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Methionyl-tRNA Synthetase from *Escherichia coli:* Active Stoichiometry and Stopped-Flow Analysis of Methionyl Adenylate Formation†

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ABSTRACT: Native dimeric methionyl-tRNA synthetase and its monomeric proteolytic fragment are shown to form and to bind 1 mol of methionyl adenylate per polypeptide chain. Moreover, at 25 °C, each monomer of the dimeric native enzyme behaves independently, exhibiting the same parameters for the methionine activation reaction as does the monomeric modified enzyme. These results were obtained using several independent methods including equilibrium and nonequilibrium dialysis, active site and tryptophan fluorescence titrations, and stopped-flow by fluorescence. Stopped-flow resolution of the reversible methionine activation reaction also demonstrates that methionine and ATP-Mg^{2+} react without coupling to form a ternary enzyme·methionine·ATP-Mg^{2+} complex. This complex readily converts to enzyme·methionyl~adenylate·PP-Mg^{2+} with a standard free energy close to zero. It is concluded that the uncoupled enzyme·methionine·ATP-Mg^{2+} complex may resemble the transition state of the reaction at the expense of the additional synergistic binding energy provided by reciprocal coupling, within the site, of the methionine molecule with the adenosine and PP-Mg^{2+} parts of the ATP-Mg^{2+} molecule (Blanquet, S., Fayat, G., and Waller, J. P. (1975), *J. Mol. Biol. 94,* 1).

Recent studies have demonstrated that several aminoacyl-tRNA synthetases exhibit extensive sequence duplication. Enzymes so far shown to possess this property are either monomeric structures composed of a polypeptide chain having twice the normal subunit size (i.e., approximately 100 000 daltons) (Kula, 1973; Koch et al., 1974; Waterson and Konigsberg, 1974; Bruton, 1975a) or else dimeric enzymes composed of two such large protomers (Bruton et al., 1974). The existence of these large protomers, which may have arisen through duplication and fusion of adjacent genes coding for normal sized subunits (i.e., approximately 45 000 daltons), raises the important issue of their functional significance.

In the case of tyrosyl-tRNA synthetase from prokaryotic origin, composed of two normal, 45 000-daltons subunits in noncovalent association, it has been shown that the subunits exhibit anticooperativity for substrate binding to the tyrosine adenylating sites, while on the other hand both subunits are able to synthesize tyrosyl adenylate at different rates (Fersht, 1975; Jakes and Fersht, 1975). In an attempt to extrapolate this property to the synthetases which possess the sequence duplication feature, these authors have performed experiments which tend to indicate that, in spite of the loss of symmetry resulting from covalent associations, remains of this anticooperativity have been preserved during the fusion process. The rationale of the two sets of binding sites for amino acid and ATP in such twice repeated sequences has been proposed in terms of a general mechanism for enhancement of specificity for the amino acid in the esterification of tRNA (Fersht, 1975).

However, the specificity of interaction with tRNA itself plays a role at least as crucial as that involving amino acid in

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determining the accuracy of protein synthesis. Therefore, repeating sequences, acquired by synthetases during evolution, may also provide an advantage for specific binding of tRNA. Indeed it has been suggested that an internal pseudo-symmetry could appear in some parts of the tRNA structure which bind both the "unit domains" of the enzymes (Kim, 1975). Such a "two-point" binding process would be capable of better discriminating between cognate and noncognate tRNAs by amplifying the difference in their free energy of binding.

The present report deals with the demonstration that, in the case of the native methionyl-tRNA synthetase composed of two large protomers, as well as in the case of its monomeric tryptic fragment, no more than one binding site for the adenylation of methionine per single polypeptide chain can be detected, at least in the absence of tRNA.

Investigations on the primary structure of native dimeric methionyl-tRNA synthetase indicate that each monomer of molecular weight 86 000 contains about 200 amino acid residues in repeated sequences (Bruton et al., 1974; Bruton, 1975b). Proteolysis of the native enzyme by trypsin generates two molecules of modified monomer with a molecular weight of 64 000 (Cassio and Waller, 1971b). Sequence analysis reveals that cleavage occurs exclusively at the carboxyl-terminal extremity of the polypeptide chain and that no more than 50 amino acid residues remain duplicated in the modified enzymatically active fragment (Bruton, 1975b). The monomeric tryptic fragment is, therefore, unlikely to have conserved intact a hypothetical second set of binding sites. The demonstration, in the present study, that this modified enzyme exhibits thermodynamic parameters which are identical with those of the native enzyme also argues against the existence of two sets of binding sites per subunit of this enzyme.

Materials and Methods

Homogeneous native methionyl-tRNA synthetase was purified using a published procedure from *E. coli* strain EM 20031 carrying the F 32 episome (Cassio and Waller, 1971a). Homogeneous trypsin-modified methionyl-tRNA synthetase was derived from the purified native enzyme as described earlier (Cassio and Waller, 1971b). The enzymes were stored in 50% glycerol at −15 °C at concentrations of the order of 15 mg/ml. Enzyme concentrations were determined from their absorbancies at 280 nm as indicated elsewhere (Blanquet et al., 1973). It was verified that the enzyme solutions were devoid of contaminating pyrophosphatase activity (Blanquet et al., 1974). Yeast inorganic pyrophosphatase (Boehringer) (EC 3.6.1.1) has a specific activity of 200 units/mg. Adenosine and ATP (Na) are purchased from Boehringer (Mannheim). L-Methionine, PP (Na), and imidazole are from Merck. The latter was recrystallized from methanol. The labeled [α-^{32}P]ATP (93 mCi/mmol) and [^{14}C]methionine (53 mCi/mmol) are from the Commissariat à l'Energie Atomique (France). Purity of the labeled methionine was checked by paper chromatography in 2-propanol–H_2O (7:3, v/v); its sulfoxide content was evaluated by counting the radioactivity of the corresponding spots. Purity of the nucleotide was verified by thin-layer chromatography on cellulose, using the solvent system isobutyric acid–water–ammonia (66:33:1, v/v/v). The [γ-^{32}P]ATP (1789 mCi/mmol) was purchased from the Radiochemical Centre, Amersham.

The experiments mentioned in this article have been performed at 25 °C in standard buffer containing 20 mM imidazole hydrochloride at pH 7.6, 0.1 mM EDTA, and 10 mM 2-mercaptoethanol. The magnesium content was 7 mM, unless specified.

Equilibrium Dialysis. Equilibrium dialysis measurements were performed in slowly rotating cells equipped with IRIS 3069 membranes (Société des Usines Rhône-Poulenc, Paris). The cell chamber which received the ligands was filled to 0.4 ml, while the enzyme chamber was filled with 0.050 ml (Blanquet et al., 1975a). In one set of experiments, 6.25 ng of yeast pyrophosphatase (Boehringer-Mannheim) was added to the enzyme chamber. In this case the labeled methionine concentration was varied from 20 to 200 μM, while in the other cases it was from 2 to 200 μM. Equilibration of ligands was achieved in 2 h. At the end of the experiments, portions from each compartment were counted in a liquid scintillation counter after addition of 12 ml of Bray's solution.

Active Site Titration. [γ-^{32}P]ATP, enzymes, and yeast inorganic pyrophosphatase (1.25 μg) were incubated at 25 °C in standard buffer (1.8 ml) containing 7 mM magnesium chloride. Synthesis of methionyl adenylate was started by adding an aliquot of methionine. Control experiments were performed in parallel without adding the requisite amino acid. From zero time, 100-μl aliquots of this mixture were periodically mixed with 2.5 ml of 0.35% perchloric acid in 50 mM sodium acetate (pH 4.5) containing 0.4% w/v activated charcoal plus 100 mM PP_i and 100 mM P_i. After adsorption of the labeled ATP, the Norit was filtered on Whatman no. 1 filter paper disks, washed, and counted on an IDL crystal scintillation counter.

Nonequilibrium Dialysis. The apparatus consists of a lucite dialysis cell (kindly provided by Dr. Pantaloni) with an open-to-the-air upper chamber containing the enzyme and labeled substrates, separated by an IRIS 3069 membrane (Société des Usines Rhône-Poulenc) from a lower chamber through which buffer is pumped at a constant rate and from which the effluent is sampled for measurements of radioactivity. The upper chamber (1 ml) was filled with 0.5 ml of a solution containing the enzyme and α-^{32}P-labeled ATP in standard buffer with 10 mM magnesium chloride and 0.1 μg of yeast inorganic pyrophosphatase.

The formation and subsequent reversion of methionyl adenylate were initiated by adding 5-μl aliquots of stock solutions (200 mM) of methionine and pyrophosphate, respectively. Pumping of the effluent into the lower chamber (0.4 ml) was ensured by a perfusor (B. Braun Melsungen S. A.) working at a constant rate (30 ml/h). This rate was controlled using a flowmeter (Sho-rate, Brooks) equipped with a sapphire ball. The dialysis cell was connected to the perfusor and to the collector with the shortest lengths of polyethylene catheter no. 1 from Biotrol (inner diameter of 0.30 mm). Stirring of the upper and lower chambers was respectively ensured by a bent rotating Pasteur pipet and a magnet. During the course of the experiment, the volume contained in the upper chamber was controlled by taking 5-μl aliquots each 15 min and comparing their radioactivity content with the measured distribution of the radioactivity between the two chambers at the same time. Finally the dialysis cell was thermostated at 10 °C. Fractions obtained during dialysis (1.5 ml) were directly counted in an SL30 Intertechnique spectrometer, using the Czerenkov effect.

Fluorescence at Equilibrium. The excitation light (295 nm) is provided by a stabilized 450-W Osram xenon source analyzed by a HRS2 monochromator (Jobin & Yvon). The emission of fluorescence is registered at 332 nm using another HRS2 monochromator followed by an EMI 6456 photomul-

[1] Abbreviations used: E, enzyme; AA, L-methionine; PP, pyrophosphate; AA~AMP, methionyl adenylate; P_i, inorganic phosphate.

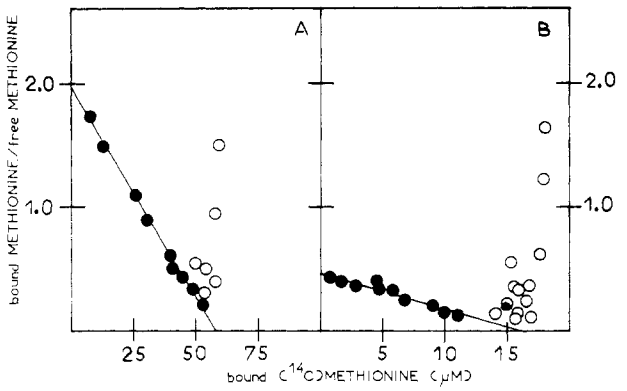


FIGURE 1: Equilibrium dialysis. Native (panel A) and trypsin-modified (panel B) methionyl-tRNA synthetases at concentrations of 27 and 19 μM, respectively, are titrated by [^{14}C]methionine in the presence of 1.85 mM ATP with 2 mM pyrophosphate (-●-) or pyrophosphatase (-○-). All experiments are performed in the presence of 7 mM magnesium chloride.

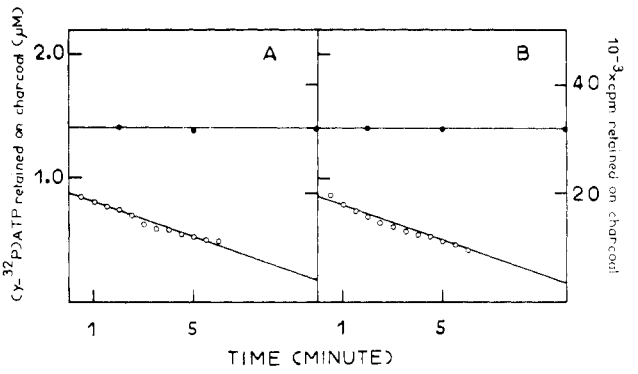


FIGURE 2: Active site titration. Native (0.25 μM) (panel A) and trypsin-modified (0.57 μM) (panel B) methionyl-tRNA synthetases have been incubated with 1.55 μM [γ-^{32}P]ATP in the presence of magnesium and yeast inorganic pyrophosphatase at 25 °C. At time zero, adenylate synthesis was started by adding a final concentration of 1.25 mM methionine. The resulting time-dependent variations of the intact labeled ATP (-○-) are plotted in comparison with the parallel experiment in which the amino acid has been omitted (-●-). Extrapolations to zero time of the steady-state ATP consumption provide initial bursts of ATP hydrolysis when methionine is added. These initial bursts correspond to 2.1 and 0.95 ATP molecules hydrolyzed respectively per molecule of native and trypsin-modified enzyme.

tiplier. Both monochromators are equipped with diffraction gratings blazed at 340 nm. The quartz cell (1 cm × 0.4 cm, Hellma) was filled with 0.8 ml of the enzyme solutions. Saturation curves were obtained by adding successive aliquots of 0.005 ml of the buffer containing the ligand under study. Levels of fluorescence are expressed as percentage of the fluorescence of the free enzyme at the beginning of titration. In the case where stabilities of the enzyme–methionyl adenylate complexes were followed as a function of time, unnecessary exposure of the synthetase complexes to the excitation light was avoided.

Stopped-Flow Fluorescence. Rapid mixing experiments are performed at 25 °C in a Durrum-Gibson stopped-flow spectrometer equipped with fluorescence detection. Excitation at 295 nm of the tryptophan fluorescence is ensured by a 450-W Osram xenon lamp followed by a Jobin-Yvon HRS2 monochromator. The slit width of the excitation monochromator is 2 mm. The observation cell is a quartz tube of 2-mm diameter and 20-mm length. The emission is observed with an aperture at 90° after passing MTO (A 340 b) and Corning CS 0.54 filters, using an Hammamatsu R 376 photomultiplier. The dead time of the spectrometer is 2.5 ms.

Relaxations were obtained by 1:1 mixing of 0.2-ml volumes of reactants. The registered oscillograms were fitted to single exponentials using a multiparameter iterative least-squares procedure on a Wang 2200 B computer.

Results

1. Stoichiometry of the Methionyl Adenylate Molecules Formed and Bound on the Dimeric Native Methionyl-tRNA Synthetase or Its Monomeric Trypsin-Modified Derivative. (a) Equilibrium Dialysis. Methionine reacts with ATP-Mg^{2+} on the enzyme to yield methionyl adenylate with concomitant release of pyrophosphate. This reaction has been carefully studied in the past using different methods (Blanquet et al., 1974). It is characterized by a central complex symbolized elsewhere by X, which contains the E·AA·ATP-Mg^{2+} and E·AA~AMP·PP-Mg^{2+} complexes in equilibrium. The pyrophosphate molecule may dissociate from the X species thus giving the E·AA~AMP complex in equilibrium with free PP-Mg^{2+}. Equilibrium dialysis experiments performed in this study deal with the stoichiometry of ^{14}C-labeled amino acid in the X and E·AA~AMP complexes formed with native and trypsin-modified methionyl-tRNA synthetases. The Scatchard plots presented in panels A and B of Figure 1 represent the formation of the X species by titrating the enzymes with amino acid in the presence of saturating amounts of ATP-Mg^{2+} and PP-Mg^{2+}. The formation of the E·AA~AMP species by titrating the E·ATP-Mg^{2+} complex by amino acid in the presence of yeast inorganic pyrophosphatase is presented on the same figure. The results clearly show that in both cases the stoichiometry of adenylate formation and binding corresponds to the subunits composition of the enzyme species, i.e., respectively two and one methionyl adenylate molecules per mole of native and trypsin-modified enzymes. These experiments are independent of the stability of the adenylate since both experiments are performed in the presence of unlimiting amounts of ATP-Mg^{2+}. Finally it must be noted that the dissociation constants of methionine from the X species, (E·ATP-Mg^{2+})·(AA)/(X), the values of which are respectively 28 and 32 μM with the native and trypsin-modified enzymes, are in excellent agreement with the values published elsewhere using the isotopic ATP-PP exchange or fluorescence titration (Blanquet et al., 1974).

(b) Active Site Titration. Such a technique has been already applied to the native methionyl-tRNA synthetase by others (Fersht et al., 1975). The resulting stoichiometry (two sites per mole of dimeric enzyme) was in excellent agreement with our previous binding stoichiometries (Blanquet et al., 1974). In the present work, we apply this method to both the native and the trypsin-modified methionyl-tRNA synthetases under our standard conditions. The results of Figure 2 demonstrate that the initial bursts of labeled ATP depletion correspond to two and one molecules of ATP consumed respectively in the case of the native enzyme and of its derivative. Moreover, in both cases the initial methionyl adenylate synthesis is followed by similar steady-state ATP hydrolysis corresponding to the lability of the enzyme–adenylate complex. The corresponding turnover rates are in good agreement with the results of the fluorescence section presented later.

(c) Nonequilibrium Dialysis. This technique provides a very rapid and useful method for titration of the active sites of activating enzymes. Such a titration experiment is presented in Figure 3 where at zero time native or trypsin-modified methionyl-tRNA synthetase, α-^{32}P-labeled ATP, and pyrophos-

phatase are mixed in the upper chamber of the dialysis apparatus in standard buffer containing magnesium. At the same time collection of the effluent from the lower chamber (0.5 ml/min) was started. The radioactivity contained in the resulting fractions was proportional to the amount of the free labeled ATP in the upper chamber since this radioactivity measures the unidirectional diffusion of the ligand across the membrane. In Figure 3 the histogram which represents the sampled radioactivity follows the free ligand concentration in the upper chamber, while the continuous line reflects the corresponding concentration of total labeled ATP concentration remaining in the chamber containing the enzyme. This line was obtained by calculation using the balance of radioactivity between the two chambers from zero time. It has been verified that, at the concentrations used, ATP-Mg^{2+} does not bind significantly the enzymes. Adenylate formation is started by adding a saturating aliquot of methionine to the solution in the upper chamber. Its synthesis results in a rapid depletion of free labeled ATP diffusing across the membrane. The magnitude of this effect allows determination of stoichiometries of 1.9 ± 0.1 and 0.9 ± 0.1 adenylate molecules respectively bound per mole of native and trypsin-modified methionyl-tRNA synthetases. In the course of the same experiment, reversion of the adenylate was performed by adding an excess of pyrophosphate to the upper chamber. Under these conditions, the bound labeled ATP is instantaneously removed as free ATP, thus increasing the rate of diffusion of this ligand across the membrane. As shown in Figure 3, the sampled radioactivity reflects this effect, the magnitude of which corresponds to the above stoichiometries.

(d) Fluorescence Titration. As was demonstrated elsewhere, the tryptophan fluorescences of native and trypsin-modified methionyl-tRNA synthetases are markedly sensitive to the combinations of their various substrates (Blanquet et al., 1972, 1974). Indeed methionine binding to the enzymes leads to a 26% increase of proteic fluorescence. Moreover, ATP-Mg^{2+} can react with the E·AA complex thus promoting a quenching of the enzyme fluorescence, the final magnitude of which is 73% of the free enzyme fluorescence. In the case of both enzymes, this latter effect has already allowed the determination of the equilibrium constant (E·AA)·(ATP-Mg^{2+})/(E·AA~AMP)·(PP-Mg^{2+}) as well as the stoichiometry for adenylate formation. This constant, symbolized by $K_{f_1} = K_{eq}/K_{AA}$ with K_{eq} = (E)·(AA)·(ATP-Mg^{2+})/(E·AA~AMP)·(PP-Mg^{2+}), was found very similar with the two forms of the enzyme while the stoichiometries of synthetized methionyl adenylate molecules were respectively 2.2 and 1.2 per mol of native or trypsin-modified enzyme (Blanquet et al., 1974). A comparison of the corresponding titration curves is presented again in Figure 4. It is now easy to perform such a titration in the presence of pyrophosphatase which will shift the above equilibrium toward the E·AA~AMP species. The corresponding active titrations are also presented in Figure 4, where it is shown that two and one molecules of adenylate are respectively formed and bound by the dimeric native and the monomeric trypsin-modified enzymes. In panels C and D, the stabilities of such formed adenylate-enzyme complexes are examined as a function of time. Respective half-life times of 11 and 14 min are derived from these experiments, the low values of which exclude a significant over-estimation of the above stoichiometries due to the unavoidable hydrolysis of the formed adenylate in the course of the titration experiments. Finally it was tested that the presence of adenosine (1.8 mM), which is known to bind synergistically the E·AA complex (Blanquet et al., 1975a), has no measurable effect on the enzyme–adenylate stabilities. This property reflects the strength of the enzyme–adenylate interaction.

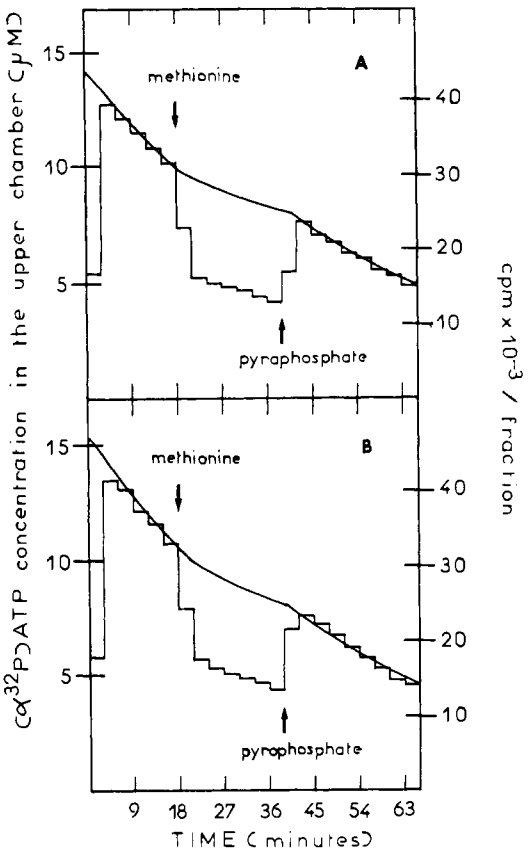


FIGURE 3: Nonequilibrium dialysis. Native (panel A) and trypsin-modified (panel B) methionyl-tRNA synthetases at respective concentrations of 2.5 and 5.2 μM are incubated with 15 μM [α-^{32}P]ATP in the presence of 0.1 μg of yeast pyrophosphatase and 10 mM magnesium chloride. Synthesis of the methionyl adenylate is started by adding methionine at a final concentration of 2 mM. Reversion of the adenylate is ensured by adding 2 mM final pyrophosphate. The histogram follows the sampled radioactivity dialyzing across the membrane (free ATP) which is expressed in cpm/fraction (one fraction is 1.5 ml/3 min). The continuous line indicates the concentration of total ATP remaining in the chamber containing the enzyme and is calculated from zero time using the balance of radioactivity across the membrane. For the sake of clarity, both lines are normalized to coincidence.

2. Stopped-Flow Analysis of Methionyl Adenylate Synthesis Catalyzed by the Dimeric Native Methionyl-tRNA Synthetase or Its Monomeric Trypsin-Modified Derivative. In the previous section it has been shown that the fluorescence phenomena resulting from the reaction of methionine with ATP-Mg^{2+} on the enzyme are fully relatable to the results obtained by the more direct isotopic methods. Consequently, the tryptophan fluorescence is used with no reservation in this article in order to monitor the reaction scheme corresponding to the enzyme-catalyzed reversible formation of methionyl adenylate from methionine and ATP-Mg^{2+}. This scheme has already been analyzed in part using isotopic ATP-PP exchange as well as fluorescence (Blanquet et al., 1974). However, in the case of these equilibrium methods, the central complex, X, of the reaction was not resolved since it contains isomers in equilibrium. Stopped-flow analysis of the reaction is a useful way to resolve this central complex by measuring its rates of isomerization, provided that reaction of substrates is not rate limiting in the observed rates. Taking into account the fact that when it is formed the adenylate does not dissociate significantly from the enzyme, the analyzed reaction may be formulated as

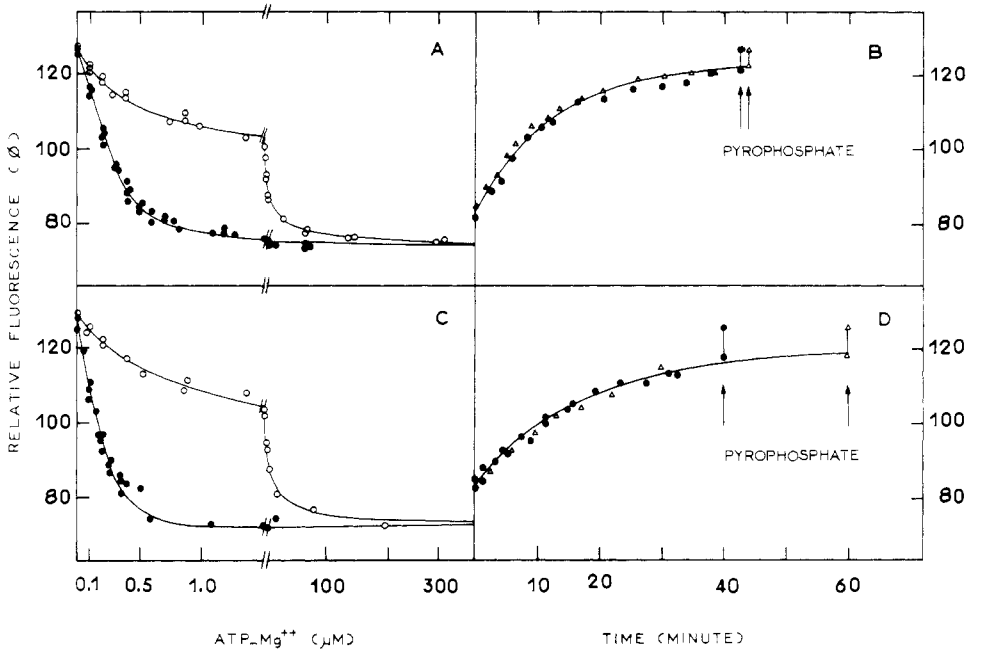
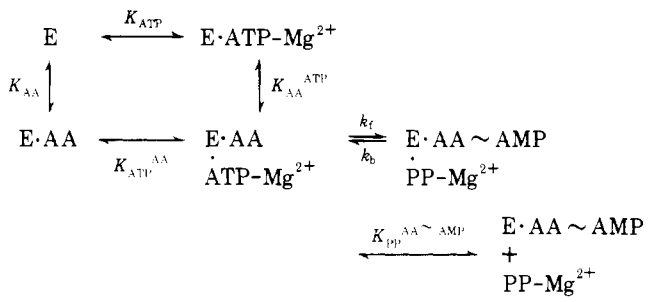


FIGURE 4: Fluorescence at equilibrium. Native (panels A and B) and trypsin-modified (panels C and D) methionyl-tRNA synthetases at concentrations of 0.25 and 0.34 μM, respectively, are titrated by ATP (panels A and C) in the presence of 1.8 mM methionine with (-●-) or without (-○-) 0.1 μg of yeast inorganic pyrophosphatase. In the presence of pyrophosphatase (-●-), the stoichiometries are obtained by calculating the ATP concentrations which correspond to the intersections of the final plateau with the initial slope of the titration curves. In panels B and D, stabilities of the enzyme-methionyl-AMP complexes are followed as a function of time in the absence (-●-) or in the presence of 1.14 mM adenosine (-△-). These complexes are previously formed by reacting native and trypsin-modified methionyl-tRNA synthetases with limiting ATP (respectively 0.5 and 0.4 μM) in the presence of 1.8 mM methionine, and yeast pyrophosphatase. At the end of kinetics, reversion to the enzyme-methionine complex was ensured by adding excess pyrophosphate (1.2 mM). In addition, all the solutions contained 10 mM magnesium chloride.

shown in Scheme I. The symbols used in this scheme are those defined in Table I.



As indicated in the previous section, when ATP-Mg^{2+} reacts with the enzyme-methionine complex, the tryptophan fluorescence of the enzyme is markedly decreased upon the formation of enzymatically bound methionyl adenylate. Thus a rate constant for adenylate formation can be measured in these conditions. According to the above reaction scheme, in the general case where each species of the reaction has a different quantum yield of fluorescence ($F_0, F_1, F_2 \ldots$), the total fluorescence, F, can be expressed as:

$$(E)_0F = (E\cdot AA\sim AMP\cdot PP)F_1 + (E\cdot AA\sim AMP)F_2 + (E\cdot AA)F_3 + (E\cdot ATP)F_4 + (E\cdot AA\cdot ATP)F_5 + (E)F_0$$

which can be transformed into the following equation:

$$(E)_0F = (A)\frac{(F_1(PP) + F_2K_{PP}^{AA\sim AMP})}{((PP) + K_{PP}^{AA\sim AMP})} + ((E)_0 - (A)) \times \frac{(F_3K_{ATP}^{AA}(AA) + F_4K_{AA}^{ATP}(ATP) + F_5(AA)(ATP) + F_0K_{AA}K_{ATP}^{AA})}{(K_{ATP}^{AA}(AA) + K_{AA}^{ATP}(ATP) + (AA)(ATP) + K_{AA}K_{ATP}^{AA})}$$

where the minimal ((E·AA~AMP·PP-Mg^{2+}) + (E·AA~AMP)) species which complexes the adenylate is symbolized by (A).

As shown in the latter formula, F is a linear function of the A variable; thus the fluorescence variations satisfy the same rate constant as the A species. This species is ruled by the following differential equation:

$$d(A)/dt = k_f((E)_0 - (A))G^{-1} - k_b(A)H^{-1}$$

with

$$G = 1 + K_{ATP}^{AA}/(ATP) + K_{AA}^{ATP}/(AA) + K_{ATP}^{AA}K_{AA}/(AA)(ATP)$$

and

$$H = 1 + K_{PP}^{AA\sim AMP}/(PP)$$

The rate constant for adenylate formation can be directly derived from this equation, provided that the substrates concentrations remain unaffected in the course of the reaction:

$$k = k_fG^{-1} + k_bH^{-1}$$

This rate constant (k) has been calculated by assuming that all of the association-dissociation processes are in rapid preequilibrium. This assumption is verified by the fact that the experimental data and the calculated rate constant (k) are subject to the same substrate dependence. In addition, in the case of methionine, we have verified that in our standard conditions 60 μM of this ligand (a concentration equal to the dissociation constant which theoretically ensures an apparent rate twice that of the kinetical dissociation constant) reacts with the enzyme within the dead time (2.5 ms) of the stopped-flow apparatus. In the case of the other substrates of the reaction, the absence of ligand-induced fluorescence changes precludes direct measurement of their reaction rate.

TABLE I: Stopped-Flow Resolution of the Methionyl-Adenylating Reaction Catalyzed by Native Methionyl-tRNA Synthetase or by Its Trypsin-Modified Form.[a]

	Native	Trypsin Modified
k_F (s^{-1})	342 ± 15	259 ± 9
k_B (s^{-1})	226 ± 6	223 ± 7
$K = k_F/k_B$	1.52 ± 0.11	1.11 ± 0.07
K_{AA} (μM)	63*	59*
K_{ATP} (mM)	1.2*	1.5*
K_{AA}^{ATP} (μM)	77 ± 5	80 ± 9
K_{ATP}^{AA} (mM)	1.50 ± 0.15	1.50 ± 0.10
$K_{PP}^{AA\sim AMP}$ (μM)	101 ± 7	214 ± 11
$K_X = K_{PP}^{AA\sim AMP}/(1 + K^{-1})$($\mu$M)	61 ± 9 70*	113 ± 11 120*
$K_{eq}K_X/K_{AA} = K_{ATP}^{AA}/(1 + K)$ (mM)	0.6 ± 0.1 0.36*	0.7 ± 0.1 0.63*
$K_{eq}K_X/K_{ATP} = K_{AA}^{ATP}/(1 + K)$ (μM)	30.5 ± 3.5 20.2*	37.9 ± 5.7 24.8*
$K_{eq}/K_{AA} = K_{ATP}^{AA}/KK_{PP}^{AA\sim AMP}$	9.7 ± 2.2 6*	6.3 ± 1.1 5.2*
$K_{eq}/K_{ATP} = K_{AA}^{ATP}/KK_{PP}^{AA\sim AMP}$	0.50 ± 0.11 0.26*	0.34 ± 0.08

[a] The rate constants (k), calculated according to the reaction scheme postulated in the text, have been fitted to the whole set of experimental data with the use of a nonlinear iterative regression procedure. The resulting parameters are presented in this table with their standard errors. For the sake of comparison, parameters determined elsewhere (Blanquet et al., 1974) are compiled on this table with asterisks. The parameters are defined as follows: k_F is the rate of isomerization from E·AA·ATP-Mg^{2+} to E·AA~AMP·PP-Mg^{2+}, while k_B is the inverse one. K_{AA} = (E)(AA)/(E·AA), K_{ATP} = (E)·(ATP-Mg^{2+})/(E·ATP-Mg^{2+}), K_{AA}^{ATP} = (E·ATP-Mg^{2+})(AA)/(E·AA·ATP-Mg^{2+}), and K_{ATP}^{AA} = (E·AA)(ATP-Mg^{2+})/(E·AA·ATP-Mg^{2+}). The central complex of the reaction which contains the sum of the minimal isomers E·AA·ATP-Mg^{2+} and E·AA~AMP·PP-Mg^{2+}, is defined by the X symbol used in other publications (Blanquet et al., 1974, 1975a). K_X = (E·AA~AMP)(PP-Mg^{2+})/(X), $K_{PP}^{AA\sim AMP}$ = (E·AA~AMP)(PP-Mg^{2+})/(E·AA~AMP·PP-Mg^{2+}), $K_{AA}^{ATP}/(1 + K)$ = (E·ATP-Mg^{2+})·(AA)/(X), $K_{ATP}^{AA}/(1 + K)$ = (E·AA)(ATP-Mg^{2+})/(X) with $K = k_F/k_B$ and K_{eq} = (E)(AA)(ATP-Mg^{2+})/(E·AA~AMP)(PP-Mg^{2+}).

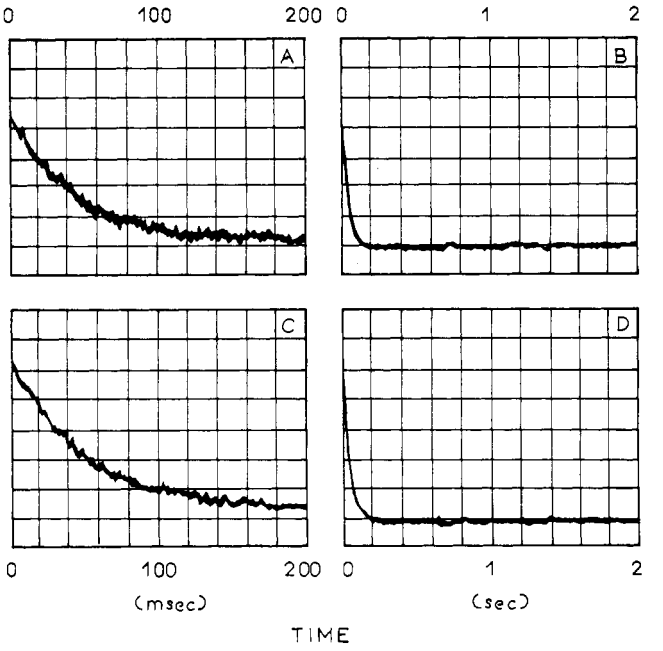


FIGURE 5: Oscillograms for the formation of methionyl-tRNA synthetase-methionyl adenylate complex after rapid 1:1 mixing of a solution containing the enzyme under study plus 2.5 mM methionine and 6.1 μM PP with a solution containing the same concentrations of methionine and PP plus 172 μM ATP. In addition, all solutions contain 7 mM magnesium chloride. Panels A and B present the relaxations obtained on two different base times with native methionyl-tRNA synthetase (concentration after mixing is 0.156 μM). Panels C and D present comparative relaxations obtained with the trypsin-modified enzyme (final concentration is 0.37 μM). Respective rise times of 1 and 5 ms are used with the 20- and 200-ms base times. In all cases the amplitude of signals at equilibrium is equal to 300 mV, after subtracting the blank signal. The voltage unit of the oscillograms is 20 mV/unit.

However, based on the results of the kinetic analysis of the ATP-PP exchange (Blanquet et al., 1974), we can safely assume that the dissociation-association processes involved are also in rapid preequilibrium. Resolution of the parameters contained in this rate constant was achieved by monitoring: (1) formation of methionyl adenylate from the E·AA complex reacting with ATP-Mg^{2+} in the presence of buffering pyrophosphate concentrations (i.e., the pyrophosphate produced in the course of the catalysis remains negligible in front of the added pyrophosphate); (2) formation of methionyl adenylate from the E·ATP-Mg^{2+} complex reacting with methionine in the presence of buffering pyrophosphate concentrations; (3) reversal of the synthetized methionyl adenylate by various amounts of pyrophosphate in the presence of buffering amino acid and ATP-Mg^{2+} concentrations. Part of the results are presented in Figure 6, where the experimental k values are plotted as a function of the ATP-Mg^{2+} concentration for different pyrophosphate concentrations. The data relative to the formation or reversion of the adenylate are shown in the same figure. At this point, it must be noted that the relaxations associated either with the formation or the reversal of the adenylate fitted very well single exponential curves. Moreover, it is shown in Figure 5 that no secondary effect occurs in the time range of seconds, in the case of either the enzymes examined. This clearly indicates that, under our catalytic conditions (at least in the absence of tRNA), no more than one set of active sites is expressed. In addition, Figures 5 and 6 demonstrate that the native and the trypsin-modified enzymes exhibit very similar rate constants, while as mentioned in the introduction, the proteolytic fragment has a poor chance to preserve intact a hypothetical second binding site.

The whole set of experimental data collected with the two forms of enzyme have been fitted to the calculated rate constant either by a series of independent linear regressions or by a general nonlinear iterative regression. Since both modes of analysis converge on the same values, we adopt the general mode in order to provide related standard errors. The isotherms derived from this fit are presented with the experimental data on Figure 6, while values for the thermodynamical parameters contained in the theoretical expression of the rate constant are summarized with their standard errors in Table I. It must be noted that reactions of the enzyme·AA complex with ATP-Mg^{2+} or of the enzyme·ATP-Mg^{2+} with AA provide identical maximal rates for adenylate formation. This fact argues either for random reaction between these substrates and (or) for the validity of the assumption of rapid preequilibria. The latter would involve equality of $K_{AA}K_{ATP}^{AA}$ and $K_{ATP}K_{AA}^{ATP}$, which is, indeed, verified in Table I.

Conclusion

In the set of experiments presented here, which are restricted to adenylate synthesis in the absence of tRNA, there is no ev-

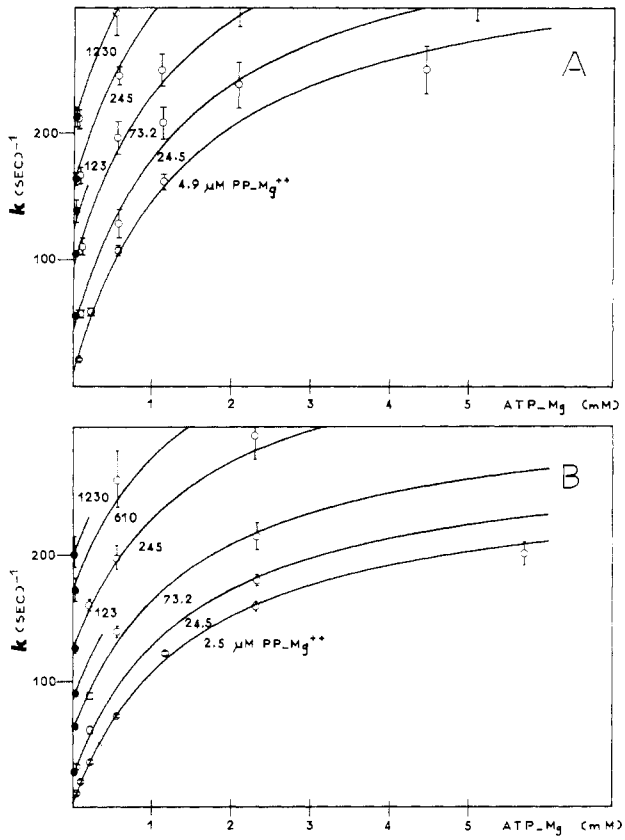


FIGURE 6: Rate constants for the formation or reversion of the methionyl-tRNA synthetase–methionyl adenylate complex. Native (panel A) and trypsin-modified (panel B) methionyl-tRNA synthetases, at the respective concentrations of 0.156 and 0.37 μM after 1:1 mixing, have been reacted in the presence of 2.5 mM methionine and 7 mM magnesium with (1) ATP in the presence of various amounts of PP (-O-); (2) PP in the presence of 22 μM ATP (-●-). The relaxations obtained have been fitted to single exponentials, the rate constants of which are presented on the figure. These data have been analyzed according to the calculated rate constant for the reaction (see Table I). The isotherms derived from this fit are presented as continuous lines with the experimental data.

idence that the existence of repeated sequence within each subunit of methionyl-tRNA synthetase is reflected by binding or reaction at a second site per polypeptide chain. Assuming that repeating sequences within subunits originate from gene duplication followed by fusion of adjacent genes, our results indicate that the process leading to covalent fusion of "monomers" has either abolished or else altered one of the original active site per subunits. In the former case, the advantage of the fusion process may reside in the acquisition of enhanced specificity toward tRNA, as discussed in the introduction. The alternative interpretation is to consider that the polypeptide chains of methionyl-tRNA synthetase derive from two originally noncovalently associated subunits exhibiting anticooperative behavior similar to that of bacterial tyrosyl-tRNA synthetase (Fersht, 1975). As a consequence of covalent linkage between these subunits, the anticooperativity may have grown into a settled "half-of-sites reactivity" due to lack of symmetry in the fused structure. The latter hypothesis would be consistent with the existence of one active site and one unavailable site per polypeptide chain. In this context, it is of interest to recall that in the aminoacylation of tRNA by native as well as trypsin-modified methionyl-tRNA synthetase, methionine displays two K_m values, whereas ATP does not (Lawrence, 1973). Similar effects have been reported in the case of several monomeric synthetases which belong to the class possessing repeated sequences (Yarus and Berg, 1969; Yaniv and Gros, 1969; Rouget and Chapeville, 1971). It is tempting to attribute this behavior to a conditional functioning of the hypothetical closed site in the course of the aminoacylation turnover. Nevertheless, alternative explanations may be suggested, for instance, in terms of competition between the aminoacyl moiety of methionyl-tRNA and free methionine for a common binding site. We intend to resolve this point by studying the methionine dependence, if any, of the transfer rate of the aminoacyl moiety from the adenylate to the tRNA, and of the kinetics of methionyl-tRNA binding.

The first part of the present study deals with the binding and consumption of ligands in the course of the adenylation reaction. Since it is found that neither the native nor the trypsin-modified enzymes express more than one active site per polypeptide chain, it was of interest to refine our analysis with the use of prestationary stopped-flow kinetics. Resolution of the kinetics of the adenylation reaction demonstrates that each of the enzymes catalyzes adenylate synthesis according to a simple scheme which involves a unique set of active sites. In agreement with this scheme, formation and reversal of methionyl adenylate follow single exponential relaxations in the case of both enzymes. This result argues for identical behavior of each of the subunits of native enzyme. Moreover, the values of Table I demonstrate that each of the subunits of the dimer displays the same thermodynamical parameters as does the modified monomer. Thus, it may be safely concluded that the native enzyme has two independent active sites for adenylate synthesis, the catalytic properties of which are unaffected by subunit assembly. Furthermore, as stated in the introduction, the modified fragment has lost a major portion of the duplicated sequence present in each native monomer and is, therefore, unlikely to have conserved a hypothetical second set of binding sites involved in the catalytic process. The identity of the catalytic parameters of the two forms of the enzyme thus provides an additional argument against the occurrence of a second set of binding sites in each monomer of the native enzyme.

Finally, the values of Table I which resolve the kinetics of the adenylation reaction have to be related with other studies dealing with synergism in the binding process of methionine plus adenosine and PP-Mg^{2+} (Blanquet et al., 1975a,b). Indeed, the determination of the K_{AA}^{ATP} and K_{ATP}^{AA} constants, compared with the K_{AA} and K_{ATP} values (Blanquet et al., 1974), demonstrates that the synergistic coupling which appeared between methionine and adenosine plus PP-Mg^{2+} is cancelled out by the introduction of the crucial α-phosphate. Therefore the expense of an additional binding energy of 3 kcal provided by the adenosine and PP-Mg^{2+} parts of the ATP-Mg^{2+} molecule enables this substrate to resemble the transition state despite the electrostatic repulsion between the phosphoryl and carboxyl groups within the reaction center. Mahler and Cordes (1969) have calculated that free acetyl-AMP has a free energy content which is approximately 6 kcal higher than that of ATP in solution. In the case of methionyl-tRNA synthetase, the difference in free energy between adenylate and ATP is strikingly decreased by synergism in the enzymic environment. Indeed, the standard free energy of the reaction is decreased to close to zero (60–240 cal) when calculated from the k_f/k_b ratio. Similar results have been obtained in the case of isoleucyl-tRNA synthetase from *E. coli* (Holler and Calvin, 1972; Holler et al., 1973). Thus it could be that synergistic leveling of the standard free energy is an essential feature of the mechanism leading to adenylate formation. On the side of the E·AA~AMP·PP-Mg^{2+} species, it is striking to note that

$PP-Mg^{2+}$ dissociates from this complex with the same equilibrium constant as in the case of the quaternary dead-end complex E·AA·adenosine·$PP-Mg^{2+}$ (Blanquet et al., 1975a). This finding clearly indicates that this quaternary complex simulates the transition state of the reaction, and that the AA~AMP molecule exerts the same synergistic coupling as the AA plus adenosine combination on $PP-Mg^{2+}$ binding to the enzyme. However, the quantum yields of fluorescence for these E·AA~AMP·$PP-Mg^{2+}$ and E·AA·adenosine·$PP-Mg^{2+}$ species are markedly different (respectively 77 and 138% of the free enzyme fluorescence). The only occurrence of a supplementary covalent link does not account for this effect since methioninyl adenylate, the alkyl analogue of methionyl adenylate, increases the fluorescence (Blanquet et al., 1972) as does methionine in combination with adenosine, or methioninol in combination with AMP (unpublished results).

The major difference between the ester and anhydride bonds exhibited by methioninyl and methionyl adenylate, respectively, resides in the atomic distances between the carbonyl carbon and the adjacent atoms. Indeed the single bonds C–C and C–O from a carbon atom are shortened if this carbon atom is double bonded to another atom. Therefore the anhydride bond will be shorter than the ester bond. This fact could be the basis for an enzyme distortion induced by methionyl adenylate which would affect the tryptophan radiative process in a way different from that of the relaxed synergistic couples or the longer methioninyl adenylate molecule.

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