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Mechanism of Aminoacylation of Transfer RNA. A Pre-Steady-State Analysis of the Reaction Pathway Catalyzed by the Methionyl-tRNA Synthetase of *Bacillus stearothermophilus*†

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ABSTRACT: The rate of formation of the product Met-tRNA^{Met}, catalyzed by the methionyl-tRNA synthetase from *Bacillus stearothermophilus,* has been determined in both the pre-steady-state and steady-state phases by rapid sampling techniques. A minimum estimate of the formation of the intermediate Met-AMP, in the presence of tRNA^{Met}, has been obtained from the kinetics of ATP–pyrophosphate exchange. The individual rate constants for the formation and interconversion of enzyme-bound intermediates have been directly measured by stopped-flow fluorescence. The two approaches have been combined to give a description of the reaction pathway. The pre-steady-state rate of methionyl transfer from E·(Met-AMP)$_2$ to tRNA was measured over a range of pH and with two species of tRNA^{Met}. Under all conditions, this rate constant was identical to the initial rate of charging of tRNA^{Met} in the steady-state phase (for example: 2.3 s^{-1} at pH 7.78 with tRNA$^{Met}_M$). Therefore, methionyl transfer, or a conformational change immediately preceding transfer, is the rate-determining step. The rate of formation of methionyl adenylate in the presence of saturating concentrations of all substrates and the rate of dissociation of tRNA^{Met} from its complex with E·(Met-AMP)$_2$ are both faster than the proposed rate-determining step (25 and 12 s^{-1}, respectively, at pH 7.78 with tRNA$^{Met}_M$). The k_{cat} for ATP–pyrophosphate exchange under the same conditions, 11 s^{-1}, is significantly less than the rate constant for the formation methionyl adenylate measured by stopped-flow fluorescence (25 s^{-1}).

The aminoacyl adenylate mechanism has been established beyond reasonable doubt for several enzymes (Fersht and Jakes, 1975; Fersht and Kaethner, 1976; Fasiolo and Fersht, 1978; Gangloff and Fersht, 1978; Mulvey et al., 1978). In this paper we have chosen one enzyme, the methionyl-tRNA synthetase from *Bacillus stearothermophilus,* for a detailed kinetic investigation. This enzyme, a dimer (2 × 82 000), possesses an editing mechanism which reduces the incidence of mischarging of tRNA^{Met} with noncognate amino acids such as homocysteine and α-aminobutyrate (Fersht and Dingwall, unpublished results). In addition, it is particularly suitable for study by pre-steady-state kinetics, since, like the equivalent enzyme from *Escherichia coli* (Hyafil et al., 1976), its intrinsic fluorescence shows great sensitivity to events at the active site. A combination of stopped-flow fluorescence and quenched-flow experiments has made possible the identification and study of the following steps in the reaction pathway: methionyl adenylate formation, tRNA binding, and aminoacyl transfer from the methionyl adenylate complex to tRNA. Since the formation of methionyl adenylate in the presence of tRNA can be followed by stopped-flow fluorescence; the rate constant for this step can be measured directly instead of inferring its value from the kinetics of pyrophosphate exchange. Comparison of the rates of these individual steps with the overall rate of aminoacylation of tRNA has established that methionyl adenylate does accumulate during the turnover of substrates and that aminoacyl transfer from this complex to tRNA is the rate-limiting step.

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0006-2960/78/0417-5591$01.00/0

Experimental Section

Materials

Crude enzyme and tRNA fractions of *B. stearothermophilus* were obtained from the Imperial College of Science and Technology, London. The methionyl-tRNA synthetase was purified, assayed, and stored as described by Mulvey and Fersht (1976). Radioactively labeled methionine was purchased from the Radiochemical Centre, Amersham.

Purification of $tRNA^{Met}$. The tRNA was fractionated by batch chromatography on DEAE[1]-cellulose followed by chromatography on benzoylated diethylaminocellulose. All species of $tRNA^{Met}$ eluted in a salt gradient (0.4–1.0 M NaCl) with a methionine acceptance of 160 pmol/A_{260}. This fraction, referred to subsequently as "enriched $tRNA^{Met}$", was used in several experiments. Further purification was achieved on DEAE-Sephadex A-50 (salt gradient of 0.38–0.53 M NaCl in 10 mM $MgCl_2$ and 20 mM Tris-HCl, pH 7.5) followed by chromatography on Sepharose 4B (reverse salt gradient from 1.3 M ammonium sulfate; Holmes et al., 1975). This last column separated $tRNA^{Met}_F$ and $tRNA^{Met}_M$.

Assay for $tRNA^{Met}_F$. tRNA fractions were charged with [^{3}H]methionine and formylated (using unlabeled formyl tetrahydrofolate) by the procedure of Leder and Bursztyn (1966). The reaction mixture was extracted with phenol and the tRNA was precipitated with ethanol and redissolved in 0.01 M acetate buffer (pH 6.0) containing 10 mM $MgCl_2$. Deacylation of the Met-tRNA was initiated by bringing a small volume of the solution to 0.17 M in Tris-Cl (pH 8.1) and 8 mM in cupric chloride. The copper ions and Tris together catalyze rapid deacylation of Met-tRNA, whereas formylated Met-$tRNA^{Met}_F$ hydrolyzes slowly. Therefore, the relative amounts of formylated and unformylated Met-tRNA can be determined by quenching small samples of the deacylation mixture in 5% trichloroacetic acid at timed intervals. The radioactivity remaining in the precipitated tRNA is measured by scintillation counting and can be extrapolated to zero time.

Methods

Experiments were performed at 25 °C in standard buffers containing 10 mM $MgCl_2$, 10 mM mercaptoethanol, 0.1 mM phenylmethanesulfonyl fluoride, and 0.144 M Tris-Cl (I = 0.1) (pH 7.78) or 0.013 M Bistris-Cl (pH 5.87). Enzyme concentration was estimated by A_{280} and active-site titration (Mulvey and Fersht, 1976) and tRNA concentrations were estimated from amino acid acceptance.

Stopped-Flow Fluorescence Measurements (Fersht et al., 1975). The fluorescence changes produced on mixing two solutions were monitored on a storage oscilloscope and an Aminco DASAR system with output to a Bryans XY/t recorder fitted with either linear or logarithmic amplifiers.

Quenched-flow experiments were performed on both the conventional and pulsed modes of the quenched-flow apparatus (Fersht and Jakes, 1975). Typically, in experiments involving the charging of tRNA with labeled amino acid, the reaction was quenched with trichloroacetic acid after incubations of 10 ms to several seconds; each sample was filtered on Whatman GF/C, washed, dried, and then counted in a toluene-based scintillant.

Rate of Formation of Methionyl Adenylate. (a) One syringe of the stopped-flow fluorimeter contained methionyl-tRNA synthetase (0.75 μM), $tRNA^{Met}_M$ (4.5 μM), and inorganic pyrophosphatase (Worthington, 2 units/mL) and the other syringe contained ATP (2.0 mM) and varying concentrations of methionine (10–200 μM). (b) In several other stopped-flow experiments, enzyme and $tRNA^{Met}_M$ concentrations were varied and the substrate syringe contained 2 mM methionine and 200 μM ATP. In this case, tRNA was included in both syringes to eliminate any small fluorescence changes due to dilution of the free tRNA and subsequent dissociation of the enzyme–tRNA complex.

Transfer of Methionine to tRNA. (a) Isolated Methionyl Adenylate Complex. Enzyme-bound [^{14}C]Met-AMP was freed from excess ligands by gel filtration on Sephadex G-25. After dilution to 0.36 μM in the standard pH 7.78 buffer, the complex was mixed in the stopped-flow fluorimeter with $tRNA^{Met}_M$, $tRNA^{Met}_F$, or enriched $tRNA^{Met}$ (2.8 μM), and the changes in fluorescence were monitored (excitation at 295 nm with 10-nm slits). The rate of formation of [^{14}C]Met-tRNA under these conditions was measured directly using the quenched-flow apparatus. Identical experiments were performed in the stopped-flow fluorimeter, except with Bistris-Cl (I = 0.1) at either pH 6.90 (0.38 M) or 5.87 (0.13 M).

(b) In Situ Formation of Aminoacyl Adenylate. Methionyl-tRNA synthetase (0.3 μM) was preincubated in one syringe of the quenched-flow apparatus with [^{14}C]methionine (15.5 μM, 60 Ci/mol), ATP (2.0 mM), inorganic pyrophosphatase (2 units/mL), and Bistris-Cl (0.013 M, pH 5.87). The other syringe contained enriched $tRNA^{Met}$ (6.2 μM), unlabeled methionine (5.0 mM), and Tris-HCl (0.288 M, pH 7.78). The two solutions were mixed and automatically quenched with trichloroacetic acid, and the precipitated [^{14}C]Met-tRNA was assayed as described previously.

Initial Rate of Aminoacylation of $tRNA^{Met}$. One syringe of the quenched-flow apparatus contained methionyl-tRNA synthetase (0.75 μM), enriched $tRNA^{Met}$ (18.2 μM), and inorganic pyrophosphatase (2 units/mL), and the other syringe contained [^{14}C]methionine (217 μM, 20 Ci/mol) and ATP (2.0 mM). The two solutions were mixed and quenched as above.

tRNA Binding. (a) Fluorescence Titration. The association of methionyl-tRNA synthetase and $tRNA^{Met}$ was measured by the quenching of protein fluorescence in a Perkin-Elmer MPF-3 fluorimeter with a excitation wavelength of 295 nm, and a path length of 0.4 cm, an emission at 340 nm, and a 1.0-cm path length. Aliquots of $tRNA^{Met}_M$ solution (typically 10–50 μM) were added from a Hamilton syringe to 1.0 mL of enzyme solution (0.05 or 2.2 μM). Correction for the internal absorption of the excitation beam by tRNA was made in two ways. The same additions of $tRNA^{Met}_M$ were made to a control solution containing 10 μM *N*-glycyltryptophan, and the degree of fluorescence quenching was used to correct the experimental data. The correction was also calculated from the A_{295} of the $tRNA^{Met}_M$ solution using the procedures of Ehrenberg et al. (1971).

(b) Stopped-Flow Fluorescence. Methionyl-tRNA synthetase (0.1 μM) was mixed in the stopped-flow fluorimeter with solutions of $tRNA^{Met}_M$ (0.1–2.0 μM). In other experiments, the free enzyme was replaced by its complex with methionyl adenylate in Bistris-Cl (pH 5.9, I = 0.01).

(c) Analytical Ultracentrifugation Following the Procedure of Krauss et al. (1975). Free and enzyme-bound tRNA were separated by velocity sedimentation in an MSE analytical ultracentrifuge Mk II using an ultraviolet scanner and 0.5–2.0-cm path-length cells. The cells were scanned alternately at 255 and 280 nm; the concentration of methionyl-tRNA synthetase was either 0.28 or 0.8 μM and $tRNA^{Met}_M$ was varied between 0.2 and 2.0 μM.

[1] Abbreviations used: DEAE, diethylaminoethyl; Tris-HCl, 2-amino-2-hydroxymethyl-1,3-propanediol hydrochloride; Bistris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-1,3-propanediol.

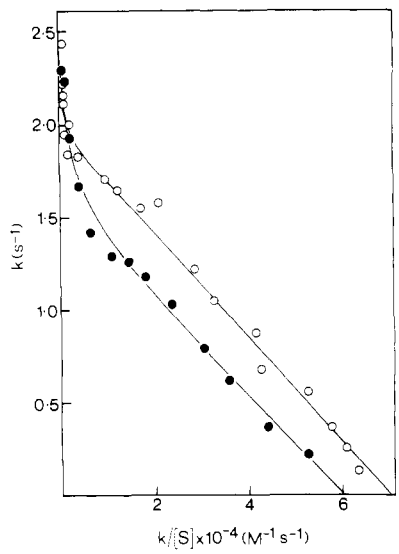


FIGURE 1: The initial rate of aminoacylation of $tRNA^{Met}$: dependence on substrate concentration. Standard conditions, 5–10 nM methionyl-tRNA synthetase and 20 μM enriched $tRNA^{Met}$: (○) 2.0 μM–2.4 mM [^{14}C]methionine (58–10 Ci/mol), 3.7 mM ATP; (●) 4 μM–4 mM ATP, 0.6 mM [^{14}C]methionine (20 Ci/mol).

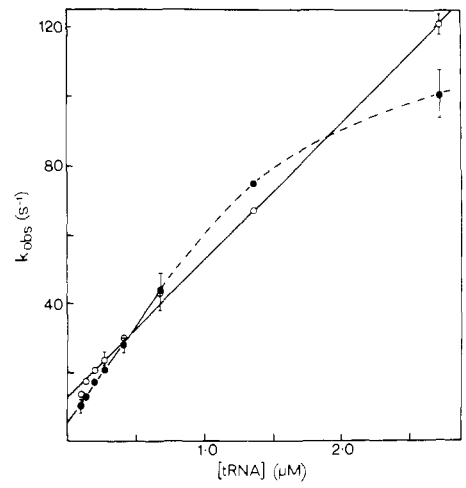


FIGURE 2: $tRNA^{Met}_M$ binding measured by stopped-flow fluorescence: dependence of rate on tRNA concentration. Standard conditions; 50 nM methionyl-tRNA synthetase (●), 50 nM enzyme-methionyl adenylate complex (○); 0.1–2.7 μM $tRNA^{Met}_M$.

Results

Purification of $tRNA^{Met}$. Fractions of "enriched $tRNA^{Met}$", obtained from the second chromatography step in the purification, were used for several experiments. All the $tRNA^{Met}$ species were present in these fractions at an amino acid acceptance of 160 pmol/A_{260}. Further purification was achieved on DEAE-Sephadex A-50 and, finally, $tRNA^{Met}_F$ and $tRNA^{Met}_M$ were separated on Sepharose 4B with amino acid acceptances of 1200 and 1580 pmol/A_{260}, respectively. Concentrations of $tRNA^{Met}$ were calculated from the amino acid acceptance.

The purity of the $tRNA^{Met}_M$ fraction was estimated in two ways. An aliquot was applied to an RPC-5 column (Pearson et al., 1971) and eluted in a salt gradient of 0.4–1.0 M NaCl (in 10 mM $MgCl_2$ and 144 mM Tris-Cl, pH 7.8). $tRNA^{Met}_{M1}$ and $tRNA^{Met}_{M2}$ eluted separately with amino acid acceptances of 1750 and 1670 pmol/A_{260}, respectively. Therefore, the $tRNA^{Met}_M$ fraction was not better than 93% pure. In a second experiment, the analytical ultracentrifuge was used to cosediment $tRNA^{Met}_M$ (1.4 μM) with an excess of methionyl-tRNA synthetase (2.1 μM). Any tRNA unable to bind the enzyme, and in excess of 10% of the total tRNA, would have been detected as a separate boundary. None was found and the $tRNA^{Met}_M$ was assumed to be at least 90% pure.

Steady-State Kinetics. Eadie–Hofstee plots of the kinetics of charging of $tRNA^{Met}$ are biphasic with respect to both ATP and methionine concentrations (Figure 1). At saturating concentrations of substrates the rate of aminoacylation of tRNA is 2.4 s^{-1} (Table I). Several other aminoacyl-tRNA synthetases show similar behavior: methionyl-tRNA synthetase of *Escherichia coli* (Lawrence et al., 1973), tyrosyl-tRNA synthetase of *E. coli* and *B. stearothermophilus* (Jakes and Fersht, 1975), phenylalanyl-tRNA synthetase of yeast (von der Haar, 1976), and valyl-tRNA synthetase of *B. stearothermophilus* (unpublished results). In the last two cases, monophasic kinetics with increased k_{cat} and K_M can be obtained at high ionic strength (e.g., in 0.2 M KCl). von der Haar (1976) has suggested that this behavior with the phenylalanyl-tRNA synthetase may be explained by a salt-dependent equilibrium between two enzyme forms which differ in their activity. This explanation may not hold for the methionyl-tRNA synthetase because the value of k_{cat} is independent of concentration of salt up to 0.25 M KCl. The K_M values for methionine and ATP in the ATP–pyrophosphate exchange reaction have been measured previously (Mulvey and Fersht, 1976). The presence of enriched $tRNA^{Met}$ (12 μM) has little effect on the exchange reaction (Table I). The values of K_M and the other steady-state constants are summarized in Table I.

TABLE I: Kinetic Data.[a]

	k_{cat} (s^{-1})[b]	K_M (μM) Met	ATP
Steady State			
ATP–PPi exch[c]	10	47	15
+ $tRNA^{Met}$	9	22	
charging reaction:			
enriched $tRNA^{Met}$	2.4	(28, >1000)[d]	(25 900)[d]
$tRNA^{Met}_F$	1.6		
$tRNA^{Met}_M$	2.2		
Pre-Steady-State			
formation of Met adenylate[c]	29	30	46
+ $tRNA^{Met}_M$	25	(25 500)[d]	
Met transfer:			
$tRNA^{Met}_M$	2.3		
$tRNA^{Met}_F$	1.5		

[a] 25 °C, pH 7.78, 10 mM $MgCl_2$. [b] Moles of product formed/mole of enzyme dimer under steady-state conditions. [c] See Mulvey and Fersht (1976). [d] Biphasic kinetics, lower K_M is estimated from linear portion of plot at low substrate concentrations.

The charging reaction was also studied by quenched-flow at a higher concentration of enzyme (0.38 μM) to look for a possible "burst" in the aminoacylation of tRNA. The rate of charging (2.1 ± 0.03 s^{-1}) is constant over four turnovers and is similar to that measured in the conventional initial rate assay at the same methionine concentration (1.8 s^{-1}; see Figure 1). The burst of aminoacylation is negligible (0.04 ± 0.03 mol of Met-tRNA/mol of enzyme), showing that the steady state is reached in the first turnover.

The Binding of tRNA. There is a 20% decrease in the fluorescence of methionyl-tRNA synthetase on binding tRNA. The pseudo-first-order rate constant for this process, k_{obsd}, can be measured by stopped-flow fluorescence. k_{obsd} increases

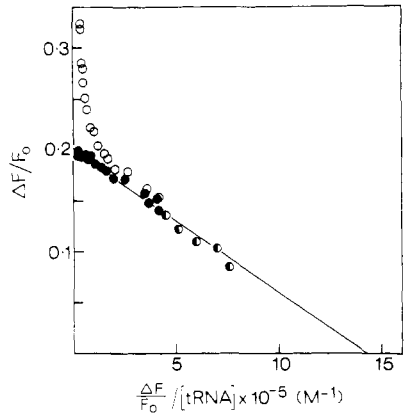


FIGURE 3: $tRNA^{Met}_M$ binding measured by fluorescence titration. Standard conditions, 50 nM methionyl-tRNA synthetase, 0.1–9.0 μM $tRNA^{Met}_M$. Internal absorption corrected by: (○) calculation, (●) experiment (see text).

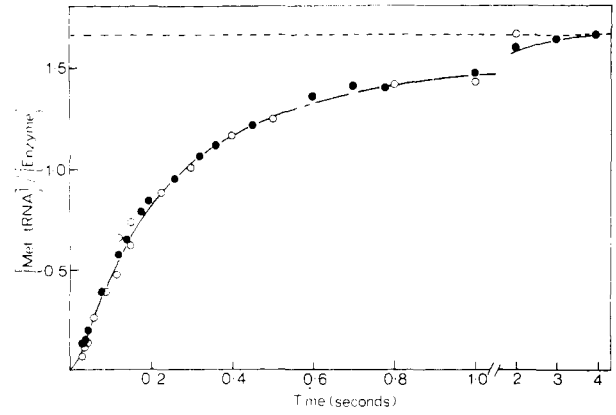


FIGURE 4: Transfer of methionine from E·(Met-AMP)$_2$ to $tRNA^{Met}$; comparison of rapid quenching (●,○) with stopped-flow fluorescence (solid curve). The complex of enzyme and [^{14}C]methionyl adenylate was isolated on Sephadex G-25 (●) or formed in situ (○). Standard conditions, 0.15 μM methionyl-tRNA synthetase, 3.1 μM enriched $tRNA^{Met}$.

TABLE II: $tRNA^{Met}_M$ Binding.[a]

method	k_1'[b] $\times 10^{-7}$ (M^{-1} s^{-1})	k_{-1}'[b] (s^{-1})	K_S (nM)	ν
stopped-flow fluorescence				
(a) free enzyme	5.7	5.0	90[c]	
(b) Met adenylate complex	4.0	12.6	310[c]	
fluorescence titration			140	1.48
anal. ultracentrifug			90	1.46

[a] 25 °C, pH 7.78, 10 mM $MgCl_2$. [b] k_1' and k_{-1}' are *apparent* rate constants for association and dissociation; see text. [c] Calculated from the ratio of k_1' and k_{-1}'.

linearly with the concentration of tRNA up to 0.7 μM (Figure 2). The deviation from linearity at high concentrations of tRNA suggests a two-step mechanism for binding, as has been established for the phenylalanyl-, seryl- and tyrosyl-tRNA synthetases from temperature-jump measurements (Riesner et al., 1976; Krauss et al., 1977). Unfortunately, our measurements had to be restricted to the concentration range shown, so that the kinetics could not be fully analyzed. However, the data from the linear regions may be analyzed to give *apparent* association and dissociation rate constants according to eq 1 and 2 (see Table II):

$$E + tRNA \underset{k_{-1}'}{\overset{k_1'}{\rightleftharpoons}} E \cdot tRNA \quad (1)$$

$$k_{obsd} = k_1'[tRNA] + k_{-1}' \quad (2)$$

k_1' is the lower limit to the real association rate constant. Measurements with other aminoacyl-tRNA synthetases have yielded values for this rate constant above 10^8 M^{-1} s^{-1} (Pingoud et al., 1973, 1975; Krauss et al., 1973; Blanquet et al., 1976), significantly higher than the value of k_1' determined here (Table II).

At low concentrations of enzyme, below 0.03 μM, the rate constants increased with decreasing enzyme, presumably as the enzyme dissociates into subunits.

The equilibrium binding of tRNA was monitored by measurements of enzyme fluorescence. The tRNA gives high internal absorption and so the fluorescence data were corrected by calculation (Ehrenberg et al., 1971). Biphasic plots of tRNA binding were obtained (Figure 3), as has been found with other aminoacyl-tRNA synthetases (Blanquet et al., 1973; Bosshard et al., 1975; Jakes and Fersht, 1975). Alternatively, the degree of fluorescence quenching by internal absorption was determined directly by the addition of tRNA to a solution of *N*-glycyltryptophan. Empirical correction of the enzyme fluorescence data was then possible and gave a simple binding curve, suggesting that correction by calculation may be inadequate. The dissociation constant (K_S) of the enzyme–tRNA complex is 0.14 μM, and the stoichiometry of tRNA binding is 1.48 (Table II).

Since fluorescence titration gave a nonintegral stoichiometry, this was redetermined in the analytical ultracentrifuge (Krauss et al., 1975). Enzyme-bound and free tRNA are separated by centrifugation, and the cell is scanned at two ultraviolet wavelengths (255 nm and 280 nm). The concentrations of free and bound tRNA can be calculated from the measured extinction coefficients of the enzyme and tRNA. The presence of impurities in either ligand will directly influence the apparent value of the stoichiometry. The methionyl-tRNA synthetase is close to 100% pure by active-site titration and gel electrophoresis (Mulvey and Fersht, 1976). The $tRNA^{Met}_M$ used in these experiments was 90% pure (see above), and the stoichiometries determined by analytical ultracentrifugation were corrected accordingly. The results agree closely with those from fluorescence titration (Table II). The failure to saturate the enzyme with 2 mol of tRNA suggests that, like the enzyme from *E. coli* (Blanquet et al., 1976), this methionyl-tRNA synthetase binds only 1 mol of $tRNA^{Met}$ tightly.

Methionyl Transfer Reaction. Methionyl-tRNA synthetase saturated with 2 mol of methionyl adenylate can be isolated from unreacted substrates on Sephadex G-25 (Mulvey and Fersht, 1977a). When this is mixed with $tRNA^{Met}$, only 80% of the methionine moiety is transferred to the tRNA. This is not due to an equilibrium effect, since the extent of transfer is not affected by the presence of alkaline phosphatase which hydrolyses the AMP released during the reaction. This can be compared with 0.99 mol of methionyl-tRNA formed/mol of ATP consumed when the enzyme is preincubated with tRNA (Mulvey and Fersht, 1977b).

On mixing the [^{14}C]methionyl adenylate complex with enriched $tRNA^{Met}$ in the quenched-flow apparatus, the transfer of radioactivity proceeds in two poorly resolved phases (Figure 4). The same reaction course is obtained whether the methionyl adenylate is first isolated on Sephadex G-25 or whether it is formed in situ. In the latter case, a large excess of unlabeled methionine is added to the tRNA solution to

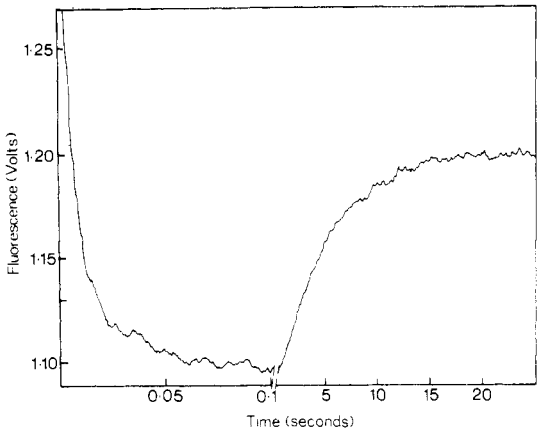


FIGURE 5: tRNA binding followed by transfer of methionine on mixing E·(Met-AMP)$_2$ and tRNA$^{Met}_M$ in the stopped-flow fluorimeter. Conditions: pH 5.9, 0.18 μM enzyme, 2.7 μM tRNA$^{Met}_M$. Excitation at 295 nm, 10-nm band-pass, time constant 2.2 ms.

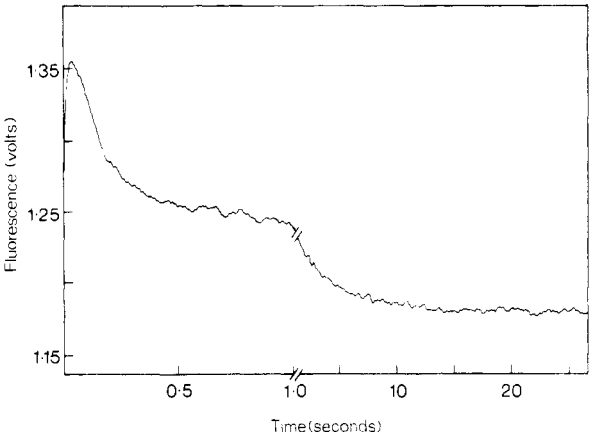


FIGURE 6: The formation of methionyl adenylate on mixing enzyme-tRNA$^{Met}_M$ complex with methionine and ATP in the stopped-flow fluorimeter. Standard conditions, 0.3 μM methionyl-tRNA synthetase, 2.0 μM tRNA$^{Met}_M$, 35 μM methionine, 1.3 mM ATP. Excitation at 295 nm, 5-nm band-pass, 10-ms time constant.

suppress the transfer of radioactive label during the steady-state phase of the reaction.

The transfer of methionine from the isolated methionyl adenylate complex to partly purified tRNA^{Met} can be followed in the stopped-flow fluorimeter. Under the reaction conditions of Figure 4 there is a rapid decrease in fluorescence (k = 40 s^{-1}), corresponding to tRNA binding, followed by a slower, biphasic increase in fluorescence as the methionine is transferred. This is plotted in Figure 4, where data from the quenched-flow experiment are superimposed on the curve from the stopped-flow fluorescence experiment. The coincidence of the two curves shows that the extent of fluorescence quenching is directly proportional to the amount of methionine transferred. Moreover, the small lag in the transfer of methionine (the first 50 ms in Figure 6) is nicely accounted for by the prior binding of tRNA, as can be calculated from the rate of the initial decrease in enzyme fluorescence.

When the stripped methionyl adenylate complex is mixed with purified tRNA$^{Met}_M$ or tRNA$^{Met}_F$ in the stopped-flow fluorimeter, the transfer reaction follows a single exponential rather than the biphasic curve. This is illustrated in Figure 5 where the transfer of methionine to tRNA$^{Met}_M$ is recorded at pH 5.9. There is first a decrease in enzyme fluorescence as the tRNA binds (k = 125 s^{-1}), followed by an increase in fluorescence (k = 0.2 s^{-1}) caused by acyl transfer to the tRNA. The rates of methionyl transfer to tRNA$^{Met}_M$ and tRNA$^{Met}_F$ differ (see Table III) and this could account for the biphasic pre-steady-state phase in the reaction with enriched tRNA^{Met} (Figure 4). The extent of methionyl transfer from E·(Met-AMP)$_2$ is 80%, as with enriched tRNA^{Met}. Since the reaction follows a single exponential, both active sites on the dimeric enzyme must react with tRNA simultaneously. The methionyl-tRNA synthetase of *B. stearothermophilus* could well resemble the corresponding enzyme from *E. coli* (Blanquet et al., 1973), which binds tRNA with equal affinity on either subunit in the presence of methionyl adenylate.

The increase in fluorescence caused by the transfer of methionine may be suppressed by adding methionine (100 μM) and ATP (2.0 μM) to the tRNA solution. Here the methionyl adenylate which reacts with tRNA is rapidly regenerated from fresh substrates, thus eliminating any change in enzyme fluorescence caused by the destruction of methionyl adenylate. This observation strongly supports the assertion, made below, that the formation of methionyl adenylate quenches the enzyme's fluorescence in the presence of tRNA.

In Table III the rate of methionyl transfer, measured by stopped-flow fluorimetry at different values of pH and with different species of tRNA, is compared with the initial rate of charging of tRNA at saturating concentrations of substrates under steady-state conditions. The initial rates were measured with 10 nM methionyl-tRNA synthetase, and pre-steady-state experiments were performed with 150–400 nM enzyme. The methionyl-tRNA synthetase of *E. coli* is known to dissociate over this range of concentrations (Blanquet et al., 1974), and the rate of binding of tRNA with the enzyme from *B. stearothermophilus* is found to increase as the concentration of enzyme is decreased below 50 nM (see above). Therefore, before comparing the pre-steady-state data with the initial rate measurements, it is essential to establish whether the latter vary with enzyme concentration in this same range. There is little variation in the value of k_{cat} for charging with 5 to 25 nM enzyme. At 109 μM methionine, the initial rate of charging of tRNA is 1.8 s^{-1} with 10 nM enzyme (Figure 1) and 2.1 s^{-1} with 380 nM enzyme. The overall rate of charging shows little change over a large range of enzyme concentrations. The data of Table III show that the initial rate of charging and the rate of methionyl transfer are closely similar under a variety of conditions.

TABLE III: Comparison of Rate Constants for Aminoacylation of tRNA and Aminoacyl Transfer.[a]

pH	tRNA	k (s^{-1}) steady state[b]	k (s^{-1}) pre-steady-state[c]
7.8[d]	"enriched"	2.4	
	tRNA$^{Met}_M$	2.2	2.3
	tRNA$^{Met}_F$	1.6	1.5
7.0[e]	"enriched"	0.75	
	tRNA$^{Met}_M$	0.69	0.81
5.9[e]	tRNA$^{Met}_M$	0.24	0.21

[a] 25 °C, 10 mM $MgCl_2$, I = 0.1. [b] k steady state: initial rate of charging of tRNA per mole of enzyme dimer at saturating concentrations of substrate. [c] k pre-steady-state: the first-order rate constant for the transfer of methionine from the isolated methionyl adenylate complex to tRNA, measured by stopped-flow. [d] Tris-Cl buffer. [e] Bistris-Cl buffer.

Formation of Methionyl Adenylate in the Presence of tRNA. We have shown previously that in the absence of tRNA

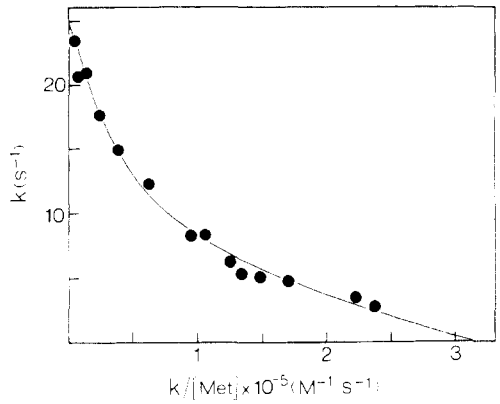


FIGURE 7: The formation of methionyl adenylate in the presence of $tRNA^{Met}$: dependence of the rate upon methionine concentration. 12 μM-12 mM methionine, reaction conditions as in Figure 6.

there is a decrease in the fluorescence of the enzyme on the formation of E·Met-AMP (Mulvey and Fersht, 1976). When methionyl-tRNA synthetase is mixed, in the presence of tRNA, with methionine and ATP there is a similar decrease in enzyme fluorescence (Figure 7). The rate of this change and its dependence on substrate concentration (Table I) suggest that it also corresponds to the formation of methionyl adenylate. The rate constant for the formation of methionyl adenylate was determined assuming the following reaction:

$$E \cdot tRNA \cdot AA \cdot ATP \xrightarrow{k_1} E \cdot tRNA \cdot AA\text{-}AMP + PP_i$$

$$\xrightarrow{k_2} E \cdot AA\text{-}tRNA + AMP \quad (3)$$

In this case the observed rate of formation of the adenylate species is given by (Fersht, 1977):

$$k_{obsd} = k_1 + k_2 \quad (4)$$

Since $k_2 = 2.3\ s^{-1}$ (see Table III), k_1 may be calculated from the stopped-flow data and this value used in the Eadie-Hofstee plot (Figure 7).

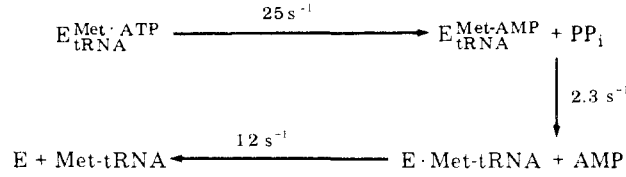
In the absence of tRNA, methionyl adenylate is formed with $k_{cat} = 29\ s^{-1}$ and K_M (Met) = 30 μM. The presence of $tRNA^{Met}_M$ reduces k_{cat} to $25\ s^{-1}$ and leads to a biphasic dependence on methionine concentration (Figure 7). These effects of tRNA on the kinetics of the formation of methionine adenylate were not detected in the kinetics of the ATP-pyrophosphate exchange reaction. This may be because the rate constant for ATP-pyrophosphate exchange is a compound function of both the forward and reverse rate constants (Mulvey and Fersht, 1976). Since the stoichiometry of methionyl adenylate formation is not known in the presence of tRNA (see Discussion), the origin of the nonlinear Eadie-Hofstee plot cannot be established.

The fluorescence change which results from the formation of E·Met-AMP is followed by a second, very slow decrease in enzyme fluorescence ($t_{1/2}$ = several seconds, Figure 6). The rate of this phase depends on both the concentration of methionine ($K_M = 38 \pm 4$ μM) and on the ratio of tRNA to enzyme, suggesting that it corresponds to complete aminoacylation of tRNA in the reaction mix.

Discussion

Proof of the aminoacyl adenylate pathway has previously rested on the following lines of evidence (Fersht and Jakes, 1975; Fersht and Kaethner, 1976): (a) the isolation and characterization of the enzyme-bound aminoacyl adenylate; (b) estimation of the rate of formation of this complex in the presence of tRNA from the kinetics of ATP-pyrophosphate exchange; (c) direct measurement of the reaction of the complex with tRNA showing that the rate of aminoacyl transfer is compatible with the value of k_{cat} for aminoacylation of tRNA.

SCHEME I



In the present study of the methionyl-tRNA synthetase, this same set of criteria have been used to establish the kinetic competence of the E·Met-AMP complex as an intermediate in the aminoacylation of $tRNA^{Met}$. The methionyl adenylate has been isolated and characterized (Mulvey and Fersht, 1977a). The kinetics of ATP-pyrophosphate exchange, aminoacylation of tRNA (Table I), and of methionyl transfer (Table III) have been determined. The results of these experiments are themselves sufficient to prove the aminoacyl adenylate pathway. However, the fluorescence of methionyl-tRNA synthetase is very sensitive to enzyme-substrate interactions, and this makes it possible to examine each step in the reaction pathway by stopped-flow fluorescence.

Changes observed in the enzyme's fluorescence were first correlated with molecular events: (a) tRNA binding in the presence or absence of other substrates leads to a rapid quenching of fluorescence. (b) The formation of methionyl adenylate is accompanied by a decrease in fluorescence both in the presence and absence of tRNA. (c) A combination of stopped-flow and quenched-flow experiments has established that the increase in fluorescence observed during the reaction of E·Met-AMP with tRNA is directly proportional to the extent of transfer of [^{14}C]methionine. (In an earlier study, Bartmann et al. (1975) were able to assign fluorescence changes, observed during a single turnover experiment with phenylalanyl-tRNA synthetase, to the formation of phenylalanyl adenylate and to the subsequent transfer of phenylalanine to tRNA.)

At saturating concentrations of substrates, the rate constant for the formation of methionyl adenylate in the presence of $tRNA^{Met}_M$ is $25\ s^{-1}$ (Figure 7), and the rate constant for methionyl transfer is $2.3\ s^{-1}$ (Tables I and III). The rate of dissociation of the enzyme-product complex is probably close to $12\ s^{-1}$, as measured for the ternary complex of enzyme, methionyl adenylate, and $tRNA^{Met}_M$ (Table II). Therefore, methionyl transfer, or a closely related conformational change, is the rate-determining step. This conclusion is confirmed by the following observations: (a) the rate of methionyl transfer is similar to the initial rate of aminoacylation of tRNA at different values of pH and with different species of $tRNA^{Met}$ (Table III); (b) there is no burst or lag in the initial rate measurement of Figure 2, where stoichiometric concentrations of enzyme and tRNA are used.

Finally, the overall agreement between the experimentally determined rate constants for each step and the initial rate measurements may be checked using Scheme I. When the individual rate constants, determined under the reaction conditions of the quenched-flow experiments, are substituted into the rate equation for this scheme (Gangloff and Fersht, 1978), they predict an initial rate of aminoacylation of $1.8\ s^{-1}$

and a "burst" of −0.10 mol of Met-tRNA/mol of enzyme. These calculated values are close to the experimental values of 1.8–2.1 s^{-1} for the initial rate and +0.04 for the burst.

The Stoichiometry of Enzyme-Bound Intermediates. It can be inferred from the kinetics of aminoacylation of $tRNA^{Met}$ that the predominant enzyme species in the steady-state is the complex of enzyme, methionyl adenylate, and $tRNA^{Met}$. This is confirmed by fluorescence measurements. On the formation of methionyl adenylate, the enzyme fluorescence is quenched and the level of fluorescence is restored when the methionine is transferred to $tRNA^{Met}$. In the steady-state phase, the fluorescence of the enzyme remains at the quenched level.

The stoichiometry of methionyl adenylate bound to the enzyme-$tRNA^{Met}$ complex in the steady state is not known. If the complex E·$(Met\text{-}AMP)_2$ is preformed, both subunits will react simultaneously with $tRNA^{Met}$ (see above). However, the behavior of the enzyme in the presence of saturating concentrations of substrate may not be relevant in vivo where the concentration of unacylated $tRNA^{Met}$ is probably very low.

The methionyl-tRNA synthetase of *E. coli* is in most respects similar to the corresponding enzyme from *B. stearothermophilus* (Mulvey and Fersht, 1976). The concentrations of methionyl-tRNA synthetase and $tRNA^{Met}$ in *E. coli* are 5 and 8 μM, respectively (Blanquet et al., 1973). Ninety-eight percent of the $tRNA^{Met}$ is aminoacylated (Cassio and Mathieu, 1974) and there is sufficient elongation factor Tu to bind all of the Met-$tRNA^{Met}_M$ (Furano, 1975). The Met-$tRNA^{Met}_F$ (3–4 μM) will be distributed between the methionyl-tRNA synthetase, the transformylating enzyme, and the 30-s ribosomal initiation complex. Thus, in vivo, the reaction pathway will be stepwise. The methionyl-tRNA synthetase will exist predominantly as the E·$(Met\text{-}AMP)_2$ complex, which will react with $tRNA^{Met}$ as it is released from the ribosome.

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