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Identification of residues involved in the binding of methionine by *Escherichia coli* methionyl-tRNA synthetase

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Comparison of the amino-acid sequences of several methionyl-tRNA synthetases indicates the occurrence of a few conserved motifs, having a possible functional significance. The role of one of these motifs, centered at position 300 in the *E. coli* enzyme sequence, was assayed by the use of site-directed mutagenesis. Substitution of the His^{301} or Trp^{305} residues by Ala resulted in a large decrease in methionine affinity, whereas the change of Val^{298} into Ala had only a moderate effect. The catalytic rate of the enzyme was unimpaired by these substitutions. It is concluded that the above conserved amino-acid region is located at or close to the amino-acid binding pocket of methionyl-tRNA synthetase.

Methionyl-tRNA synthetase; Methionine; Spectrophotofluorimetry

1. INTRODUCTION

The elucidation of the structure–function relationships of the *E. coli* methionyl-tRNA synthetase (MTS) has recently benefited the combination of site-directed mutagenesis, genetic screening and structural studies. Residues and regions involved in tRNA recognition or in the amino-acid activation step have been identified (reviewed in [1]). However, few data are yet available about the residues involved in the amino-acid recognition. This situation contrasts with that of the *B. stearothermophilus* tyrosyl-tRNA synthetase where, thanks to the availability of co-crystals, the tyrosine discrimination by this synthetase was studied in depth [2, and references therein].

By comparing all known MTS sequences [3], three conserved motifs emerged (regions 255, 300 and 355 in the *E. coli* enzyme sequence) in addition to those of known functional significance. Although the functional importance of these three motifs has to be established, it may be expected that one or a combination of them correspond to the methionine binding site.

Chatton et al. [4] have shown that an elevation of the K_m for methionine for yeast cytoplasmic MTS was caused by the change of Gly^{502} into Asp. It is noticeable that the 500 region of the yeast enzyme aligns with the residues of the 300 region of the *E. coli* MTS (Fig. 1). This 300 region is in the vicinity of a cavity, visible on the recently solved crystallographic structure of the *E. coli* MTS:ATP complex [5] (Fig. 2). This pocket was suggested to correspond to the binding site for methionine [5]. In this study, experiments were therefore performed in order to possibly establish a link between the 300 region and the capacity of MTS to bind and/or activate methionine.

As evidenced by the comparison of the ATP complexed 3-D structure of MTS to that of free MTS [5, and S. Brunie, unpublished results], the orientation of His^{301}, a residue located on the border of the above cavity, is markedly sensitive to the complexation of ATP by MTS. The His^{301} side chain rotates by about 180° upon ligand binding. Such a localised structural change might deal with the reciprocal couplings which methionine and ATP exert when they are bound to the enzyme [6,7]. Rearrangements of the MTS 3-D structure upon methionine binding and adenylate formation are strongly suggested by associated changes of the intrinsic fluorescence of the protein [8]. In this context, on the border of the cavity, a tryptophane residue (Trp^{305}) appears as a candidate to be involved in the major increase of fluorescence occurring upon methionine binding. In addition, this residue, changed into Ala by site-directed mutagenesis, has recently been shown to participate in methionine binding and/or activation [9]. Altogether, these data prompted us to use the site-directed mutagenesis technique for further studying the role of Trp^{305}, particularly in the fluorescence of the enzyme, and for determining the importance of His^{301} in the reactions sustained by the *E. coli* MTS.

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2. MATERIALS AND METHODS

2.1. *Site-directed mutagenesis, enzyme purification and activity measurements*

Mutant genes encoding the W305A, W305F and H301A MTS were generated through oligonucleotide site-directed mutagenesis as described elsewhere [10]. Variant enzymes were overproduced and purified, as described [11]. Enzyme concentrations were calculated using a specific extinction coefficient at 280 nm equal to 1.72 cm^2/mg [8]. However, in the case of the W305A and W305F, a 6.6% lower extinction coefficient value was used to take into account the lack of Trp^{305}. Isotopic [^{32}P]PP_i–ATP exchange was assayed in standard buffer (20 mM Tris- HCl, pH 7.6 (25°C), 10 mM 2-mercaptoethanol, 0.1 mM EDTA, 7 mM $MgCl_2$) containing 2 mM of each [^{32}P]PP_i, ATP and methionine [12]. For K_m measurements, ATP was varied from 0 to 2 mM, and methionine from 0 to 30 mM. The reaction of tRNA methionylation was assayed in standard buffer containing 150 mM KCl, 2 mM ATP, 0 to 125 μM [^{14}C]-L-methionine (1.8 TBq/mol) and 8 μM $tRNA_f^{Met}$ (200 pmol/A_{260} unit) from a crude tRNA extract of $tRNA_f^{Met}$ overproducing cells [13].

2.2. *Fluorescence at equilibrium*

Intrinsic MTS fluorescence was measured at 25°C in 20 mM Tris-HCl (pH 7.6), 0.1 mM EDTA, 10 mM 2-mercaptoethanol, 2 mM free $MgCl_2$, as described elsewhere [14,15]. In the case of titration with $tRNA_m^{Met}$ (prepared as in [13]), total $MgCl_2$ was kept constant (8 mM). The dissociation constants of complexed substrates, as well as the amplitudes of fluorescence variations, were deduced by least-square fitting the theoretical curves to the titration data [14,16].

3. RESULTS

To study the role of Trp^{305} and that of His^{301}, the *met*G547 gene was used as the starting material. This gene expresses M547, a fully active truncated monomeric form of *E. coli* MTS [10]. The Trp residue was substituted by either Phe (W305F enzyme), or Ala (W305A), and the His residue was changed into an Ala (H301A). In addition, during a PCR amplification of the *met*G547 gene, the mutation of the GTT codon for Val^{298} into GCT which encodes Ala, as well as two other silent mutations were obtained accidentally. The resulting V298A enzyme variant was also studied.

The michaelian parameters of the homogeneous H301A, W305F, W305A and V298A enzymes in the [^{32}P]PP_i-ATP exchange and $tRNA_f^{Met}$ aminoacylation reactions were determined (Table I). The K_m's for methionine of the W305A enzyme were markedly increased (23-fold and 16-fold, respectively, in the two

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E. coli      291HFIGKDIVYFHSLFWP-AML309
B. stearo.   257HLVGKEIVRFHTIYWP-IML275
T. therm.    255HLIGKDILKPHAVFWP-TML273
S. c. mit.   400HVIGHDIAKFHTVYWP-SFL418
S. c. cyt.   482QFMGKDNVPFHTVVFPGSQL501
Consensus    H[L,F]IGKDIV[R,K]FHTV[F,Y]WP-[S,T]ML
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Fig. 1. Conservation of the *E. coli* MTS motif 300 within the known methionyl-tRNA synthetases sequences. The sequences of MTS from *E. coli* [18], *Bacillus stearothermophilus* [3], *Thermus thermophilus* [19], *Sacharomyces cerevisiae* mitochondrion [20] and *Sacharomyces cerevisiae* cytoplasm [21] were aligned. Shown at the bottom is a consensus sequence obtained through the selection of those residues conserved in at least three out of the five compared sequences. At locations which do not satisfy the above rule, two alternative consensus residues are proposed.

MTS catalysed reactions), whereas the K_m for ATP, as measured in the exchange reaction, did not vary by more than 50%. In the case of the H301A enzyme, the K_m for methionine was more deeply affected, with a 345-fold increase in the exchange reaction, and an immeasurably high value in the aminoacylation reaction. The corresponding V/K_m value was $10^3\ s^{-1}\cdot M^{-1}$, as compared to $0.9 \times 10^6\ s^{-1}\cdot M^{-1}$ for the M547 control enzyme. The effect of the V298A mutation was rather small, with a K_m for methionine only 4-fold greater than that of the control enzyme. Contrasting with the above data, the W305F change resulted in K_m for methionine smaller than those measured in the case of M547.

In all the cases studied, the V values were not significantly lowered. This precludes that the mutations have disorganised the catalytic centre of the enzyme, and indicates that the V298A, W305A and H301A substitutions specifically act at the level of methionine binding.

To probe this conclusion, the binding constants of methionine to the enzymes were determined spectrophotofluorimetrically (Fig. 3). The results in Table II confirmed that the V298A and W305A substitutions affect the primary binding of methionine. The affinities of the amino acid for the mutant enzymes were reduced by factors of 3.8 and 42, respectively. A parallel effect was observed for the binding of methioninol, thus demonstrating that the loss of affinity for methionine was indifferent on the presence or the absence of the carbo-

Table I

K_m (μM) and V (s^{-1}) values for the tRNA aminoacylation and [^{32}P]PP_i–ATP isotopic exchange reactions catalysed by the MTS variants

		M547	V298A	W305F	W305A	H301A	
$tRNA_f^{Met}$ aminoacylation	K_m of methionine	3.4±0.8	25±7	2.5±0.7	55±5	>300
	V at infinite methionine	3.1±0.4	2.2±0.6	3.5±0.4	2.0±1.0	>0.3
[^{32}P]PP_i-ATP isotopic exchange	K_m of methionine	22±2	95±10	20±2	510±50	7200±1200
	V at infinite methionine	50±2	47±2	58±2	49±4	30±4
	K_m of ATP	300±30	650±120	320±60	410±60	850±120[a]
	V at infinite ATP	50±5	52±6	53±7	48±5	43±3[a]

[a]Measured in the presence of 35 mM methionine.

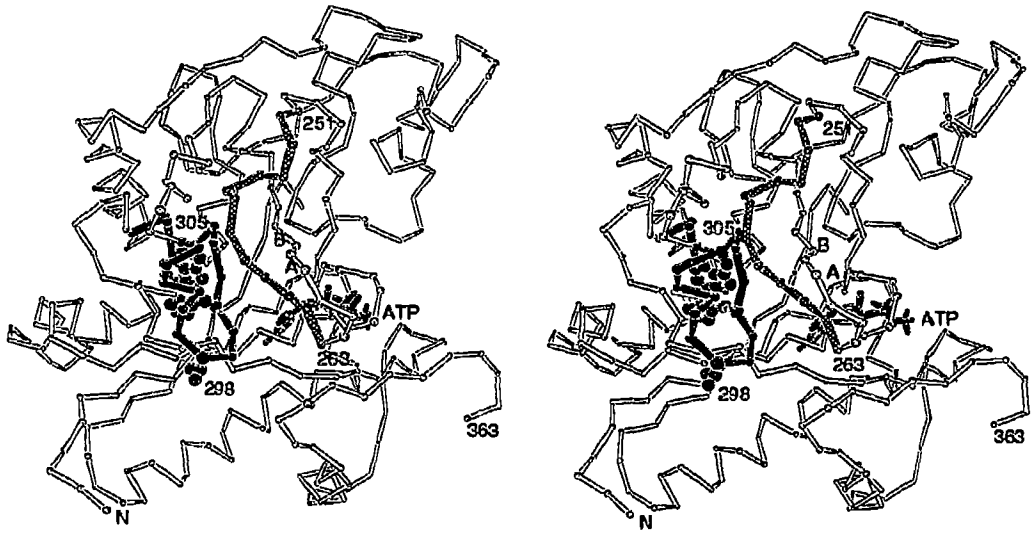

Fig. 2. Stereo view of the putative methionine binding cavity of ATP-complexed MTS. The site is limited in the back with the A and B strands of the β-sheet of the nucleotide-binding fold, and on the left with the side-chain of Trp^{305}. The front border is composed of two distinct peptides: the conserved stretch of sequence around region 300 is shown in black; the peptide containing the stretch of region 255 [3] is shaded grey. In the free enzyme structure, the imidazole ring of His^{301} is rotated by about 180°, partly locking the accessibility to the site. Note that Lys^{335} [16] and Arg^{233} [9] were found to participate to the stabilisation of the transition state on the way to methionyl-adenylate formation. The figure was drawn using the MAXIMAGE/PREMA programmes kindly provided by Mark Rould (Yale University, USA).

xylate moiety of the amino acid. Concerning the H301A enzyme, the affinity of methionine as well as that of methioninol were immeasurably low, consistently with the major effect of this substitution on the K_m values of the amino acid. On the other hand, in the case of the W305F enzyme, the affinity for methionine as well as that for methioninol were slightly enhanced by a same factor of 1.3, in good agreement with the lowered K_m values of methionine reported above. Finally, it should be noted that the dissociation constant of the enzyme:$tRNA_m^{Met}$ complex was not significantly modified by any of the studied mutations.

In the case of MTS, the formation of the reactive complex E:met:ATP-Mg^{2+}, which precedes the activation of the amino acid, depends on the establishment of positive couplings between the methionine and ATP sites on the enzyme [6,7]. These couplings are believed to more or less compensate for the expected electrostatic repulsion between the negative charges carried by the carboxylate of methionine and the α-phosphoryl group of ATP [15]. Therefore, the free energy of coupling can be made measurable by suppressing one of these two negative charges, when following the effect of the occupation of the methionine site on ATP-Mg^{2+} binding, and vice versa. For instance, methioninol can be used in place of methionine, or a combination of adenosine plus PP_i-Mg^{2+} can mimic the ATP-Mg^{2+} molecule devoid of the α-phosphate charge.

To further analyse the enzymes, the binding of methionine was studied in the presence of 9.4 mM adenosine or of 9.4 mM adenosine plus 2 mM PP_i-Mg^{2+}, as well as that of methioninol in the presence of 8 mM ATP-Mg^{2+}. The addition of adenosine plus PP_i or of ATP rendered measurable the binding constants of methionine or methioninol to the H301a enzyme (Table II). This shows that H301A has retained some capacity to couple between the sites for the two substrates of the methionine activation reaction. The main effect of the His^{301} substitution is thus a loss of affinity for methionine. In addition, the results in Table II demonstrated that the synergistic coupling factors between the site for the nucleotide and that for the amino acid were only slightly sensitive to the other mutations studied. Consequently, the V298, H301 and W305 residues appear not to participate in the transition state stabilisation on the way to methionyl-adenylate formation. Such a conclusion is in agreement with the insensitivity of the V values of MTS to the mutations studied.

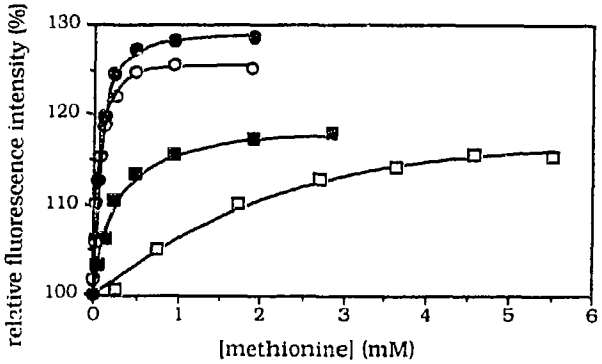

Fig. 3. Titration of the studied enzymes (0.8 μM) by methionine. The fluorescence (% of that of the enzyme without methionine) is plotted as a function of total added methionine. The curves calculated from the dissociation constants in Table II are drawn. The molar free enzyme fluorescences, in % of that of the control M547 enzyme (full circles), were: W305F (open circles), 88%; W305A (open squares), 106%; V298A (full squares), 96%. The relative increase in enzyme tryptophane fluorescence upon methionine saturation were 30 ± 2%, 26 ± 2%, 23 ± 3% and 20 ± 2%, for M547, W305F, W305A and V298A, respectively.

Table II

Equilibrium binding parameters of ligands to the MTS variants. Equilibrium constants (μM) for methionine, methioninol, and $tRNA_m^{Met}$ were obtained by spectrophotofluorometric titrations, as described in section 2. Coupling factors between the methionine, adenosine and pyrophosphate sites were evaluated through the measurement of the apparent dissociation constants of methionine in the presence of either 9.4 mM adenosine (K_{met}^{ado}) or 9.4 mM adenosine plus 2 mM PP_i-Mg^{2+} ($K_{met}^{ado\text{-}PP}$). K_{metol}^{ATP}, the apparent dissociation constant of methioninol was measured in the presence of 8 mM ATP-Mg^{2+}. Coupling constants representative of the effect of adenosine on methionine binding, of that of PP_i on methionine binding in the presence of adenosine, and of that of ATP on methioninol binding, are calculatable from the various dissociation constants. The coupling constants C_{met}^{ado}, $C_{met}^{ado\text{-}PP}/C_{met}^{ado}$ and C_{metol}^{ATP} correspond to K_{met}/K_{met}^{ado}, $K_{met}^{ado}/K_{met}^{ado\text{-}PP}$ and $K_{metol}/K_{metol}^{ATP}$, respectively.

	M547	V298A	W305F	W305A	H301A
K_{met}	50±5	190±15	40±4	2100±500	n.m.[a]
K_{met}^{ado}	11±2	81±4	9.6±3.8	500±80	n.m.
$K_{met}^{ado\text{-}PP}$	0.23±0.04	2.0±0.1	0.22±0.05	15±2	460±59
K_{metol}	550±60	2500±200	410±70	12000±4000	n.m.
K_{metol}^{ATP}	2.7±0.2	21±2	3.4±0.4	95±8	900±90
K_{tRNA}	0.8±0.4	1.3±0.4	1.3±0.4	1.4±0.5	1.1±0.4
C_{met}^{ado}	4.5±1.3	2.4±0.3	4.2±2.1	4.2±1.7	n.m.
$C_{met}^{ado\text{-}PP}/C_{met}^{ado}$	48±22	41±7	44±32	33±18	n.m.
C_{metol}^{ATP}	204±37	120±21	200±58	126+53	n.m.

[a]n.m.: not measurable.

Finally, the variations of fluorescence following methionine saturation of the studied mutants were compared (Fig. 3). The extents of these variations, expressed as percentages of the fluorescence of the free enzyme species, (26%, 23% and 20% for W305F, W305A and V298A, respectively) remained comparable to the variation recorded with the M547 control enzyme (30%). A central role of Trp^{305} in the methionine-dependent fluorescence stimulation of MTS can be therefore excluded.

4. CONCLUSION

The localisations of His^{301} and Trp^{305} residues in the MTS 3-D structure and the effect of their mutations on the methionine binding parameters reinforce the idea that the 7Å cavity noted in [5] actually is the methionine binding pocket. Val^{298}, located in a segment (residues 296–302) connecting helixes H3 and HD, does not belong to the residues surrounding this cavity. The small variation of the methionine binding free energy caused by the V298A transition could be thus explained by an indirect perturbation of the methionine site, rather than by direct involvement into the methionine pocket.

We propose here that the main role of the His^{301} and Trp^{305} side-chains is to interact with that of methionine. Recently, an alignment of class I aminoacyl-tRNA synthetases sequences [17] was performed (C. Landès, J.J. Perona, S. Brunie, M.A. Rould, C. Zelwer, T.A. Steitz and J.L. Risler, to be published). Interestingly, this work made visible some conservation of the 300 motif region of *E. coli* MTS within the isoleucyl-, leucyl- and valyl-tRNA synthetases sequences. However, in this comparison, His^{301} of MTS is not shared by other synthetases, whereas Trp^{305} can also be found in the compilated valyl-tRNA synthetases sequences. Taking into account the larger effect of His^{301} substitution on methionine binding, as compared to the Trp^{305} substitution, it may be speculated that the capacity of MTS to discriminate among amino acids whose side-chains possess an aliphatic character is rather based on the presence of His^{301}, whereas Trp^{305} would stabilise the enzyme:methionine complex in a less specific manner, through hydrophobic contact. Consistent with this, the aromatic character of residue 305 is enough to insure methionine binding. Substitution of Trp^{305} by a phenylalanine does not lower methionine affinity. Noticeably, the *S. cerevisiae* cytoplasmic enzyme sequence aligning with the *E. coli* enzyme 300 region contains a Phe instead of a Trp at the corresponding position.

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