme bound to α-Met can catalytically give detectable accumulation of the [32P]PPi reaction product. It is known that MetRS displays large variations of intrinsic fluorescence when binding methionine or methionyl adenylate from which useful thermodynamic parameters can be derived. Throughout this study, we used a Histagged MS47 monomeric version of E. coli MetRS, fully active, both in vitro and in vivo (Schmitt et al., 2009). Addition of β3-Met to MetRS caused an increase of intrinsic tryptophan fluorescence, similar to the increase caused by titration with α-Met. A dissociation constant of 1.2 ± 0.2 mM for the MetRS: β3-Met complex was derived from the titration data (Fig. 2 and Table 1). This value was only 24-fold lower than the Kd of MetRS: α-Met. According to the 0.1% contamination level of α-Met in our β3-Met sample, the concentration of α-Met in the titration experiments did not exceed 4 µM. Because this value was low as compared to the Kd of α-Met for MetRS (50 µM, Table 1), we concluded that the contaminating α-Met could not influence the measured Kd value of β3-Met. Consistent with this conclusion, no detectable effect on the dissociation constant of the MetRS: β3-Met complex was observed if β3-Met was treated with MGL prior to the measurement (Fig. 2). This latter result also showed that the non-canonical amino acid was not affected by MGL treatment.

2.3. Interpretation of the [32P]ATP-PPi exchange assay is obscured by amino acid contamination

Measurement of the apparent catalytic parameters (kcat-app and Km-app for β3-Met) of the [32P]ATP-PPi isotopic exchange reaction using the commercial β3-Met sample showed that as compared to α-Met, kcat-app was lowered by a factor of 42 (1.2 s−1 as compared to 50 s−1) whereas Km-app was increased by a factor of 35 (630 µM as compared to 18 µM). Overall, the catalytic efficiency of the isotopic exchange reaction was reduced by an apparent factor of ca 1500 when α-Met was replaced by β3-Met. Notably, these results were at odd with those of a study reporting that MetRS was very tolerant to the substitution of α-Met by β3-Met, with only a modest (2-fold) preference for Met in ATP-PPi exchange (Melo Czekster et al., 2016). Moreover, in the presence of 2 mM β3-Met treated with MGL, the isotopic exchange rate dropped to 0.07 s−1 as compared to 0.9 s−1 in the presence of 2 mM untreated β3-Met (a value in agreement with kcat-app of 1.2 s−1 and Km-app of 0.63 mM). This result showed that, at least with the commercial sample, the β3-Met-dependent [32P]ATP-PPi exchange rate mostly reflected activation of contaminating α-Met. The exchange rates measured when varying the concentration of untreated β3-Met were therefore interpreted using a competitive inhibition scheme (Fig. 3). In this scheme, β3-Met binds the enzyme at a site overlapping the α-Met site but cannot be activated. Fitting showed that the experimental results were consistent with activation of β3-Met occurring at a negligible rate and β3-Met being contaminated with 0.09% α-Met (mol/mol; Fig. 3). Notably, this level of contamination was compatible with the results of mass spectrometry analyses of β3-Met. Finally, traces of α-Met were also evidenced in enzyme preparations. Attempts to diminish the contamination to a level where β3-Met-dependent [32P]ATP-PPi exchange rates can be accurately measured were unsuccessful. Because of the high sensitivity of ATP-PPi isotopic exchange to the presence of traces of α-Met, we concluded that this method was not suitable to detect a possible activation of β3-Met by MetRS.
2.4. β3-Met is a poor substrate of MetRS

As described above, fluorescence titration can be used to follow the bindings of α-Met or β3-Met to MetRS. It is also known that addition of ATP in the presence of magnesium ions to the MetRS:Met complex leads to a large decrease of fluorescence intensity reflecting synthesis of methionyl adenylate. Hence, a MetRS: β3-Met complex formed in the presence of MGL-treated β3-Met was mixed with ATP-Mg2+. We observed a slow increase in fluorescence (rate constant of the order of 0.05 s−1), contrasting with the fast decrease observed in the case of α-Met adenylate formation. However, importantly, this increase in fluorescence was reversed by the subsequent addition of

![Fig. 1. LC-HRMS analysis of β3-Met (blue) and MGL-treated β3-Met (yellow) samples. Extracted ion current (EIC) chromatograms of (A) m/z 164.074 ± 0.001 corresponding to [β3-MetH]+ (C6H14NO2S) and (B) m/z 150.058 ± 0.001 corresponding to [α-MetH]+ (C5H12NO2S). The blue chromatograms were shifted to the right for clarity. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

![Fig. 2. Intrinsic enzyme fluorescence titration of MetRS with β3-Met. Panel A: MetRS (0.5 µM) was titrated with increasing amounts of commercial β3-Met, as described in Materials and Methods. The experimental points show the measured fluorescence as a function of the concentration of β3-Met. Data were fitted to a simple equilibrium model using standard least-square procedures. The curve shows the resulting fit (Kd = 1.2 mM). Panel B: Same experiment but β3-Met was treated with MGL before titration (Kd = 1.2 mM).

![Table 1](en)

| Enzyme/Substrate | WT/α-Met | WT/β3-Met | V298I/α-Met | V298I/β3-Met |
|------------------|----------|-----------|-------------|-------------|
| K_Met (mM)       | 0.05 ± 0.01 | 1.2 ± 0.2 | 0.029 ± 0.001 | 0.56 ± 0.01 |
| K_MetATP (mM)    | 0.070 ± 0.004 | 0.30 ± 0.04 | 0.063 ± 0.004 | 0.15 ± 0.02 |
| kₐ (s⁻¹)         | 260 ± 10 | 0.051 ± 0.003 | 262 ± 12 | 0.057 ± 0.005 |
| K_P (mM)         | 0.075 ± 0.025 | 1.0 ± 0.25 | 0.06 ± 0.01 | 0.8 ± 0.2 |
| k_b (s⁻¹)        | 189 ± 23 | 1.2 ± 0.3 | 195 ± 30 | 0.95 ± 0.2 |
| Aminoacylation rate (s⁻¹) | 0.52 ± 0.07 (5.6 ± 0.3) 10⁻⁴ | n.d. | n.d. |

Aminoacylation rates were measured in the presence of 2 µM tRNA, 2 mM ATP and 2 mM of either α-Met or β3-Met. Results are the mean ± sd from two independent experiments.

2.4. β3-Met is a poor substrate of MetRS

As described above, fluorescence titration can be used to follow the bindings of α-Met or β3-Met to MetRS. It is also known that addition of ATP in the presence of magnesium ions to the MetRS:Met complex leads to a large decrease of fluorescence intensity reflecting synthesis of methionyl adenylate. Hence, a MetRS: β3-Met complex formed in the presence of MGL-treated β3-Met was mixed with ATP-Mg2+. We observed a slow increase in fluorescence (rate constant of the order of 0.05 s⁻¹), contrasting with the fast decrease observed in the case of α-Met adenylate formation. However, importantly, this increase in fluorescence was reversed by the subsequent addition of
with MetRS: β3-Met using LC-HRMS (Fig. 4A). These experiments pyrophosphate, suggesting that it could be due to the formation of β3-Met. Data (average from three independent experiments) were fitted to a classical competitive inhibition scheme, where isotopic exchange depends on contaminating α-Met. Adjusted parameters for fitting were the contamination rate λ (α-Met/[β3-Met]) and the inhibition constant KI. The curve shows the best fit to the experimental data, with a contamination rate of (8.9 ± 0.3)·10\(^{-4}\) and an inhibition constant of 479 ± 21 µM. Michaelian parameters of the α-Met-dependent exchange were measured with the same batch of enzyme (k_{cat} = 50 s\(^{-1}\) and k_{cat} = 18 µM) and kept constant in the fitting procedure. The theoretical equation giving the initial rate (vi) as a function of the concentration of β3-Met ([S]) was: 

\[
vi = k_{cat} [E] [S] + \frac{k_{cat} [E]}{K_m + [S]} (1 + \frac{K_I}{[S]})
\]

pyrophosphate, suggesting that it could be due to the formation of β3-Met adenylate. In order to unambiguously confirm the formation of β3-Met adenylate, we analyzed the product of the reaction of ATP-Mg\(^{2+}\) with MetRS: β3-Met using LC-HRMS (Fig. 4A). These experiments clearly identified an ion at m/z 493.127 with elemental composition C\(_{49}\)H\(_{62}\)N\(_5\)O\(_{12}\)P corresponding to protonated β3-Met adenylate. This product was not present in the control sample where the reaction was stopped immediately after addition of MetRS (Fig. 4A). As expected, the corresponding ion for α-Met (m/z 479) was not observed. To obtain structural characterization of this compound, LC-HRMS/MS experiments have been carried out on the molecular ion (Fig. 4B). Three major ions were obtained. The ion at m/z 348.07 was formed from m/z 493.127 by elimination of C\(_{49}\)H\(_{62}\)N\(_5\)O\(_{12}\)P according with its elemental composition C\(_{49}\)H\(_{62}\)N\(_5\)O\(_{12}\)P and corresponded to protonated AMP. Ions at m/z 164.074 (C\(_{49}\)H\(_{62}\)N\(_5\)O\(_{12}\)P) and m/z 136.062 (C\(_{49}\)H\(_{62}\)N\(_5\)O\(_{12}\)P) were formed from the molecular ion and corresponded to the protonated β3-Met and adenine, respectively. A fragmentation mechanism of the m/z 493 ion is proposed in the Supplementary Scheme S1. These results clearly confirmed the identification of the reaction product as β3-Met adenylate.

The experiments described above showed that the increase in intrinsic enzyme fluorescence observed during reaction of MetRS with MGL-treated β3-Met and ATP-Mg\(^{2+}\) indeed reflected the synthesis of β3-methionyl adenylate. We therefore measured the rates of fluorescence variation in the presence of 2 mM ATP-Mg\(^{2+}\) and increasing amounts of MGL-treated β3-Met in order to derive the thermodynamic constants of the reaction, K β3-Met, the dissociation constant of β3-Met from the MetRS:ATP-β3-Met complex and k β, the rate of β3-methionyl adenylate synthesis (Scheme 1, Supplementary Fig. S1). The results (Table 1) showed that affinity of β3-Met for the MetRS:ATP-Mg\(^{2+}\) complex was reduced by one order of magnitude as compared to that of α-Met, and that the rate of activation of the β amino acid was 5200-fold lower than that of canonical methionine. Similarly, the enzyme remained able to catalyze the reversion of enzyme-bound β3-methionyl adenylate by PPI-Mg\(^{2+}\), though at a rate reduced by a factor of 157 as compared to the reversion of α-methionyl adenylate. Overall, these experiments showed that MetRS was indeed able to activate β3-Met, but with a catalytic efficiency (kcat/K_m[ATP]) reduced by four orders of magnitude as compared to canonical methionine. This low catalytic efficiency was mainly caused by small kcat and k_m kinetic constants, reflecting a non-optimal stabilization of the transition state of the activation reaction according to the Arrhenius law.

Finally, we examined the ability of MetRS to transfer β3-Met onto tRNA\(_{Met}\). This was first done by analyzing the products of aminoacylation reactions (see methods) using mass spectrometry after digestion with S1 nuclease (Hartman et al., 2007, 2006). The results showed the presence of a product corresponding to AMP esterified with β3-methionyl-AMP (m/z 493.127) whereas no α-methionyl-AMP was detected (Fig. 4C). In order to prevent deacylation during sample preparation and to have a better signal in MS/MS, an aliquot of the aminoacyl-tRNA was N-acetylated using acetic anhydride prior to S1 nuclease digestion. Consistent with our expectations, N-acetyl-β3-methionyl-AMP (MH\(^+\) m/z 535.138) was found in the acetylated sample whereas N-acetyl-α-methionyl-AMP (MH\(^+\) m/z 521.121) was absent (Fig. 4C). Reference experiments with α-Met instead of β3-Met gave the expected results (Fig. 4D). Identifications of N-acetyl-β3-methionyl-AMP and N-acetyl-α-methionyl-AMP were further confirmed using HR-MS/MS of protonated compounds. Supplementary Table S1 brings together the detected fragments ions for both compounds. These results allowed us to propose the fragmentation scheme of protonated molecules reported in Supplementary Scheme 2.

We finally measured the rate of tRNA aminoacylation with β3-Met using the assay based on [3′-32P]-labelled tRNA (Ledoux and Uhlenbeck, 2008). In the presence of 2 mM MGL-treated β3-Met and 2 mM ATP, MetRS catalyzed the aminoacylation of tRNA\(_{Met}\) (2 µM) at a rate three orders of magnitude lower than the rate measured in the control experiment with α-Met (Fig. 5 and Table 1). It should be underlined that the rate value measured with β3-Met must be taken cautiously since, even after treatment with MGL, traces of α-Met remain. Nevertheless, taking into account the mass spectrometry experiments, the results show that MetRS can transfer β3-Met onto tRNA though at a reduced rate, in agreement with the reduced efficiency of the β3-Met activation step as compared to the Met activation step.

2.5. Structural study of β3-Met binding to MetRS

The results described above raise the question of how β3-Met is recognized by MetRS. Understanding this may also guide efforts aimed at engineering the enzyme for more efficiency and specificity towards the β amino acid. We therefore crystallized the enzyme in the presence of β3-Met. This was done by microseeding a supersaturated (1.08 M ammonium citrate, 6 °C) enzyme solution containing 20 mM β3-Met and 1.2 Å resolution. The previously published structure of the MetRS: α-Met complex was indeed determined at a lower 1.85 Å resolution (PDB id 1FL4, (Serre et al., 2001)). To avoid any bias, the two structures were solved by molecular replacement using the structure of unliganded MetRS as a search model (PDB id IQQT, (Mechulam et al., 1999a)). In both cases, the presence of the ligand was obvious in the molecular replacement density map. The structures were then refined using successive rounds of manual rebuilding in Coot and energy minimization in Phenix. Final R/Free-R values were 0.092/0.126 (MetRS: β3-Met) and 0.145/0.171 (MetRS: α-Met complex; Table 2). The enzyme is made up of an N-terminal Rossmann fold containing the catalytic centre (Mechulam et al., 1991; Schmitt et al., 1994) and a C-terminal helix-bundle domain responsible for recognition of the CAU anticodon of tRNAs\(_{Met}\).

As previously described (Crepin et al., 2003; Serre et al., 2001; Tanrikulu et al., 2009), binding of α-Met caused a dramatic re-arrangement of aromatic residues. The side chains of W229, W253, F300 and F304 rotate such that W229 stacks on F304 and W253 stacks on F300. Furthermore, Y15 rotates, acting as a lid that locks the amino acid.
acid in its binding cavity (Fig. 6). These induced rearrangements reduce the size of the cavity and allow accommodation of the methionine side-chain. The α-Met cavity is bordered by L13, W253, P257, Y260 and I297. The amino group of the substrate is held by the side chain of D52 and by the main chain carbonyl of L13. The carboxylate is adequately positioned for reacting with the α-phosphoryl group of ATP, as suggested from comparison with MetRS structures bound to methionyl adenylate or analogues (Fig. 6, Crepin et al., 2003; Larson et al., 2011).

Such a precise positioning of the carboxylate group with respect to ATP was also observed in class 2 aminoacyl-tRNA synthetases (Schmitt et al., 1998).

Comparison of the MetRS:β3-Met structure with the MetRS:α-Met ones showed very high similarity. The side chain of β3-Met was bound in the same induced pocket as that of α-Met, without any significant positional difference (Fig. 6 and Supplementary Fig. S2). In particular, the rearrangement of aromatic residues (W229, W253, F300 and F304) occurred identically. Also, the amino group was located at the same position as that of α-Met, bound to D52 and L13. However, because of the insertion of a methylene group, the carboxylate of β3-Met is positioned differently as compared to α-Met (Fig. 6). More precisely, two alternative positions differing by a rotation of about 87° around the Cα-Cβ bond were observed for the carboxylate of β3-Met (Fig. 6 and Fig. S2). In the major conformation (70% occupancy), the carboxylate oxygens were oriented away from the active site. In this conformation, Y15 cannot reach the position where it locks the active site. Accordingly, Y15 was observed at several alternative positions, ranging from the open position seen in the unliganded enzyme to the closed position seen in the MetRS:α-Met complex (Fig. 6 and Fig. S2). In contrast, in

**Fig. 4.** LC-HRMS analyses of MGL-treated β3-Met activation and transfer onto tRNAMet. Panel A: Analysis of the products of the activation reaction. The red and blue chromatograms in correspond to the sample in which MetRS was reacted with ATP and MGL-treated β3-Met. Extracted ion current (EIC) chromatograms of m/z 479.11 ± 0.001 (red, corresponding to [α-Met adenylateH]+ C15H24N6O8PS) and of m/z 493.126 ± 0.001 (blue, corresponding to [β3-Met adenylateH]+ C16H26N6O8PS). The pink chromatogram corresponds to m/z 493.126 ± 0.001 ([β3-Met adenylateH]+) for the zero reaction time control sample. Panel B: HR-MS/MS (Rcol = 15 eV) of the m/z 493.1263 ion obtained at 2.6 min retention time (blue chromatogram in Panel A). The proposed mechanism for the decomposition of the molecular ion is indicated on the Supplementary Scheme 1. Panel C: LC-HRMS analysis of the aminoacylation reaction in the presence of MGL-treated β3-Met. The samples were submitted to S1 nuclease cleavage as indicated in Materials and Methods. The panel shows the extracted ion current chromatograms (EIC) of m/z 479.11 ± 0.001 (corresponding to [α-methionyl-AMP]H+; C33H32N6O8PS; red) and m/z 493.126 ± 0.001 (corresponding to [β3-methionyl-AMP]H+; C34H34N6O8PS; purple). The two other chromatograms correspond to the samples acetylated prior to S1 cleavage, showing the EIC of m/z 521.121 ± 0.001 (corresponding to [N-acetyl-α-methionyl-AMP]H+; C35H36N6O8PS; orange) and m/z 535.138 (corresponding to [N-acetyl-β3-methionyl-AMP]H+, C36H38N6O8PS; green). The panel shows the presence of the compounds expected from the transfer of β3-Met onto tRNA and the absence of detectable aminoacylation with α-Met. Panel D: Reference aminoacylation experiment using α-Met instead of β3-Met, with (red) or without (yellow) acetylation. The panel shows the EIC of m/z 479.11 ± 0.001 (corresponding to [α-methionyl-AMP]H+; C15H24N6O8PS; yellow) and m/z 521.121 ± 0.001 (corresponding to [N-acetyl-α-methionyl-AMP]H+, C17H26N6O9PS; red). The ions at m/z 535.138 ([N-acetyl-β3-methionyl-AMP]H+) and m/z 521.121 ([N-acetyl-α-methionyl-AMP]H+) were further characterized by HR-MS/MS (Supplementary Table 1 and Supplementary Scheme 2). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
the minor conformation, one carboxylate oxygen of β3-Met is located only at 1.3 Å from the ideal position observed in α-Met and full rotation of Y15 is possible.

With α-Met, the electrostatic hindrance between the carboxylate group and the α-phosphoryl of ATP is known to be compensated by strong synergistic binding such that K α-Met and K α-MetATP have similar values (Table 1; Blanquet et al., 1975; Fayat et al., 1977). With β3-Met, the affinity of the amino acid was 4-fold higher in the presence of S1 nuclease. The resulting digests were analyzed by TLC. The figure shows the results of a typical experiment with the fluorogram in the upper part and the corresponding image analysis in the bottom part. The figure shows that the rates differ by three orders of magnitude. The average rates from two independent experiments are reported in Table 1.

2.6. Attempt to enhance activity of MetRS towards β3-Met

Interestingly, on the side of the amino acid-binding pocket opposite to the active centre, a supplementary β3-Met minor site with an occupancy of 0.7 was observed. The second β3-Met molecule (hereafter termed β3-Met2) was bound between loop 295–297 connecting β D to αD on the one hand and helix α4 in the connective polypeptide (CP) on the other hand (Fig. 7). The secondary binding cavity is bordered by V214, K217, W221, K295, V298 and Y325. As described above, Y325 is connected to D296, thereby showing a physical link between the active site and the secondary β3-Met site. We therefore wondered whether occupation of the β3-Met2 site may affect the activity of MetRS towards β3-Met. In order to impair β3-Met2 binding, we mutated V298 into I. Measurement of the catalytic parameters of β3-Met activation showed a two-fold increase in affinity of β3-Met for the V298I mutant, both in the presence and in the absence of ATP (Table 1). This effect was however paralleled by a two-fold increase in α-Met affinity, at least in the absence of ATP. Therefore, selectivity of the V298I mutant towards β3-Met as compared to α-Met was not enhanced by a factor greater than 2.

The structure of the V298I mutant was solved in the apo form and bound to either α-Met or β3-Met (Fig. 7B,C,D). As expected, the β amino acid was found only in the main site. However, in the V298I: β3-Met structure, D296 was still observed in the “in” and “out” conformations. Moreover, both conformations of D296 were also observed in the apo and α-Met-bound forms of the V298I mutant. Hence, occupation of the β3-Met2 cavity by the Ile side-chain was unfortunately sufficient to cause destabilization of the D296 position, meaning that the mutation did not have the desired structural effect. However, in the apo form, alternative conformations for W229 and W253, two residues involved in the conformational rearrangement of aromatic residues induced by α-Met or β3-Met binding were observed (Supplementary Fig. S3). Therefore, the enhanced affinity of the V298I mutant for both β3-Met and α-Met may have been caused by a better propensity to adopt a conformation corresponding to the conformation induced by Met binding.

2.7. Concluding remarks

This study shows that E. coli MetRS is able to use β3-Met as a substrate in both the amino acid activation and tRNAβ3Met aminoacylation reactions. These results are in keeping with previous studies showing that several β amino acids, including β3-Met, could be transferred onto tRNA by the corresponding aminoacyl-tRNA synthetase (Hartman et al., 2007, 2006). The efficiency of the reaction appears however very low as compared to what was observed in a recent study (Melo Czekster et al., 2016). This discrepancy might be due to the presence of contaminating α-Met in commercial β3-Met samples. The high-resolution structure of the MetRS: β3-Met complex explains why the non-canonical amino acid has a rather good affinity for the enzyme but is activated ca 5000-fold more slowly. This is due to a non-optimal positioning of the carboxylate moiety, rendering reaction with the α-phosphoryl group of ATP difficult. β3-Met can also bind in a secondary site, though less efficiently as suggested by partial occupancy in the structure in spite of the high concentration of amino acid used for crystallization (20 mM). Disrupting the secondary site had little, if any, influence on the efficiency of MetRS towards β3-Met. Nevertheless, this study provides structural and functional foundations for MetRS engineering to obtain a more active and specific enzyme towards β3-Met. Such an enzyme would open the possibility of directing site-specific incorporation of β3-Met in vivo.

3. Materials and methods

3.1. Site-directed mutagenesis and enzyme purification

The gene encoding M547 from pBSM547+ (Mellot et al., 1989;
was subcloned into pET15b (Guillon et al., 2005) in order to add an N-terminal His-tag to the MetRS product. The QuickChange method (Braman et al., 1996) was used to generate site-directed mutations. The M547 variants were produced in BLR(DE3) cells. Cultures were grown overnight at 37 °C in autoinducible TBAI medium containing 50 µg/mL of ampicillin. The cell pellet was suspended in purification buffer (10 mM Hepes-NaOH pH 7.5, 500 mM NaCl, 3 mM 2-mercaptoethanol) and cells were disrupted by ultrasonic disintegration. After centrifugation, the extract was loaded onto Talon affinity resin (Clontech). The resin was washed with purification buffer containing 10 mM imidazole and the protein eluted by increasing imidazole concentration to 125 mM. The eluate was diluted ten-fold before further purification on a Q-Hiload (GE-Healthcare) ion exchange column. The procedure yielded 15–20 mg of homogeneous protein for a 250 mL culture. Purified proteins were stored either at 4 °C in 10 mM Hepes-NaOH, 10 mM 2-mercaptoethanol or at −20 °C in the same buffer plus 55% glycerol.

Table 2

| Data collection | MetRS: β3-Met | MetRS: α-Met | VI298 | VI298: β3-Met | VI298: α-Met |
|----------------|--------------|--------------|-------|--------------|--------------|
| Space group    | P2₁          | P2₁          | P2₁   | P2₁          | P2₁          |
| a, b, c (Å)    | 78.2, 45.23, 86.25 | 78.2, 45.19, 85.89 | 78.5, 45.3, 86.31 | 78.3, 45.11, 86.21 | 78.4, 45.15, 86.23 |
| Resolution (Å) | 1.45         | 1.2          | 1.5   | 1.48         | 1.38         |
| R_{sym} (%)    | 3.8 (16.0)   | 10.5 (164)   | 7.6 (96.4) | 11.7 (103) | 6.8 (114) |
| Completeness (%)| 98.4 (95.1) | 99.0 (94.8)  | 99.2 (97.4) | 99.6 (98.3) | 99.1 (95.6) |
| ReLU (%)       | 3.3 (3.1)    | 6.4 (6.2)    | 4.7 (4.6) | 6.8 (6.6)   | 6.8 (6.4)   |
| CC₁/₂ (%)      | 99.9 (96.9)  | 99.8 (32.6)  | 99.8 (95.2) | 99.8 (59.7) | 99.9 (52.3) |

A single crystal was used for data collection. 

* R_{sym}(I) = \frac{\sum_i \sum_{hkl} |I(hkl)| - |I(hkl)|}{\sum_i \sum_{hkl} |I(hkl)|}, where i is the number of reflections hkl.

* Values in parentheses are for highest-resolution shell.

* CC(1/2) is the correlation coefficient between two random half data sets (Karplus and Diederichs, 2012).

* R_{work} = \frac{\sum |F_{obs} - F_{calc}|}{\sum |F_{obs}|}; R_{free} is calculated with 5% of the reflections.

* ligands and solvent molecules other than water (citrate and glycerol).

Fig. 6. Binding of β3-Met to MetRS (panel A) compared to the binding of α-Met (panel B). Relevant side chains are shown in sticks and nearby water molecules in red spheres. Occupancies of alternative conformations (%) are indicated in blue. MetRS is drawn as a ribbon. The structures of MetRS: β3-Met, MetRS: α-Met (this study) and MetRS:methionyl-sulfamoyl-adenosine (PDB id 1PFY; (Crepin et al., 2003; Larson et al., 2011)) were superimposed. Panel C compares the conformations of the ligands after superimposition. Major and minor conformations of the carboxylate of β3-Met are indicated. Hydrogen bonds are shown with yellow dots. Figs. 5 and 6 were drawn with PyMOL (The PyMOL Molecular Graphics System, Schrödinger, LLC). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
3.2. Isotopic \(^{32}\text{P}\)PPi-ATP exchange

Amino acid-dependent \(^{32}\text{P}\)PPi-ATP exchange activity was measured at 25 °C in standard buffer (20 mM Tris-HCl pH 7.6, 7 mM MgCl\(_2\), 10 mM 2-mercaptoethanol, 0.1 mM EDTA) containing 2 mM ATP and 2 mM \(^{32}\text{P}\)PPi as described (Blanquet et al., 1974; Schmitt et al., 1994). For \(K_m\) measurements, concentrations of \(\alpha\)-Met or \(\beta 3\)-Met were varied between 8 µM and 2 mM or 0.125 mM to 8 mM, respectively.

3.3. Treatment of \(\beta 3\)-Met with methionine gamma lyase (MGL)

\(L\)-\(\beta 3\)-Met was purchased from Fluorochem Ltd. (UK), dissolved in standard buffer and adjusted to pH 7.5 with NaOH. For \(\alpha,\gamma\)-elimination of contaminating methionine to \(\alpha\)-ketobutyrate, methanethiol and ammonia, 100 µL batches of \(\beta 3\)-Met (160 mM) were incubated overnight at 37 °C with 1 unit of \(Pseudomonas\ putida\) MGL recombinantly expressed in \(E. coli\) (Biovision Inc., Milpitas, USA) in the presence of 150 µM phosphoenolpyruvate. A control sample was made by omitting MGL; this control treatment had no effect on the rates of \(^{32}\text{P}\)PPi-ATP isotopic exchange.

3.4. tRNA aminoacylation assay

Aminoacylation of \(E. coli\) tRNA\(^{\text{Met}}\) produced and purified as described (Mechulam et al., 2007) was assayed using the \([3'\text{-}^{32}\text{P}]\)tRNA\(^{\text{Met}}\)-labelling method (Ledoux and Uhlenbeck, 2008). tRNA\(^{\text{Met}}\) was labelled by exchange of the 3' terminal adenosine (Ledoux and Uhlenbeck, 2008) catalyzed by \(E. coli\) tRNA nucleotidyltransferase overexpressed from a recombinant plasmid (kind gift from Dr. Kozo Tomita, University of Tokyo, Japan). The His-tagged nucleotidyltransferase was purified by affinity on Talon resin. The labelling reaction (50 µL) contained tRNA (1 µM), sodium pyrophosphate (50 µM), MgCl\(_2\) (10 mM), glicyn (pH 9; 50 mM), \([\alpha\text{-}^{32}\text{P}]\) ATP (0.3 µM, 111 TBq/mmol) and 0.02 µM nucleotidyltransferase. After 5 min at 37 °C, 5 µL of a solution containing 1 µM CTP and 0.1 U of pyrophosphatase were added. After 2 min at 37 °C, the reaction was phenol and then ether extracted. The aqueous phase was run over a Micro Bio-Spin P-6 molecular sieving column (Bio-Rad) to remove nucleotides and in particular unreacted ATP. After ethanol precipitation, labelled tRNA was redissolved in standard buffer containing 7 mM MgCl\(_2\) and 20 µM unlabelled tRNA.

Aminoacylation activity was measured in 20 µL reactions at 25 °C in 20 mM Tris-HCl (pH 7.6), 7 mM MgCl\(_2\), 10 mM 2-mercaptoethanol, 0.1 mM EDTA, 7 mM MgCl\(_2\), 150 mM KCl, in the presence of 2 mM ATP, 2 µM \([3'\text{-}^{32}\text{P}]\) tRNA\(^{\text{Met}}\), and 2 mM of either \(\alpha\)-Met or MGL-treated \(\beta 3\)-Met. M547 enzyme diluted in reaction buffer containing 200 µg/mL BSA was used at a concentration of 1 nM in the case of \(\alpha\)-Met and 1 µM in the case of \(\beta 3\)-Met. At desired incubation times, 3 µL of the reaction mixture were withdrawn and mixed with an equal volume of SI nuclease (NEN from laboratory stocks, 2 units/µL in 0.3 M Na-acetate, pH 4.8). The digestion products were run on a PEI cellulose TLC plate (Bio-Rad) in glacial acetic acid/1 M NH\(_4\)Cl/water (5:10:85). Plates were phosphorimaged with a Typhoon scanner (GE Healthcare). Images were processed with ImageJ (Schneider et al., 2012) in order to determine for each incubation time the fraction of aminoacylated tRNA, from which aminoacylation rates were derived. A typical experiment is shown in Fig. 5.

3.5. Fluorescence at equilibrium

Variations of the intrinsic fluorescence of M547 and its variants (0.5 µM) upon titration with substrates were followed at 25 °C in 20 mM Tris-HCl (pH 7.6), 10 mM 2-mercaptoethanol, 2 mM MgCl\(_2\) and 0.1 mM EDTA as described (Mechulam et al., 1991; Schmitt et al., 1994). Measurements were done in a Hellma 1 cm × 0.4 cm cuve with
an FP-8300 JASCO spectrofluorometer (λexc = 295 nm, λem = 340 nm). All titration curves were corrected for dilution by multiplying the measured fluorescence by the ratio of the current volume in the cuvette over the initial volume before titration. Concentrations of α-Met or β3-Met (untreated or MGL-treated) were varied from 3 μM to 1 mM and from 0.06 μM to 4 mM, respectively. Data were fitted to simple saturation curves from which the corresponding dissociation constants were derived using the Origin software (OriginLab Corp.; see Fig. 2).

3.6. Fluorescence at the pre-steady state

Fluorescence measurements at the pre-steady-state were performed as described (Hyafil et al., 1976; Schmitt et al., 1994) using an SX20 stopped flow apparatus (Applied Photophysics, UK). All experiments were performed in 20 mM Tris-HCl (pH 7.6), 10 mM 2-mercaptoethanol, 2 mM MgCl2, and 0.1 mM EDTA. The formation of α- or β3-methionyl adenylate was initiated by mixing 1:1 (v/v) an enzyme solution (1 μM for α-Met or 2 μM for β3-Met) containing ATP-Mg2+ (2 mM) and PPI (10 μM) with a solution containing the same concentrations of ATP-Mg2+ plus variable amounts of the amino acid (10 μM to 640 μM for α-Met or 25 μM to 2 mM for β3-Met). For the reverse reaction, enzyme:adenylate complexes were pre-formed by incubating the enzyme with ATP-Mg2+ and the amino acid for a few minutes at 25 °C (1 μM enzyme, 0.1 mM ATP-Mg2+ and 500 μM α-Met or 2 μM enzyme, 2 mM ATP-Mg2+ and 25 μM β3-Met). The solution was then mixed 1:1 (v/v) with a solution containing the same concentrations of ATP-Mg2+ and amino acid plus variable amounts of pyrophosphate (20 μM to 1.28 mM for α-Met or 20 μM to 2 mM for β3-Met). After mixing, fluorescence was recorded and fitted to single exponentials from which the rate constants were derived (Supplementary Fig. S1). Each rate was determined three times. Kinetic parameters (kcat and km, see Scheme 1) and equilibrium parameters (KmMet, ATP and KpP) were deduced from the fit of the measured rate constants to the theoretical saturation curves (Hyafil et al., 1976; Mechulam et al., 1991; Schmitt et al., 1994) using Origin. Each experiment was performed at least twice independently. Results in Table 1 are expressed as mean ± either standard deviation from the independent experiments or standard error from the fitting procedure, whichever the greater. All experiments were performed using MGL-treated β3-Met.

3.7. Preparation of samples for mass spectrometry experiments

Amino acid samples (160 mM α-Met, β-Met or MGL-treated β-Met) were acidified by adding formic acid (10% v/v final concentration). Samples were then diluted 1000-fold in 0.1% formic acid prior to analysis. Aminoacyl adenylate samples were prepared in 0.8 mL solutions (20 mM Tris-HCl pH 7.6, 2 mM MgCl2, 10 mM 2-mercaptoethanol, 0.1 mM EDTA) containing 0.5 μM MetRS, 1 mM MGL-treatment β-Met and 2 mM ATP-Mg2+. The reaction at 25 °C was monitored by following the fluorescence as described above. After 15 min, reaction was nearly complete and 10% (v/v) formic acid and precipitated material was removed by centrifugation prior to analysis. Analysis of tRNA aminoacylation after S1 nuclease digestion was performed essentially as described (Hartman et al., 2007, 2006). tRNAMet was first aminoacylated in 1 mL samples containing 20 mM Tris-HCl (pH 7.6), 7 mM MgCl2, 10 mM 2-mercaptoethanol, 0.1 mM EDTA, 2 mM ATP, 1 μM MetRS and either 0.4 mM of α-Met or 1 mM of β-Met. After incubation for 30 min at 25 °C, the reaction was divided into 4 samples each of which was precipitated with ethanol. One sample was dissolved in 100 μL of 3 mM Na-acetate (pH 5.5) containing 1 mM zinc acetate and tRNA was digested with 10 units of S1 nuclease for 20 min at 37 °C. Formic acid (10% v/v final concentration) was then added before mass spectrometry analysis. Another sample was submitted to acetylation as follows. After dissolution of the pellet in 100 μL of 5 mM sodium acetate (pH 5.5), 100 μL DMSO, 20 μL of glacial acetic acid and 20 μL of acetic anhydride were added. After a 30 min incubation on ice, the solution was ethanol precipitated and tRNA was digested with S1 nuclease, as described above.

3.8. Mass spectrometry

Chromatographic grade solvents (99.99% purity), acetonitrile (ACN) and formic acid (FA), were purchased from Sigma Aldrich. Liquid chromatography/high-resolution mass spectrometry (LC-HRMS) analyses were performed with the timsTOF mass spectrometer coupled with an Elute HPLC system (Bruker Daltonics, Bremen, Germany).

For all experiments, 10 μL of the sample were injected and separated on an Atlantis T3 column (3 μm, 150 × 2.1 mm; Waters, Saint Quentin, France). The effluent was introduced at a flow rate of 0.2 mL min−1 into the interface with a gradient increasing from 10% of solvent B to 90% in 8 min to achieve 100% at 10 min (A: water with 0.1% formic acid (FA); B: acetonitrile with 0.1% FA). From 10 min to 12 min, the percentage of solvent increased until 90%. The gradient was then set at 10% of B for the last 6 min.

Electrospray ionization was operated in the positive ion mode. Capillary and end plate voltages were set at −4.5 kV and −0.5 kV, respectively. Nitrogen was used as the nebulizer and drying gas at 2 bar and 8 L/min, respectively, with a drying temperature of 220 °C.

In MS/MS experiments, the precursor ion was selected with an isolation window of 1 Da and the collision induced dissociation was performed using collision energies (Ecol) ranging from 7 to 25 eV. Tuning mix (Agilent, France) was used for calibration. The elemental compositions of all ions were determined with the instrument software Data Analysis. The precision of mass measurement was better than 5 ppm.

3.9. Crystallization and structure determination

Crystals of M547 or of its variants were obtained in a solution containing 30 mM KPO4, 4 mM 2-mercaptoethanol, 1.08 mM ammonium citrate (pH 7.0) and 3.6 mg/mL of protein by microseeding with crystals of M547 (Mechulam et al., 1999b). For ligand binding studies, the amino acid was added to the crystallization medium prior to microseeding. Final concentrations during crystallization were 2 mM for α-Met or 20 mM for β3-Met. For data collection, crystals were quickly soaked in a solution containing 1.4 M ammonium citrate, 30 mM potassium phosphate (pH 7.0) and 25% v/v of glycerol before flash-cooling in liquid nitrogen. Data were collected at the Proxima 1 and Proxima 2 beamlines at SOLEIL synchrotron (Gif sur Yvette, France). Diffraction images were analyzed with the XDS program (Kabsch, 1988; Collaborative Computational Project n°4, 1994) and the data further processed using programs from the CCP4 package (Collaborative Computational Project n°4, 1994). Data statistics are summarized in Table 1. Each structure was solved by rigid body refinement of the M551 model (PDB id 1QQT), using PHENIX. Refinement statistics are summarized in Table 1. Crystals were processed using programs from the CCP4 package (Collaborative Computational Project n°4, 1994). Data statistics are summarized in Table 1. Each structure was solved by rigid body refinement of the M551 model (PDB id 1QQT), using PHENIX. Refinement statistics are summarized in Table 1.

4. Accession numbers

Models coordinates and reflection files were deposited at the PDB with identification numbers 6SPN (MetRS-β3-Met), 6SP0 (MetRS-α-Met), 6SP5 (MetRS_V298I), 6SPQ (MetRS_V298I-αMet), 6SPR (MetRS_V298I-β3-Met).

CRediT authorship contribution statement

Giuliano Nigro: Investigation, Methodology, Writing - review & editing. Sophie Bourcier: Investigation, Methodology, Writing - review
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