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Subunit Interactions in the Methionyl-tRNA Synthetase of *Bacillus stearothermophilus*†

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ABSTRACT: The methionyl-tRNA synthetase from *Bacillus stearothermophilus* is shown to be a dimer of 2 × 82,000 with identical subunits. It exhibits negative cooperativity in substrate binding and "virtual" half-of-the-sites reactivity. The enzyme binds only 1 mol of methionine in the absence of other ligands, but several methods show that 2 mol of methionyl adenylate are bound per enzyme dimer. However, one of these adenylates is formed 480 times faster than the other (k_1 = 29 sec^{-1} and k_2 = 0.06 sec^{-1}). While the rapid phase of the reaction follows normal saturation kinetics with respect to substrate concentration, the rate of the slow phase is independent of substrate concentrations down to 1 μM. It is suggested that the very slow rate of formation of the second adenylate reflects a rate limiting conformational change which precedes a more rapid chemical step on the second subunit.

The aminoacyl-tRNA synthetases exhibit a considerable diversity in their structural and catalytic properties. Despite this they may be grouped into a small number of classes based upon their subunit structures (see Loftfield, 1972, for table and references). The first group, exemplified by the valyl-, leucyl- and isoleucyl-tRNA synthetases from *Escherichia coli* and *Bacillus stearothermophilus*, are monomeric enzymes with a molecular weight of about 110,000. A second group is comprised of dimeric enzymes with identical and relatively small subunits: the tryptophanyl enzymes (mol wt 2 × 37,500) and tyrosyl enzymes (mol wt 2 × 45,000 from *E. coli* and *B. stearothermophilus*). The dimeric methionyl-tRNA synthetase from *E. coli* is a member of a third group with identical but large subunits (mol wt 2 × 85,000). Finally, the phenylalanyl and glycyl ligases are in a fourth group with an $\alpha_2\beta_2$ subunit structure.

Previous work in this laboratory has concentrated on the first two groups of synthetases. It has been shown that the two groups are kinetically very similar; the monomeric enzymes act functionally as dimers. For example, the tyrosine and valine activating enzymes both possess two sets of binding sites for ATP and amino acid but exhibit negative cooperativity in substrate binding. They bind one aminoacyl adenylate, although there is evidence that the second site on each enzyme slowly forms another mole of adenylate which does not accumulate owing to rapid hydrolysis.

In this study we extend our investigations to the third group of synthetases using the methionyl-tRNA synthetase from *B. stearothermophilus*. We are interested in characterizing the enzyme and determining its kinetic and binding behavior to see if the subunit interactions we have found in the first two classes also apply here. The *E. coli* enzyme has been reported to involve independent active sites (Blanquet et al., 1974) but results from this laboratory (Bosshard et al., 1975) indicate that the enzyme from *B. stearothermophilus* binds only one methionine per dimeric unit.

Experimental Section

Materials

All radiochemicals were purchases from the Radiochemical Centre, Amersham. Other reagents were obtained commercially. Methionyl-tRNA synthetase from *E. coli* was a gift from Dr. C. J. Bruton. The partially purified ligase of *B. stearothermophilus* was obtained from the Microbiological Research Establishment, Porton Down, Wilts. This was further purified by the procedure of Koch et al. (1975) which involved three chromatographic steps: (1) DEAE-Sephadex A-50 with a salt gradient of 100–300 m*M* Tris-HCl (pH 7.5); (2) hydroxylapatite with a gradient of 50–200 m*M* potassium phosphate buffer (pH 6.8); (3) gel filtration on Sephadex G-150 in 100 m*M* Tris-HCl (pH 7.5). The purified enzyme was dialyzed into 50% glycerol and 144 m*M* Tris HCl (pH 7.78) and stored at −20°C. All buffers contained 10 m*M* mercaptoethanol and 0.1 m*M* phenylmethanesulfonyl fluoride. Enzyme purity was monitored by polyacrylamide gel electrophoresis (see below), by its specific activity in the pyrophosphate exchange assay, and by its active site content measured by the assay of Fersht et al. (1975a).

Methods

Unless otherwise stated all experiments were carried out under standard conditions of 25.0 ± 0.1°C and a buffer of 100 m*M* Tris-HCl, 44 m*M* Tris (pH 7.78) containing 10

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mM $MgCl_2$, 10 mM mercaptoethanol, and 0.1 mM phenylmethanesulfonyl fluoride. Concentrations of methionyl-tRNA synthetase, *B. stearothermophilus*, were estimated using $E_{280}(1\%)$ 15.5 (Bosshard et al., 1975) and a molecular weight of 164,000 (see Results section). Where applicable results are quoted with their standard error.

Polyacrylamide Gel Electrophoresis. Electrophoresis of the enzyme under native conditions was carried out in a slab gel apparatus using the system of Davis (1964) with 7.5% acrylamide. Three different systems were used for judging the purity of the enzyme under denaturing conditions. Slab gels were prepared according to the method of Laemmli (1970), containing 10% acrylamide and 0.1% sodium dodecyl sulfate and including a stacking gel. This system was also used for the comparison of electrophoretic mobilities of the denatured ligase and the following markers: oyster paramyosin and valyl-tRNA synthetase *E. coli* 110,000; bovine serum albumin 66,000; catalase 60,000; tyrosyl-tRNA synthetase *B. stearothermophilus* 45,000; ovalbumin 42,000; tryptophanyl-tRNA synthetase *B. stearothermophilus* 35,000; chymotrypsinogen 25,000. Disc gels, without a stacking gel, were 10% in acrylamide and 0.1% in dodecyl sulfate and were run in either the phosphate buffer system of Weber and Osborn (1959) or Tris-bicine[1] buffer (0.1 M, pH 8.3). All gels were stained with 0.6% Coomassie Blue in methanol–acetic acid–water (5:1:4) at 45°C for 2 hr. Destaining was carried out in the same solvent at 45°C and the gels were stored in 10% acetic acid.

Sequence Analysis. The methionyl-tRNA synthetase from *B. stearothermophilus* (10.5 mg, 64 nmol) was reduced and carboxymethylated by the method of Kolb et al. (1974). The sequence analysis by automated Edman degradation was kindly performed by Dr. J. Bridgen using a Beckman 890B sequencer as described by Bridgen et al. (1975).

Stopped-Flow Fluorescence. The protein fluorescence changes produced during aminoacyl adenylate formation were monitored by stopped-flow fluorescence, as described by Fersht et al. (1975a). Methionyl-tRNA synthetase (0.4 μM) was preincubated under standard conditions with either saturating ATP or methionine and mixed with an equal volume of methionine or ATP. The resulting decrease in fluorescence was followed at wavelengths greater than 325 nm. The reverse reaction of pyrophosphate with enzyme-bound methionyl adenylate was also studied by stopped-flow fluorescence. The aminoacyl adenylate was formed in situ by incubating 0.35 μM enzyme with 0.1 mM ATP and 0.1 mM methionine under standard conditions. This solution was mixed with an equal volume of a solution containing ATP and methionine at the same concentrations and pyrophosphate (0.01–2.0 mM).

Quenched-Flow Studies. The hydrolysis of ATP during the formation of methionyl adenylate was followed by the pulse quenched-flow technique (Fersht and Jakes, 1975). One syringe of the quenched flow contained 0.38 μM ligase, 10 mM methionine, and inorganic pyrophosphatase (5 units/ml). The other syringe contained [γ-^{32}P]ATP (2.03 μM, 2000 Ci/mol). These solutions were incubated under standard conditions, mixed, and then quenched automatically with 7% perchloric acid at predetermined time intervals (0.15–100 sec). The quenched reaction mixture was then analyzed for labeled ATP and pyrophosphate as described by Fersht and Jakes (1975).

Pyrophosphate–ATP Exchange Assay. The method of Calendar and Berg (1966) was used routinely to measure [^{32}P]pyrophosphate–ATP exchange activity. The K_M of methionine was determined using a reaction mixture of 2.5 mM [^{32}P]pyrophosphate (0.06 Ci/mol), 0.9 mM ATP, 0.025 μM enzyme, 28 mM mercaptoethanol, and methionine (1 μM–2.4 mM). The mixture was incubated under standard conditions of pH and temperature except that the concentration of $MgCl_2$ was 5 mM. The incorporation of counts into ATP was followed by withdrawing 0.2-ml samples at timed intervals and quenching with 0.1 ml of 7% perchloric acid. The ATP was then absorbed onto charcoal and its radioactivity determined on a Nuclear Chicago gas flow counter.

The pyrophosphate exchange assay was also used to determine the K_M of ATP. This procedure was satisfactory while the ATP concentration was similar to that of the pyrophosphate (i.e., 0.2–2 mM). At ATP concentrations below 0.2 mM the exchange of label from [γ-^{32}P]ATP into pyrophosphate was followed over the exponential time course to equilibrium. The reaction mixture was 2.0 mM pyrophosphate, 5.6 mM methionine, 0.01–0.1 μM enzyme, and varying concentrations of ATP (0.8–800 Ci/mmol). The loss of counts from [γ-^{32}P]ATP was monitored as described above.

Equilibrium Dialysis: Methionine Binding. The equilibrium dialysis apparatus used has been described previously (Fersht, 1975). One chamber of each cell contained methionyl-tRNA synthetase (0.06–0.09 mM) and the other contained [^{3}H]methionine (0.05–2.92 mM, 52.2–6.5 Ci/mol). These solutions were equilibrated under standard conditions across a Sartorius membrane (SM 11539). After 2 hr triplicate samples of 5 μl were withdrawn from each chamber and counted in a scintillant containing 9 g of 2,5-di(5-*tert*-butyl-2-benzoxazolyl)thiophene in 0.5 l. of methoxyethanol and 1.5 l. of toluene. The binding of methionine was also measured in the presence of either 1 mM ATP and 2.5 mM pyrophosphate or 10 mM ATP and 1 unit/ml of inorganic pyrophosphatase.

Equilibrium Gel Filtration: ATP Binding. The binding of ATP to methionyl-tRNA synthetase was determined by the method of Hummel and Dreyer (1962). A column of Sephadex G-25 (0.5 × 6 cm) was equilibrated and eluted with a ligand solution containing [^{14}C]ATP and in some cases other reagents (see below); 100 μl of an enzyme solution, containing reagents at the same concentrations as in the ligand solution, was passed down the column. Individual drops were collected and assayed for [^{14}C]ATP, as described in Fersht and Jakes (1975). ATP binding was examined in the following systems: (a) 103–163 μM ATP, no additional ligands, and 45 μM enzyme; (b) 41–163 μM ATP, 5 mM methionine, either 2 mM pyrophosphate *or* 5 units/ml of inorganic pyrophosphatase, and 13.6–55 μM enzyme.

Nitrocellulose Disk Filtration of the Methionyl-tRNA Synthetase–Methionyl Adenylate Complex. Enzyme-bound methionyl adenylate was assayed under a variety of conditions by nitrocellulose disc filtration, as described by Yarus and Berg (1970). A solution (0.1 ml) of 5 μM methionyl-tRNA synthetase, 1.0 mM ATP, 0.11 mM [^{14}C]methionine (62 Ci/mol), and 0.02 unit of inorganic pyrophosphatase was incubated for 2 min at standard conditions. Duplicate samples (25 μl) were filtered through Schleicher and Schüll BA 85 nitrocellulose filters and washed with 3 ml of cold 10 mM $MgCl_2$ and 10 mM bis-

[1] Abbreviations used are: bicine, *N,N'*-bis(2-hydroxyethyl)glycine; bis-tris, *N,N*-bis(2-hydroxyethyl) iminotris (hydroxymethyl) methane.

tris (pH 6.0). The filters were dried and bound ^{14}C was monitored using a toluene-based scintillant (Fersht et al., 1975a). This assay was repeated in the absence of inorganic pyrophosphatase and with various amounts of added pyrophosphate (0–2.5 m*M*).

Results

Enzyme Purity. The material received from the Microbiological Research Establishment, Porton, possessed methionyl-tRNA synthetase activity of 36 units/mg (1 unit = 1 μmol of [^{32}P]pyrophosphate exchanged in 15 min at 37°C). The enzyme was purified by the procedure described in the Experimental Section to a specific activity of 455 units/mg. Polyacrylamide gel electrophoresis under native conditions showed only one band and dodecyl sulfate gels indicate a high degree of purity (Figure 1). The active site assay of Fersht et al. (1975a) gave $n = 2.00 \pm 0.07$ active sites per molecule of enzyme, averaged from many measurements. The enzyme showed no deterioration or loss of activity over several months of storage at −20°C.

Subunit Structure and Molecular Weight. The methionyl-tRNA synthetases from *E. coli* and *B. stearothermophilus* have the same electrophoretic mobility on all the denaturing gel systems examined (e.g., Figure 1). Several slab gels were run under denaturing conditions in which the mobility of these synthetases was compared with a number of proteins of known molecular weights (see Experimental Section). The subunit molecular weight estimated by this method is 82,000 ± 2000. This is in close agreement with the molecular weight of the enzyme from *E. coli* determined by Koch and Bruton (1974). However, it differs from the estimate of the molecular weight of the *B. stearothermophilus* enzyme made by Koch et al. (1974). The ligases from both species of bacteria have identical elution volumes on a Sephadex G-200 column (1 × 150 cm, eluted with 144 Tris-Cl (pH 7.78), 10 m*M* $MgCl_2$, 0.1 m*M* phenylmethanesulfonyl fluoride, and 10 m*M* mercaptoethanol). Therefore, it may be assumed that the methionyl-tRNA synthetase of *B. stearothermophilus* is a dimer (2 × 82,000) like the enzyme of *E. coli* (Koch and Bruton, 1974). On limited digestion with subtilisin the *B. stearothermophilus* ligase yields a fragment of mol wt 64,000 again showing the same behavior as the enzyme from *E. coli*.

The continuous dodecyl sulfate gels run in the phosphate and Tris-bicine buffers showed a single band for the methionyl-tRNA synthetase of *B. stearothermophilus*. When run on the discontinuous gel system of Laemmli (1970) this enzyme consistently gave two poorly resolved bands differing by less than 4000. This result is almost certainly an artefact of the discontinuous gel system as other members of this laboratory have found similar difficulties with other proteins. Determination of the N-terminal sequence of the enzyme shows that it has identical subunits, the first 54 residues being:

Met-Glu-Lys-Lys-Thr-Phe-Tyr-Leu-Thr-Thr-Pro-Ile-
Tyr-?-Pro-Ser-?-Lys-Leu-His-Ile-Gly-His-Ala-Tyr-
Thr-?-Val-Ala-Gly-Asx-Ala-Met-Ala-Arg(?)-Tyr-Lys-
Lys-Leu-Arg-Gly-Tyr-Asx-Val-Met-Tyr(?)-Leu-Thr-
Gly-Thr-Asx-Glx-His-Gly

(positions 1, 10, 20, 30, 40, 50, 54)

This sequence agrees with the results of G. L. E. Koch (personal communication). The repetitive yield from the sequencer, based on the yields of Ile-12 and Leu-19, is 98%. This extrapolates to an absolute yield at step 1 of 1.6 mol of N-terminal amino acid/mol of protein.

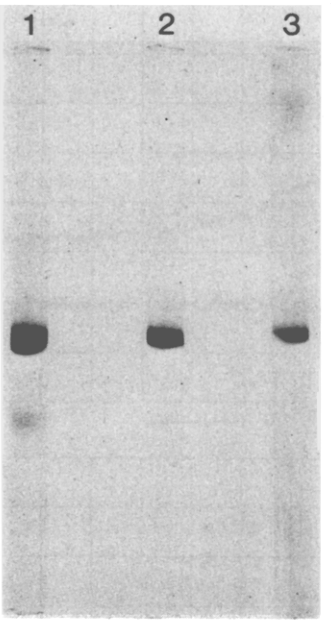

FIGURE 1: Sodium dodecyl sulfate polyacrylamide gels (in the phosphate buffer system) of methionyl-tRNA synthetase from: *E. coli* 40 μg (1); *B. stearothermophilus* 40 μg (2), and 5 μg (3).

Methionyl Adenylate Formation. When the enzyme–methionine complex is mixed with ATP in the stopped-flow fluorimeter there is a biphasic decrease in fluorescence in which the two relaxations are well separated (Figure 2). The fast relaxation corresponds to a fluorescence change of 13% (based on the fluorescence of the enzyme–product complex) and follows saturation kinetics with respect to ATP concentration, giving a K_M of 46.3 ± 2.2 μ*M* and a k_{cat} of 29.4 ± 1 sec^{-1} (see Figure 3). In contrast, the rate of the slow relaxation (9% change) is independent of ATP concentration and gives a k_{cat} of 0.06 sec^{-1} and K_M for ATP below 1 μ*M*. A biphasic decrease in fluorescence is also observed when the enzyme–ATP complex is reacted with methionine. Again the fast decrease (7%) follows saturation kinetics (K_M methionine, 29.8 ± 1.2 μ*M* and k_{cat}, 27.8 ± 0.6 sec^{-1}), while the slow relaxation (~4%) shows no dependence on methionine concentration down to 2 μ*M*.

To demonstrate that both relaxations accompany aminoacyl adenylate formation the reaction was examined by the pulse quenched-flow technique (Fersht and Jakes, 1975). On mixing γ-labeled [^{32}P]ATP with a solution of methionyl-tRNA synthetase, methionine, and inorganic pyrophosphatase the reaction products were sampled at various times up to 1 min and analyzed for labeled ATP and phosphate. The steady-state hydrolysis of ATP, due to adenylate turnover (0.004 sec^{-1}), was determined under identical conditions and subtracted from the pre-steady-state data. The results are shown in Figure 4. The curve in this figure was calculated according to

$$\frac{[P_i]}{[E_0]} = 2 + \left(\frac{k_2}{k_1 - k_2} - 1\right) e^{-k_1 t} - \frac{k_1}{k_1 - k_2} e^{-k_2 t} \quad (1)$$

which is the rate equation for the sequential reaction in Scheme I. $[E_0]$ in eq 1 is the total enzyme concentration, k_1 and k_2 are pseudo-first-order rate constants which depend on k_{cat}, K_M, and substrate concentrations. The magnitudes

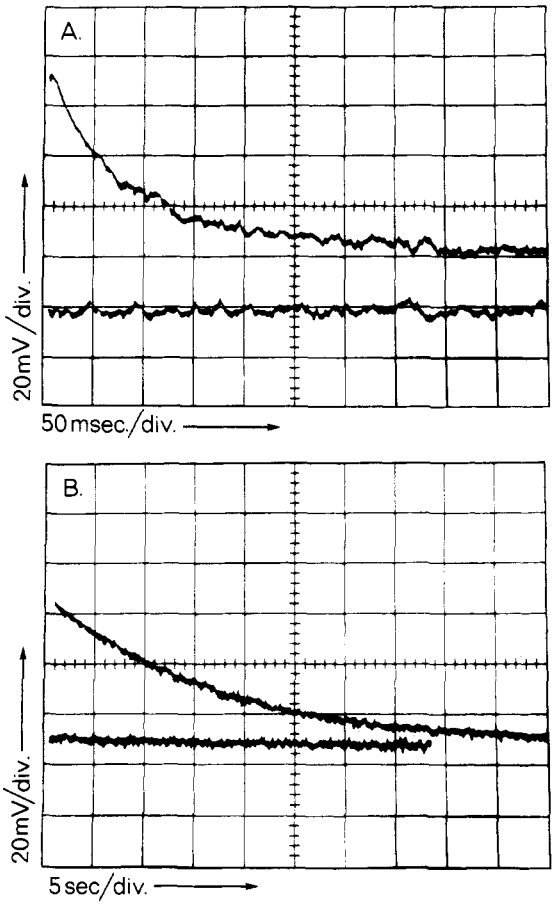

FIGURE 2: Decrease in fluorescence on mixing equal volumes of 0.4 μM enzyme incubated with 2.0 mM methionine and 86 μM ATP under standard conditions. Delayed pulse triggered at 60 sec giving an end-point signal of 1.07 V. Excitation at 295 nm; 5-nm band pass; time constants: (A) 5 msec and (B) 0.1 sec.

Scheme I

$$E \xrightarrow[\text{ATP, Met}]{k_1} E{\cdot}Met{\sim}AMP + PP_i \xrightarrow[\text{ATP, Met}]{k_2} E{\cdot}(Met{\sim}AMP)_2 + PP_i$$

$$PP_i \rightarrow 2P_i \qquad PP_i \rightarrow 2P_i$$

of k_1 and k_2 (see the legend of Figure 4) were determined from the biphasic fluorescence change, obtained in the stopped-flow fluorimeter using the same enzyme and ATP solutions as in the quenched flow experiment. In Figure 4 the chemical change is biphasic and coincides precisely with the fluorescence change.

The reverse reaction of pyrophosphate with the enzyme-adenylate complex is accompanied by an increase in fluorescence giving a single relaxation. The dependence of rate on pyrophosphate concentration gives a K_M for pyrophosphate of 85 ± 6 μM and k_{cat} of 75 ± 3 sec^{-1}. The magnitude of the fluorescence change also varies with pyrophosphate concentration. At 10 μM pyrophosphate it is 3.5% but at concentrations above 200 μM it is 9%. This variation reflects the different extents to which the reverse reaction takes place at low concentrations of pyrophosphate (see below).

Pyrophosphate-ATP Exchange Kinetics. The dependence of the initial velocity of the pyrophosphate-ATP exchange on methionine concentration shows saturation kinetics giving a single K_M for methionine of 47 ± 6 μM and

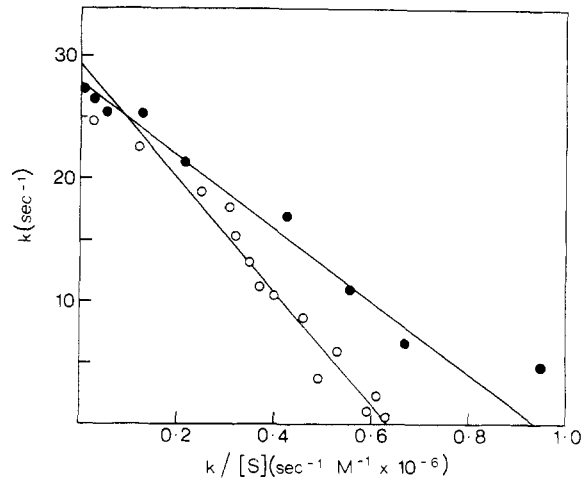

FIGURE 3: Variation of the rate constant of aminoacyl adenylate formation (rapid phase) with substrate concentration. ATP varied (1 μM–1 mM) with 10 μM methionine (○); methionine varied (1 μM–10 mM) with 5 mM ATP (●). Enzyme concentration 0.2 μM.

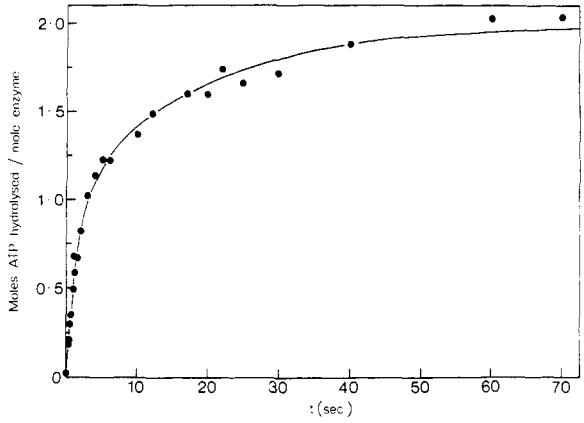

FIGURE 4: Pulsed quenched flow analysis of the hydrolysis of [γ-^{32}P]ATP (measured by phosphate release) during the pre-steady-state formation of methionyl adenylate. Equal volumes of enzyme (0.4 μM) and [γ-^{32}P]ATP (2.0 μM) were mixed in the presence of 5 mM methionine and inorganic pyrophosphatase. The solid curve was calculated, as described in the text, for a biphasic change using the observed rate constant of the fluorescence changes determined with identical substrate concentrations (k_1 = 0.577 sec^{-1} and k_2 = 0.052 sec^{-1}).

k_{cat} of 10 ± 1.0 sec^{-1}. This K_M compares very closely with the apparent K_S of methionine determined by equilibrium dialysis under the conditions of pyrophosphate-ATP exchange (K_S = 37 μM, see below). The dependence of the rate of exchange on ATP concentrations was measured both by initial velocity and by following the reaction to completion (i.e., isotopic equilibrium). In the latter case the exchange of label from [γ-^{32}P]ATP to pyrophosphate was determined and the data were analyzed according to the isotope exchange kinetics developed by Boyer (1959) and Cole and Schimmel (1970). The loss of label from ATP follows the rate equation:

$$\frac{[ATP^*]}{[\overline{ATP^*}]} = 1 + e^{-k_{obsd}t} \qquad (2)$$

where ATP* is the specific activity at time t and the bar denotes the specific activity at isotopic equilibrium, and where

$$k_{obsd} = V\frac{[ATP] + [PP_i]}{[ATP][PP_i]} \qquad (3)$$

In this way a K_M, ATP of 15.4 ± 0.8 μM, and k_{cat} of 8.2 ±

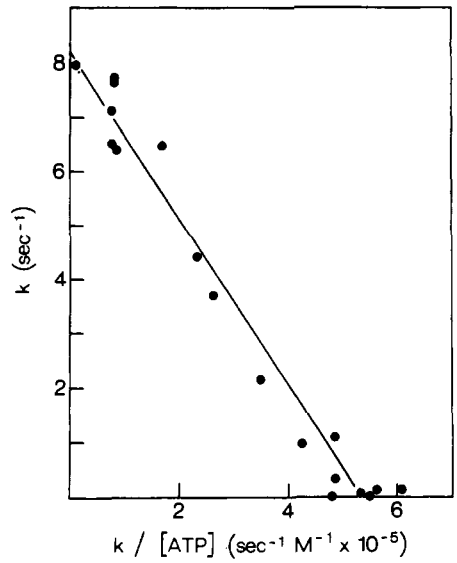

FIGURE 5: Dependence of the rate constant of ATP-pyrophosphate exchange (measured from exponential time course to isotopic equilibrium) on ATP concentration (0.02 μM–2.0 mM) with 5.6 mM methionine and 0.01–0.1 μM enzyme.

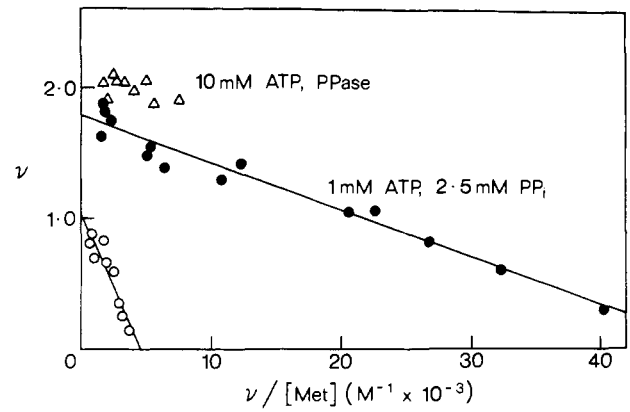

FIGURE 6: Binding of methionine to methionyl-tRNA synthetase measured by equilibrium dialysis: 120 μM enzyme and 0.04–1.4 mM methionine (○); 83 μM enzyme, 2.5 mM pyrophosphate, 1.0 mM ATP, and 0.008–1.2 mM methionine (●); 83 μM enzyme, 10 mM ATP, inorganic pyrophosphatase, and 0.4–1.0 mM methionine (△). ν = number of moles of methionine bound per mole of enzyme.

0.3 sec^{-1} were determined (see Figure 5). The results of both the pre-steady-state and the pyrophosphate exchange kinetics are summarized in Table I.

The rate-determining step in the pyrophosphate exchange reaction is not the formation of the aminoacyl adenylate, as is usually found, but also includes the pyrophosphorolysis step. This is inferred from the accumulation of 0.5 mol of adenylate under the conditions of the experiment in Figure 8 showing that the formation and pyrophosphorolysis of the adenylate occur at equal rates. This may also be deduced from the comparison of the pre-steady-state determination of the k_{cat} for the formation of the aminoacyl adenylate (29 sec^{-1}) with that for the pyrophosphate exchange (10 sec^{-1}). If the formation and pyrophosphorolysis rate constants are each about 20 sec^{-1} then the overall rate constant for pyrophosphate exchange is 10 sec^{-1}, with 0.5 mol of adenylate accumulating. The K_M values derived from the latter experiments are not the dissociation constants but are "kinetic" values.

Methionine Binding. The results of the equilibrium dialysis experiments are shown in Figure 6. In the absence of other ligands methionine binds to methionyl-tRNA synthetase with a dissociation constant, K_S, of 227 ± 35 μM and a stoichiometry of ν = 1.0 ± 0.1 mol of ligand/mol of enzyme. The highest concentration of free methionine examined was 1.4 mM and there was no indication of binding of a second amino acid molecule. In the presence of 1 mM ATP and 2.5 mM pyrophosphate the "apparent" dissociation constant is decreased to 37.0 ± 3.4 μM and ν = 1.76 ± 0.1. At first sight it would appear that two molecules of methionine bind with equal affinity under the conditions of pyrophosphate exchange. But it will be shown later that one is bound in the form of methionyl adenylate. In a control experiment saturating concentrations of methionine (above 0.4 mM free amino acid) were equilibrated in the presence of 10 mM ATP and inorganic pyrophosphate. It can be calculated from the steady-state turnover of ATP (0.004 sec^{-1}) that only 10% of the ATP will be hydrolyzed during the course of equilibration. In these controls a stoichiometry of close to ν = 2 was observed (Figure 6).

ATP Binding. The binding of ATP to methionyl-tRNA synthetase was determined by gel filtration both in the presence and absence of methionine and pyrophosphate. The degree of saturation of the enzyme with ATP was measured from the areas of the peak and the trough in the elution profile (Figure 7). With ATP concentrations of 103 and 163 μM and no additional ligands the degree of saturation (ν) was respectively 0.31 and 0.6. If only 1 mol of ATP binds at saturation the K_S would be in the region of 100–200 μM. (If the stoichiometry is higher the K_S must be correspondingly higher.)

In several experiments in which the binding of ATP (41–163 μM) was measured in the presence of 5 mM methionine and 2 mM pyrophosphate an average value of ν = 1.5 ± 0.1 was obtained. Similarly, when the binding was measured in the presence of 5 mM methionine and inorganic pyrophosphatase ν was found to be 1.6 ± 0.1. Under the latter conditions it is known that two molecules of methionyl adenylate bind to the enzyme (i.e., from the results of the active site titration, the quenched-flow experiment, and methionine binding studies). Apparently the gel filtration experiment underestimates the stoichiometry of binding. It may be concluded, therefore, that the stoichiometry of ATP binding is close or equal to 2 under conditions of both adenylate formation and pyrophosphate exchange. The results of these binding experiments are summarized in Table II.

Table I: Rate Constants for the Formation and Pyrophosphorolysis of Methionyl Adenylate Complexes.[a]

Reaction	k_{cat} (sec^{-1})	K_M (μM) Met	ATP	PP_i
Pre-steady-state formation of E·Met~AMP	29	30	46	
Pre-steady-state formation of E·(Met~AMP)$_2$	0.06	<1.0	<1.0	
Pre-steady-state pyrophosphorylysis of E·(Met~AMP)$_2$[b]	75			85
Pyrophosphate exchange	10	47	15	

[a] Standard buffer (Tris–$MgCl_2$). [b] To give $E\cdot\frac{Met\sim AMP}{Met,ATP}$

Table II: Stoichiometry of Ligand Binding.[a]

Form of Enzyme	Ligand	Additional Ligands	Stoichiometry and Dissociation Constant	Method
E	Met		1 (0.23 m*M*)	Eq. dialysis
E	ATP		1(?) (0.1–0.2 m*M*)	Gel filtration
E·Met~AMP[b]	Met	ATP	1	Kinetic, eq. dialysis
E·Met~AMP[b]	ATP	Met	1	Kinetic, gel filtration
E[c]	Met~AMP		2	Eq. dialysis, gel filtration, nitrocellulose disk filtration

[a] Standard buffer (Tris–$MgCl_2$). [b] Measured prior to formation of adenylate at second site (k = 0.06 sec^{-1}) in pre-steady state or in the presence of pyrophosphate in the steady state. [c] Methionyl adenylate formed in situ in the presence of inorganic pyrophosphatase.

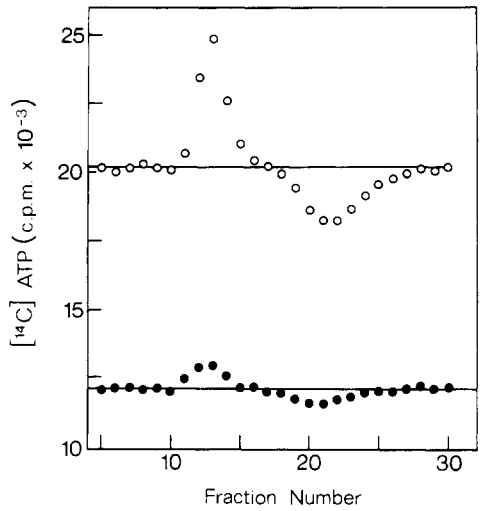

FIGURE 7: Binding of ATP to methionyl-tRNA synthetase measured by equilibrium gel filtration: 103 μM ATP and 45 μM enzyme (●); 163 μM ATP and 45 μM enzyme in the presence of 5 mM methionine and 2 mM pyrophosphate ○).

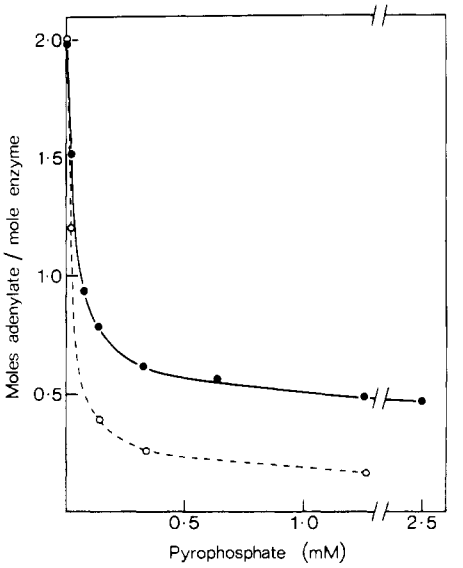

FIGURE 8: Dependence of the stoichiometry of enzyme-bound methionyl adenylate on pyrophosphate concentration. Measured by nitrocellulose disc filtration with 5 μM enzyme, 110 μM methionine, and 1 mM ATP.

Pyrophosphorolysis of Enzyme-Bound Methionyl Adenylate. In the presence of inorganic pyrophosphatase two aminoacyl adenylates are formed per dimer as shown by nitrocellulose disk filtration. In the absence of inorganic pyrophosphatase the accumulation of about 7–10 μM pyrophosphate causes the stoichiometry to drop to 1.5. The addition of increasing quantities of pyrophosphate causes the stoichiometry to plateau at 0.5 for the enzyme from *B. stearothermophilus* and 0.15 for that from *E. coli* (Figure 8).

Discussion

Subunit Structure. The methionyl-tRNA synthetase from *B. stearothermophilus* is a dimer with apparently identical subunits; we have shown that both chains have the same N-terminal sequence for 54 residues. Redetermination of the molecular weight in this study (2 × 82,000) shows that it is similar to the corresponding ligase from *E. coli*. Limited proteolysis of both enzymes yields catalytically active fragments of molecular weight about 64,000 (Cassio and Waller, 1971; Koch et al., 1975). Enzymes from both sources contain regions of internal duplications of sequence (Bruton et al., 1974; Koch et al., 1974).

Interactions between Active Sites. In the absence of other ligands the methionyl-tRNA synthetase of *B. stearothermophilus* binds 1 mol of methionine/enzyme dimer. However, two aminoacyl adenylates are formed on the enzyme as shown by active site titration (using the ATP depletion method, Fersht et al., 1975a) and by isolation on nitrocellulose filters. Stopped-flow fluorescence studies combined with quenched-flow analysis of the reaction products show that the two adenylates are formed at vastly different rates. The first is formed with a rate constant of 29 sec^{-1} and K_M values of 30 and 46 μM for methionine and ATP, respectively. The rate constant for the second is 0.06 sec^{-1} with K_M values close to or less than 1 μM for each substrate (see Table I).

The biphasic nature of adenylate formation was confirmed from the binding of the methionyl adenylate–enzyme complex to nitrocellulose filters (Yarus and Berg, 1970). Two minutes after mixing the enzyme and substrates at 25°C the production of adenylate is complete and 2.0 adenylates are bound per mol of enzyme. However, at 13 sec after mixing only 1.65 ± 0.06 mol of methionyl adenylate accumulate per mol of enzyme, consistent with the slow formation of the second adenylate at about 0.08 sec^{-1}. At 0°C the reaction is retarded and it is possible to detect a burst of 1 mol of adenylate followed by the slow formation of the second adenylate with a rate constant of 0.02 sec^{-1}.

Subunit interactions are also indicated by the negative cooperativity in substrate binding; only one methionine

binds in the accessible concentration range with a dissociation constant of 0.23 m*M*. Therefore, though the enzyme has chemically identical subunits, they behave asymmetrically in substrate binding and product formation. It should be noted that the steady-state kinetics will not indicate subunit interactions if the binding of the second set of ligands occurs after the rate-determining chemical step on the first subunit, as shown previously for the tyrosyl-tRNA synthetase (Fersht et al., 1975b).

The Mechanism of Aminoacyl Adenylate Formation. The disparity in the rates of formation of aminoacyl adenylates on the two enzyme subunits may be described loosely as half-of-the-sites reactivity even though two adenylates are eventually formed. Several mechanisms could account for the extremely low K_M values for methionine and ATP in the formation of the second adenylate. These fall into two types: (a) in which rapid adenylate formation on one subunit leads to a decrease in K_S values for both substrates on the second subunit to below 1 μM and the chemical step becomes rate limiting with its rate constant reduced to 0.06 sec^{-1}; or (b) where the rapid formation of adenylate on one subunit induces a slow rate-limiting conformational change at 0.06 sec^{-1} followed by a more rapid chemical step on the second subunit. In the latter case the low K_M values do not necessarily reflect the dissociation constants of the substrates. If the chemical step at the second site is rate limiting at 0.06 sec^{-1}, then the value of k_{cat}/K_M for pyrophosphate (see Table I) suggests that adenylate formation at this site would be reversed by 0.07 μM pyrophosphate. The results of Figure 8 show that this is not the case; at 10 μM pyrophosphate 1.5 adenylates remain bound. Therefore it is more likely that the second type of mechanism with a rate-limiting conformational change is involved here.

The General Occurrence of This Mechanism. Though the methionyl-tRNA synthetases from *B. stearothermophilus* and *E. coli* have similar molecular weights and subunit structures there are some differences in their interactions with substrates. The *E. coli* enzyme binds 2 mol of methionine in the absence of other ligands and 4 mol of ATP (Blanquet et al., 1972). Blanquet et al. (1974) suggest that there are no interactions between the subunits of this enzyme during catalysis. The methionine activating enzymes from both sources form two aminoacyl adenylates. However, this study has shown that with one of these enzymes their rates of formation can differ by several orders of magnitude. Clearly stoichiometry alone is not a sufficient criterion for effective full site activity.

The methionyl-tRNA synthetase of *B. stearothermophilus* resembles the dimeric tyrosyl-tRNA synthetase and the monomeric valyl-tRNA synthetase in its catalysis of aminoacyl adenylate formation. With the latter enzymes aminoacyl adenylate is formed rapidly at one active site and very slowly at the second. Its rate of hydrolysis at the second site is faster than its rate of formation so that it does not accumulate, and its evanescent existence was inferred indirectly (Fersht, 1975). With the methionyl-tRNA synthetase the formation rate constant on the second subunit is faster than the hydrolytic rate constant and the second adenylate can be observed and studied directly.

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