Kinetic Mechanism of *Escherichia coli* Carbamoyl-Phosphate Synthetase

Frank M. Raushel, Paul M. Anderson, and Joseph J. Villafranca*

**ABSTRACT:** The kinetic mechanism of *Escherichia coli* carbamoyl-phosphate synthetase has been determined at pH 7.5, 25 °C. With ammonia as the nitrogen source, the initial velocity and product inhibition patterns are consistent with the ordered addition of MgATP, HCO₃⁻, and NH₃. Phosphate is then released and the second MgATP adds to the enzyme, which is followed by the ordered release of MgADP, carbamoyl phosphate, and MgADP. With glutamine as the ammonia donor, the patterns are consistent with a two-site mechanism in which glutamine binds randomly to the small molecular weight subunit producing glutamate and ammonia. Glutamate is released and the ammonia is transferred to the larger subunit. Carbamoyl-phosphate synthetase has also been shown to require a free divalent cation for full activity.

**Carbamoyl-phosphate synthetase** from *Escherichia coli* catalyzes the following reaction:

\[
2\text{MgATP} + \text{HCO}_3^- + \text{L-Gln} \rightarrow 2\text{MgADP} + \text{carbamoyl-P} + \text{P}_1 + \text{L-Glu} \quad (1)
\]

Ammonia can also be used as the nitrogen source. Anderson & Meister (1965) have proposed that the enzyme catalyzes the overall reaction through a series of three partial reactions:

\[
\text{MgATP} + \text{HCO}_3^- \rightarrow \text{MgADP} + \text{OOCOPO}_3^{2-} \quad (2)
\]

\[
\text{OOCOPO}_3^{2-} + \text{NH}_3 \rightarrow \text{NH}_2\text{COO}^- + \text{P}_1 \quad (3)
\]

\[
\text{NH}_2\text{COO}^- + \text{MgATP} \rightarrow \text{NH}_3\text{COOPO}_3^{2-} + \text{MgADP} \quad (4)
\]

Carbamoyl-phosphate synthetase has a molecular weight of approximately 180 000 and is a dimer of two nonidentical subunits (Matthews & Anderson, 1972; Trotta et al., 1971). The smaller subunit of molecular weight 48 000 contains the binding site for glutamine. The larger subunit of molecular weight 130 000 contains the binding sites for the rest of the substrates and allosteric modifiers (Trotta et al., 1971).

Carbamoyl-phosphate synthetase is a challenging enzyme to study by kinetic techniques because the large number of substrates and products in the overall reaction significantly increases the number of possible kinetic schemes. Since there are two molecules of MgATP used in the reaction sequence, it is also of interest to determine if there are two separate MgATP sites and if these sites can be distinguished kinetically.

Previous kinetic studies with carbamoyl-phosphate synthetase have been limited to the enzyme from frog and bovine liver (Fahien & Cohen, 1964; Guthörlin & Knappe, 1969; Kerson & Appel, 1968; Elliot & Tipton, 1974b,c). The liver enzyme uses only ammonia as the nitrogen source and is activated by N-acetyl-L-glutamate. In the most complete kinetic study, Elliot & Tipton (1974b,c) have proposed an ordered quad-quad mechanism for bovine liver carbamoyl-phosphate synthetase based on initial velocity and product inhibition experiments. A different kinetic mechanism for the glutamine-dependent carbamoyl-phosphate synthetase from *E. coli* is proposed in this report. The mechanism is also based on initial velocity and product inhibition experiments. The proposed mechanism is consistent with all of the kinetic data and the partial reactions catalyzed by carbamoyl-phosphate synthetase from *E. coli*.

**Materials and Methods**

Carbamoyl-phosphate synthetase was isolated from *E. coli* according to the method of Matthews & Anderson (1972). Ornithine transcarbamoylase was a gift from Dr. Margaret Marshall. All other compounds were obtained from Sigma.

**Enzyme Assays.** Carbamoyl-phosphate synthetase activity was measured spectrophotometrically using a pyruvate kinase-lactate dehydrogenase coupling system. A Beckman DU monochrometer equipped with a Gilford 2000 optical density converter and a 10-mV recorder was used to follow the reaction at 340 nm.
For activity measurements in the absence of added MgADP, each 1.0-mL cuvette contained 50 mM Hepes, pH 7.5, 33 µg of each salt-free lactate dehydrogenase and pyruvate kinase, 1 mM phosphoenolpyruvate, 0.2 mM NADH, 100 mM KCI, 10 mM ornithine, 15-20 mM excess MgCl₂, and various amounts of the different substrates and inhibitors. When high concentrations of NH₄⁺ or phosphate were used, the ionic strength was kept constant with KCl. Assays were conducted at 25 °C and the carbamoyl-phosphate synthetase (1-10 µg) was added last to initiate the reaction.

In the presence of added MgADP, activity was measured by coupling the production of carbamoyl phosphate with ornithine transcarbamoylase. Each 1.0-mL reaction vial contained 50 mM Hepes, pH 7.5, 10 mM ornithine, 15 mM excess MgCl₂, 100 mM KCl, 10 units of ornithine transcarbamoylase, and various amounts of substrates and inhibitors. At equal time intervals, aliquots of the reaction mixture were quenched with 0.25 mL of 10% trichloroacetic acid. The citrulline concentration was measured colorimetrically at 460 nm (Ceriotti, 1974).

Data Analysis. The kinetic data were analyzed using the Fortran programs of Cleland (1967). Initial velocity data conforming to a parallel pattern were fitted to eq 5 and a parallel pattern to eq 6. Competitive, uncompetitive, and noncompetitive inhibition patterns were fitted to eq 7, 8, and 9, respectively. When the type of pattern was in doubt (parallel or intersecting), both possible fits were tried and a comparison of the values (square root of average residual least square) was made to determine the best fit. In no case did a set of data fit both patterns equally well. The nomenclature used in this paper is that of Cleland (1963).

\[
\frac{v}{V} = \frac{K_{II}K_{II} + K_{II}A + K_{II}B + AB}{K_{II}K_{II} + K_{II}A + K_{II}B + AB}
\]

(5)

\[
\frac{v}{V} = \frac{K_{II}A + K_{II}B + AB}{K_{II}A + K_{II}B + AB}
\]

(6)

\[
\frac{v}{V} = \frac{K(1 + (I/K_{II})) + A}{K + A(1 + (I/K_{II}))}
\]

(7)

\[
\frac{v}{V} = \frac{K(1 + (I/K_{II})) + A(1 + (I/K_{II}))}{K(1 + (I/K_{II})) + A(1 + (I/K_{II}))}
\]

(8)

\[
\frac{v}{V} = \frac{K(1 + (I/K_{II})) + A(1 + (I/K_{II}))}{K(1 + (I/K_{II})) + A(1 + (I/K_{II}))}
\]

(9)

\[
Abbreviations used: Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; EPR, electron paramagnetic resonance, PEP, phosphoenolpyruvate.

Results

Divalent Cation Requirements. Shown in Figure 1 is the effect on the reaction rate when the concentration of Mg²⁺ or Mn²⁺ is varied at an ATP concentration of 10 mM. With Mg²⁺ it is clear that carbamoyl-phosphate synthetase requires more Mg²⁺ for full activity than required to complex all of the nucleotide. With Mn²⁺, however, the optimal level of Mn²⁺ is about equivalent with the ATP concentration and additional Mn²⁺ is inhibitory. These data suggest the presence of an additional metal ion binding site other than the metal ATP site(s). Thus, all kinetic experiments were conducted with excess Mg²⁺ present in order to complex the nucleotides and to also produce the additional enzymatic activation.

Variation of MgATP. The variation of activity with MgATP concentration at 15 mM excess Mg²⁺ is shown in Figure 2 at saturating and non-saturating levels of HCO₃⁻. As can be seen from the data, the double-reciprocal plot is linear from 10 to 300 µM MgATP.

Initial Velocity Patterns. To establish the kinetic mechanism of E. coli carbamoyl-phosphate synthetase, the various kinetic patterns were determined for every possible substrate pair. Glutamine vs. either HCO₃⁻ or MgATP gives parallel initial velocity patterns. Intersecting patterns are obtained with ammonia vs. either HCO₃⁻ or MgATP. However, if the HCO₃⁻ level is raised from 1 mM to 30 mM, the NH₄⁺ vs. MgATP pattern changes to one that is parallel. The MgATP vs. HCO₃⁻ pattern is intersecting. The kinetic constants from fits of the data to eq 5 or 6 are listed in Table I. There is good agreement for the $K_m$ values of a given substrate in the various experiments.

Product Inhibition Patterns. Product inhibition studies were also carried out in order to establish the kinetic mechanism. Inorganic phosphate is competitive with MgATP and noncompetitive with HCO₃⁻ at low glutamine. At saturating glutamine, phosphate is an uncompetitive inhibitor with HCO₃⁻. Carbamoyl phosphate is uncompetitive with MgATP, HCO₃⁻, and NH₄⁺. MgADP is a linear noncompetitive inhibitor vs. MgATP, HCO₃⁻, and NH₄⁺. At saturating HCO₃⁻, the MgADP vs. NH₄⁺ pattern is uncompetitive. The inhibition of Pᵢ vs. NH₄⁺ could not be done because of the formation of a precipitate. Glutamate did not significantly inhibit the reaction at concentrations up to 100 mM. The kinetic constants from fits of the data to eq 7, 8, and 9 appear in Table II.

Discussion

From the data presented in this paper, it is apparent that carbamoyl-phosphate synthetase has a requirement for free Mg²⁺ in addition to MgATP for full activity under the assay conditions.
TABLE I: Initial Velocity Patterns.  

<table>
<thead>
<tr>
<th>varied substrates</th>
<th>fixed substrate</th>
<th>pattern type</th>
<th>app Michaelis constants (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgATP vs. HCO₃⁻</td>
<td>Gln, 10 mM HCO₃⁻</td>
<td>intersecting</td>
<td>0.25 ± 0.01 1.8 ± 0.1</td>
</tr>
<tr>
<td>MgATP vs. Gln</td>
<td>HCO₃⁻, 20 mM HCO₃⁻</td>
<td>parallel</td>
<td>0.19 ± 0.01 0.13 ± 0.01</td>
</tr>
<tr>
<td>MgATP vs. NH₄⁺</td>
<td>HCO₃⁻, 30 mM HCO₃⁻</td>
<td>parallel</td>
<td>0.20 ± 0.01 180 ± 10</td>
</tr>
<tr>
<td>MgATP vs. NH₄⁺</td>
<td>HCO₃⁻, 1 mM HCO₃⁻</td>
<td>intersecting</td>
<td>0.21 ± 0.07 130 ± 40</td>
</tr>
<tr>
<td>HCO₃⁻ vs. Gln</td>
<td>MgATP, 5 mM MgATP</td>
<td>parallel</td>
<td>2.0 ± 0.1 0.16 ± 0.1</td>
</tr>
<tr>
<td>HCO₃⁻ vs. NH₄⁺</td>
<td>MgATP, 10 mM MgATP</td>
<td>intersecting</td>
<td>2.3 ± 0.5 170 ± 30</td>
</tr>
</tbody>
</table>

*a pH 7.5, 25 °C, 100 mM KCl, 15 mM excess Mg²⁺. App, apparent.

TABLE II: Product Inhibition Constants.  

<table>
<thead>
<tr>
<th>variable substrate</th>
<th>inhibitor</th>
<th>fixed substrate (mM)</th>
<th>inhibition</th>
<th>Kᵢ (mM)</th>
<th>Kₐ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgATP</td>
<td>phosphate</td>
<td>MgATP, 0.05</td>
<td>NC</td>
<td>67 ± 10</td>
<td>77 ± 17</td>
</tr>
<tr>
<td>HCO₃⁻</td>
<td>phosphate</td>
<td>MgATP, 0.10</td>
<td>UC</td>
<td>129 ± 6</td>
<td></td>
</tr>
<tr>
<td>HCO₃⁻</td>
<td>phosphate</td>
<td>MgATP, 0.05</td>
<td>UC</td>
<td>12.2 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>MgATP</td>
<td>carbamoyl-P</td>
<td>HCO₃⁻, 1.0</td>
<td>UC</td>
<td>11.1 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>HCO₃⁻</td>
<td>carbamoyl-P</td>
<td>HCO₃⁻, 1.0</td>
<td>UC</td>
<td>13 ± 1</td>
<td></td>
</tr>
<tr>
<td>NH₄⁺</td>
<td>carbamoyl-P</td>
<td>MgATP, 2.0</td>
<td>UC</td>
<td>2.1 ± 0.1</td>
<td>0.15 ± 0.01</td>
</tr>
<tr>
<td>MgATP</td>
<td>MgADP</td>
<td>MgATP, 0.05</td>
<td>NC</td>
<td>0.9 ± 0.1</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>HCO₃⁻</td>
<td>MgADP</td>
<td>MgATP, 0.10</td>
<td>NC</td>
<td>2.6 ± 0.1</td>
<td>11 ± 2</td>
</tr>
<tr>
<td>NH₄⁺</td>
<td>MgADP</td>
<td>MgATP, 1.33</td>
<td>NC</td>
<td>0.99 ± 0.07</td>
<td></td>
</tr>
<tr>
<td>NH₄⁺</td>
<td>MgADP</td>
<td>MgATP, 5.00</td>
<td>UC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a pH 7.5, 100 mM KCl, 25 °C. Kᵢ, slope inhibition constant; Kₐ, intercept inhibition constant; C, competitive inhibition; NC, noncompetitive inhibition; UC, uncompetitive inhibition.

conditions described. At 10 mM ATP, at least 5 mM excess Mg²⁺ is required for maximal activity (Figure 1). With Mn²⁺ as the divalent metal, maximal activity is attained at a concentration of Mn²⁺ approximately equivalent to the ATP concentration. At this concentration of Mn²⁺, the proposed free divalent metal ion site must be tighter with Mn²⁺ than with Mg²⁺ because Mn²⁺ and Mg²⁺ have about equal affinities for ATP (Sillen & Martell, 1971). In preliminary binding studies with Mn²⁺ using EPR techniques, we find that carbamoyl-phosphate synthetase binds 1 mol of Mn²⁺ with a dissociation constant of approximately 40 μM, and there are also a number of other much weaker sites. The tight site is assumed to be the divalent cation site needed for activity, and the weaker sites are those that cause inhibition at higher concentrations of Mn²⁺. The specific function of this free metal ion site has not been determined, nor has the subunit to which it binds. The presence of a free divalent metal ion binding site in addition to a MgATP binding site has also been demonstrated for pyruvate kinase (Gupta et al., 1976), glutamine synthetase (Hunt et al., 1975), and PEP-carboxy kinase (Foster et al., 1967). A requirement for excess Mg²⁺ for maximum activity of carbamoyl-phosphate synthetase from other sources has been reported (Elliott & Tipton, 1974b; Kerson & Appel, 1968) and similar preliminary observations have been noted with the E. coli enzyme (Anderson, 1977; Powers & Meister, 1978).

When the excess Mg²⁺ concentration is maintained at 15 mM, the double-reciprocal plot for ATP is linear down to at least 10 μM either at saturating or nonsaturating levels of HCO₃⁻. Earlier reports of nonlinear reciprocal plots (Anderson & Meister, 1966) are probably due to inadequate Mg²⁺ levels to saturate both the enzyme and ATP. Thus, inhibition by free ATP may have produced the observed nonlinearity. Since two molecules of ATP are used in the overall reaction, nonlinear reciprocal plots could be expected because of squared terms for ATP in the rate equation unless there is an irreversible step (either product release or saturation of an intervening substrate) between the addition of the two molecules of ATP. Since linear reciprocal plots are obtained, the addition of ATP is not the rate-limiting step.

Kinetic Mechanism. The following kinetic mechanism is consistent with all of the initial velocity and product inhibition data.

By using the rules formulated by Cleland (1963), the reasoning used to derive the above mechanism is as follows:

1. The initial velocity patterns for bicarbonate vs. both MgATP and NH$_4^+$ are intersecting and the MgATP vs. NH$_4^+$ pattern is intersecting at low HCO$_3^-$ but parallel at saturating HCO$_3^-$ levels. These results demonstrate that HCO$_3^-$ binds to the enzyme between the addition of MgATP and NH$_4^+$.

2. The MgATP double-reciprocal plot is linear at both saturating and nonsaturating levels of HCO$_3^-$, indicating that a product release step must separate the addition of the two molecules of MgATP.

3. Phosphate is a competitive inhibitor vs. MgATP and thus binds to the same enzyme form.

4. Phosphate is a noncompetitive inhibitor vs. HCO$_3^-$ at low glutamine and uncompetitive at saturating glutamine indicating that the ammonia derived from glutamine binds between the addition of HCO$_3^-$ and the release of Pi.

5. Carbamoyl phosphate is an uncompetitive inhibitor vs. MgATP, all three substrates indicating that there is a product released immediately before and after the release of carbamoyl phosphate.

6. The inhibition patterns for MgADP are linear noncompetitive vs. MgATP, HCO$_3^-$, and NH$_4^+$, but the NH$_4^+$ vs. MgADP inhibition pattern is uncompetitive if the HCO$_3^-$ level is raised to 50 mM from 1 mM. These results show that the binding of HCO$_3^-$ precedes the binding of ammonia. Since all of the inhibition patterns are linear, the release from the enzyme of the two molecules of MgADP must be separated by release of another product (in this case carbamoyl phosphate).

7. The glutamine vs. MgATP and HCO$_3^-$ initial velocity patterns for the two substrates are both parallel. This is different from the result when ammonia is used. The reason for the parallel patterns is the fact that the binding site for glutamine is on a different subunit than the rest of the reaction and the ammonia must therefore be transferred from one subunit to the other. The parallel pattern results from the relaxation of glutamate before the NH$_3$ reacts at the other subunit. This situation is similar to the biotin enzymes which have a two site ping-pong mechanism (Northrop, 1969; McClure et al., 1971). In this case ammonia is being transferred instead of carboxy biotin. The results with glutamine are consistent with the initial velocity data using ammonia as a substrate if it is proposed that the ammonia is not transferred until ATP and HCO$_3^-$ have reacted on the other subunit. The rate equation for the proposed kinetic mechanism using NH$_3$ as the nitrogen source has been derived using the method of King & Altman (1956). The predicted initial velocity and product inhibition patterns from the derived equation agree with the experimental patterns. The initial velocity and product inhibition patterns for the particular mechanism have also been derived by Elliot & Tipton (1974a). The proposed kinetic mechanism is consistent with the three partial reactions catalyzed by carbamoyl-phosphate synthetase (Anderson & Meister, 1966).

\[
\text{MgATP} + \text{HCO}_3^- \rightarrow \text{MgADP} + \text{Pi} \quad (10)
\]

\[
\text{carbamoyl-P} + \text{MgADP} \rightarrow \text{MgATP} + \text{HCO}_3^- + \text{NH}_3 \quad (11)
\]

\[
\text{glutamine} \rightarrow \text{glutamate} + \text{NH}_3 \quad (12)
\]

The strong inhibition of carbamoyl-phosphate synthetase by diadenosine pentaphosphate (AP$_5^-$A) (Powers et al., 1977) suggests two ATP sites and this is confirmed by the above kinetic mechanism. Also consistent with the existence of two ATP sites is an experiment (P. M. Anderson, unpublished experiment) that shows that the ATPase and carbamoyl phosphate synthetase reactions can be inhibited under certain conditions with Ellman's reagent, while the ATP synthesis reaction is increased almost twofold.

A recent pulse-chase type experiment has been reported by Powers & Meister (1978) which indicates that at high ATP concentrations (14 mM) it is possible to populate both ATP sites before the addition of ammonia. However, in those experiments it cannot be determined whether P$_i$ has left the enzyme surface before the addition of the second ATP (with bound CO$_2^-$ as the activated form of HCO$_3^-$). It has been suggested by Sauer et al. (1975) that bound CO$_2^-$, rather than carboxy phosphate, is the intermediate that reacts with the ammonia to form carbamate.

An alternative explanation is that the mechanism is partially random at the point of addition of the two molecules of ATP. However, the pathway in which both ATP molecules bind before NH$_3$ cannot be predominant because there is no evidence for nonlinearity in the MgATP double reciprocal plots.

It should also be mentioned that the pulse chase experiments of Powers and Meister were conducted in phosphate buffer and at low Mg$^{2+}$ levels, while the experiments reported here were done in Hepes buffer, 15 mM excess Mg$^{2+}$, and 10 mM ornithine. All of these reagents are known to affect the binding of ATP to carbamoyl-phosphate synthetase. It is therefore possible for each pathway to predominate under different reaction conditions.

The proposed mechanism in this paper is different from the one suggested by Elliot & Tipton (1974b,c). They have proposed the ordered addition of ATP, HCO$_3^-$, ATP, and NH$_3$ followed by the ordered release of carbamoyl phosphate, ADP, ADP, and P, for the beef liver enzyme. Their mechanism is based in part on a nonlinear reciprocal plot for MgATP at low bicarbonate and nonlinear inhibitions by MgADP. These results are not seen with E. coli enzyme. Since their results were obtained with a free Mg$^{2+}$ concentration of 1 mM, the observed nonlinear plots could be due to inhibition effects by uncomplexed nucleotides and/or nonsaturation of the free metal ion site. In addition, their mechanism does not account for the synthesis of ATP from ADP and carbamoyl phosphate in the absence of P$_i$; since P$_i$ is proposed to be the last product to leave the enzyme in the forward reaction. (It must therefore be the first to add in the reverse reaction.) However, these enzymes are from different sources and they have been shown to be different in many respects. Therefore, although they catalyze the same reaction, it is quite possible that the liver enzyme and the E. coli enzyme have different kinetic mechanisms.

In summary E. coli carbamoyl-phosphate synthetase has been shown to require free divalent metal ions for full activity. The initial velocity and product inhibition patterns are consistent with the ordered addition of MgATP, HCO$_3^-$, and NH$_3$ followed by the release of P$_i$. The second molecule of MgATP adds to the enzyme which is followed by the ordered release of MgADP, carbamoyl phosphate, and MgADP. With glutamine as the nitrogen source, the data are consistent with a two-site mechanism in which glutamine binds randomly to the small subunit, glutamate is released, and NH$_3$ is transferred to the large subunit after the reaction of MgATP and HCO$_3^-$.

References

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

Roderick S. Mulvey* and Alan R. Fersht*

ABSTRACT: The rate of formation of the product Met-tRNAMet, 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 tRNAMet, 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 tRNAMet. Under all conditions, this rate constant was identical to the initial rate of charging of tRNAMet in the steady-state phase (for example: 2.3 s⁻¹ at pH 7.78 with tRNAMetM). 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 tRNAMet 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 tRNAMetM). The kcat 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).

† From the MRC Laboratory of Molecular Biology, Cambridge CB2 2QH, England. Received May 12, 1978; revised manuscript received August 8, 1978.

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 tRNAMet 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 (Hyaff 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.