Structure–activity relationships of methionyl-tRNA synthetase: graphics modelling and genetic engineering

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The 3D structure of the methionyl-tRNA synthetase from E. coli *has been investigated using X-ray analysis[1,2] at a resolution 1.8Å. 90% of the molecule is now well defined and the zinc atom has been identified in a buried region of the molecule, close to the active site. At the same time, the refinement of the complex ATP–MetRS at 2.5Å has been carried out. The crystallographic* R *factor has been assigned a value of 25% at 2.5Å with an overall temperature factor of 9Å² and 22% when the individual temperature factors are refined. A Fourier difference map clearly reveals the electron density of the bound ATP, showing the phosphate groups deeply plunging into the active site. In parallel, the synthetase gene has been used to probe some of the enzyme structure–activity relationships. A series of 60 modified enzymes truncated at the C-terminus have been constructed* in vitro *and assayed for activity. In agreement with the graphics model, the results show that a minimum of 534 residues is necessary to sustain the aminoacylation reaction. A programme of site-directed mutagenesis is in progress: residues thought to be important for the catalytic activity, the metal coordination and tRNA interaction are being modified. Preliminary results are discussed in the light of the crystallographic model.*

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Aminoacyl-tRNA synthetases are responsible for an essential step in protein biosynthesis. In the presence of ATP, they catalyse the specific transfer of an amino-acid to its cognate tRNA, thus ensuring the fidelity of genetic code translation. Aminoacyl-tRNA synthetases are therefore interesting models that can be used in the study of protein–nucleic acid recognition. The synthetase–tRNA interaction has been extensively studied using various physical and biochemical approaches (reviewed in Schimmel and Söll in Reference 3):

- Recently, crosslinking experiments have provided information about the chemical interaction of *E. coli* methionyl-tRNA synthetase (MetRS) with the CCA terminus[4] and the anticodon[5] of the $tRNA_f^{Met}$.
- The complete sequences of 17 aminoacyl-tRNA synthetase genes of various origins are presently known.
- The X-ray 3D stucture of tyrosyl-tRNA synthetase (TyrRS) and MetRS have been undertaken at high resolution while a tRNA–enzyme complex of the Asp system from yeast[7] is now the subject of an extensive X-ray study.

This family of enzymes exhibits a wide variety of quaternary structures and molecular weights. The comparison of their primary sequences shows little homology. However, a comparison of the 3D backbones of the methionyl- and tyrosyl-tRNA synthetases has shown that the homology between their 3D structures is larger than the homology obtained from sequence comparison[8]. Observation of the 3D structure of these synthetases has shown a 'Rossmann fold' at the aminoacylation site, which is characteristic for nucleotide-binding proteins[9]. Recently, the study of some tyrosyl-tRNA synthetase mutants, obtained by *in vitro* mutagenesis, has shown the role of specific side chains of this 'nucleotide-binding domain' involved in the interaction with ATP and tyrosine[10–13]. The two proteins are organized in two distinct structural domains: the first one contains the Rossmann fold, the N-terminus and the active site; the second domain, is totally helical in MetRS. Such a biglobular organization could be applied to other synthetases.

Another interesting feature to be mentioned is the high homology of sequence (about 200 residues) observed in the N-terminal domain, between the MetRS from *E. coli*[14,15] and its homologous enzyme from yeast[16]. Such an homology is also observed between the TyrRS from *B. stearothermophilus*[17] and that from *E. coli*[18].

3D STRUCTURE OF THE METHIONYL-tRNA SYNTHETASE

Two active forms of the MetRS from *E. coli* have been characterized: a native dimeric form with a molecular

weight of 2 × 76 000 and a monomeric form, with a molecular weight of 64 000, obtained by mild trypsin proteolysis[19]. This tryptic fragment retains full specificity towards methionine and $tRNA_f^{Met}$ and full activity in both the activation and aminoacylation reactions. This form has been crystallized[20] and studied[1,2,21] by X-ray diffraction.

As an initial step in the determination of the structure of MetRS, a Richards's box model allowed a rough interpretation of the 2.5Å Multiple Isomorphous Replacement (MIR) map, the identification of about two thirds of the main chain and the existence of a so-called Rossmann fold[1,2]. The existence of ill-defined areas in the electron density map, alternating with interpretable areas, made the alignment with the sequence very difficult. In fact, only the first 55 residues were correctly aligned on the model. From these preliminary results, the molecule was assumed to be made up of three domains with a disordered C-terminus in the crystals; the β-sheet of the Rossmann fold was considered to be composed of five parallel strands, a third domain being inserted between the third and fourth strands.

A nonnegligible improvement of the MIR map was obtained with a set of data collected using synchrotron radiation, coupled to the development of a new algorithm to process the solvent flattening in the electron density[22]. Nevertheless, now that the refinement of the structure has almost been completed, it must be emphasized that such a large structure could not have been solved without the help of conjugate techniques: graphics modelling, biochemical data and site-directed mutagenesis.

As a starting point, affinity labelling by oxidized $tRNA_f^{Met}$ has led to the identification of two reactive lysines (61 and 335) at the CCA binding site of the tRNA[4]. In addition, possible involvement of the Lys465 in the enzyme–anticodon interaction has been revealed by chemical crosslinking[5]. Thus, it was expected that Lys335 would be close to the active site and Lys465 would be as far as possible from this active site. With these two anchor points, the authors have been able to align the amino-acid sequence in the helical domain, representing approximately 200 residues.

The extensive use of the interactive modelling program Frodo[23] implemented initially on an MPS and later on an Evans & Sutherland PS300 graphics system allowed the identification of 75% of the atoms. In the areas where the sequence alignment was uncertain, only the peptide backbone and the first atoms of the side chains were placed. Then, the refinement of the structure started, using the Hendrickson and Konnert procedure[24] interrupted by frequent graphic adjustments. This continuous graphic revision was required to fit the molecule to the density, and also to correct the geometric errors (e.g. when the torsion angles did not satisfy the Ramachandran plot) and, step by step, to add more atoms. Calculations were made on a Cray-1 computer and a Star 100 parallel processor.

Nucleotide binding domain

Realigning the sequence of amino-acids in the model led to some reinterpretation of the backbone of the molecule. Thus, it turns out that the structure comprises

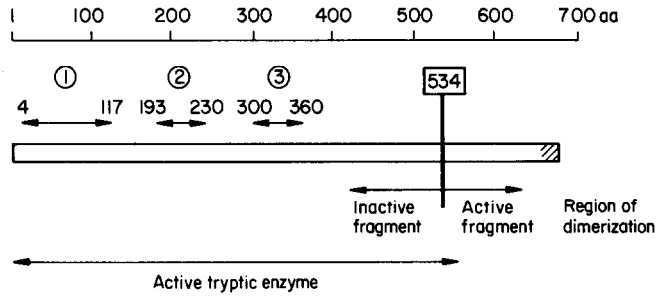

Figure 1. Structural properties of MetRS. The three horizontal arrows numbered ①, ② and ③ indicate the regions involved in the Rossmann fold. The length of the active monomeric tryptic fragment is represented by a horizontal arrow. From a set of 60 modified enzymes truncated at the C-terminus, the results have shown that a minimum of 534 amino-acids are necessary to sustain both activation of methionine and the aminoacylation of $tRNA^{Met}$

two domains (see Colour Plate 1): the first domain, containing the Rossmann fold, ends at residue 360. This is the starting point of the second, helical domain. Moreover, the β-sheet of the Rossmann fold appears to be composed of six parallel strands instead of five. The only homology between the 3D structures of MetRS and TyrRS lies in the superposition of the first five strands of the β-sheet of the Rossmann fold; the sixth short strand of MetRS is in the prolongation of the first one of TyrRS.

Length of the tryptic fragment

The crystallographic study was performed with the monomeric tryptic fragment. For years, a puzzling problem has been the identification of the exact length of this molecule.

The determination of the minimum length of the molecule required for activity and enzyme specificity has been helpful to solve part of the structure, on the active site side, for which the quality of the MIR map was badly resolved. There is a 'bulge' of density in which the C-terminus is buried. The C-terminal peptide is well indentified up to Tyr531, which is very close to the active site, and probably involved in the interaction with the CCA acceptor arm of tRNA. In close agreement with this observation, preliminary results concerning deletion mutants strongly suggest that Leu530, Tyr531, Asn532 and Arg533 are directly implicated in catalysis. The results show that a minimum of 534 residues are necessary to sustain the aminoacylation reaction (see Figure 1). From the electron density map, it can be assumed that the C-terminal residue is Lys547. With such an assumption, the C-terminal backbone folds back towards the N-terminus domain, on the other side of the central β-sheet, making a link between the two domains (this agrees with Reference 25), and shaping a long channel into which the 3′ acceptor arm of the tRNA can bind.

Localization of the zinc atom

The MetRS is a metalloenzyme with one zinc atom per subunit whose structural role has been confirmed[26]; a functional role of the zinc is more hypothetical, since removing the zinc may affect the environment of the active site.

Table 1. Distances between the zinc atom and its closest neighbours

Residue	His28(Nδ)	Thr82(Oγ)	Asp83(Oε)	His95(Nε)
Distance from zinc atom (Å)	2.82	3.09	2.94	3.65

When the crystallographic *R* factor had reached the value of 30%, a strong peak on the difference Fourier map could be assigned to the zinc atom. This assumption was confirmed during the course of the refinement; calculations were performed using the scattering diffusion factor of Zn^{++}. Its individual temperature factor *B* was assumed to be 15Å^2. However, when Zn^{++} is replaced by a water molecule in the calculations, its individual *B* becomes negative.

The best candidates for ligands of the metal atom appear to be: Thr82, Asp83, His95, His28 and, possibly, a water molecule. Table 1 gives the distances between the zinc atom and the closest neighbours; the van der Waals surface of the environment of the zinc atom is shown in Colour Plate 2.

In order to clairfy the catalytic and/or structural role of the zinc atom, all of the putative coordinating residues are currently being modified by mutagenesis. The effect of each mutation will be determined after purification of each mutated form of the MetRS.

Localization of the ATP site

After about 90% of the structure had been identified, it was decided to pursue the refinement of the structure of the ATP–enzyme complex obtained by diffusion of ATP into crystals of MetRS. The experimental data for this complex was collected using synchrotron radiation. The crystallographic factor *R* has been found to be 25% with an overall temperature factor of 9Å^2, and 22% when the individual temperature factors were refined. The root-mean-square deviation of the bond length from ideality is 0.028Å. The solvent has not yet been added.

At this stage of the refinement of the ATP complex, the electron density of the ATP molecule became clearly visible on a Fourier difference map. The three phosphate groups deeply enter the active site: the γ-phosphorus is close to Cys11, and the β-phosphorus is close to His28. Since His28 is a possible ligand of the zinc atom, it might form a gate between the zinc and the ATP sites and play a role in both the ATP and zinc binding processes. Experiments aimed at modifying His28 by site-directed mutagenesis are currently in progress.

Colour Plate 3 shows the graphics modelling of the ATP molecule on the electron density map; the density of the adenine is not yet well defined. However, the location of ATP in the active site is in agreement with the observation made earlier from the Fourier difference map between the native enzyme and a bromo-ATP derivative used as a heavy atom derivative in the phase determination[2]. Although the quality of this difference map was poor, it was possible to assume that the phosphates were located in the active site of the enzyme, a situation different from that of AMP when bound to TyrRS. In fact, the ATP complex of TyrRS has not yet been obtained, the concentration of bound ATP being too low in the crystals[27].

INTERACTION BETWEEN MetRS and $tRNA_f^{Met}$

In order to identify the possible residues involved in the contact of MetRS with $tRNA_f^{Met}$, a hypothetical model of the interaction of MetRS and tRNA was constructed graphically; because the coordinates of $tRNA^{Met}$ were not available, those of yeast $tRNA^{Phe}$ were used in the modelling process. Recently, another tentative model of the interaction between TyrRS and $tRNA^{Tyr}$ has been proposed[28]. However, a large part of the X-ray structure (about 100 residues) of the C-terminal domain of the TyrRS is still unknown, thus the proposed model only considers the interaction of the acceptor arm of $tRNA^{Tyr}$ with the enzyme. The main difference between the two synthetases, with respect to their interaction with tRNA, is their size. The distance between the active site of MetRS and the tip of the molecule in the helical domain agrees well with the distance between the acceptor arm and the anticodon (about 70Å). In the case of the TyrRS, because of its smaller molecular weight this distance is shorter and the interaction with the $tRNA^{Tyr}$ must involve the two subunits of the dimer as shown by neutron scattering experiments[29].

The docking of MetRS with $tRNA^{Phe}$ has been made using the program Hydra that allows an online monitoring of the energy (van der Waals and electrostatic) of interaction; this docking uses two anchor points determined biochemically as explained previously. The anticodon must be in the vicinity of Lys465 and the acceptor arm must be closed to both Lys61 and Lys335, lying in the channel shaped on one side of the active site by the C-terminus of the enzyme (see Colour Plate 4). This interpretation appears to be consistent with the results obtained by mutagenesis; substitution of Lys335 for a Glu results in complete inactivation, whereas replacement of Lys61 with Gln alters the Michaelis constant of ATP in the aminoacylation reaction.

Admittedly, this model is still preliminary. However, it looks reasonable and may represent a good starting point for the location of all the possible contacts between the two molecules in order to indicate the residues to be engineered. Of course, further docking will have to be performed using the $tRNA_f^{Met}$ model from *E. coli*, or possibly that from yeast, whose structure is presently under refinement[30].

CONCLUSION

MetRS is one of the few systems that has been studied using techniques such as crystallography, biochemistry and mutagenesis. In addition, the authors are convinced that the complex interactions between a protein and a large nucleic acid such as tRNA, deserves to be studied using molecular dynamic methods. This will be their goal as soon as the refined high resolution structure is available.

The main problem when changing residues by site-directed mutagenesis is to ascertain whether the resulting modifications (e.g. changes in affinity or kinetic parameters) reflect a direct or an indirect effect. Currently, no well-defined engineering procedure has been estab-

lished. The strategy usually adopted is a modification of the van der Waals volume of the residue by either maintaining the hydrophilic character of the residue and altering the P_k, or changing the hydrophilic character and conserving the P_k. Nevertheless, this technique emerges as a useful tool in the determination of enzymatic mechanisms even if the mutations are still often difficult to interpret. While such a technique requires the precise knowledge of the 3D structure, it is noticeable that, in turn, it can provide help in solving 3D structures.

In conclusion, the authors believe that identification of the amino-acid residues and the nucleotides that are essential in the MetRS–tRNA interaction may help to define universal rules which govern the specific recognition between synthetases and tRNA.

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