Serine activation is the rate limiting step of tRNA\textsuperscript{Ser} aminoacylation by yeast seryl tRNA synthetase

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ABSTRACT

Using the quenched flow technique the mechanism of seryl tRNA synthetase action has been investigated with respect to the presteady state kinetics of individual steps. Under conditions where the strong binding sites of the enzyme are nearly saturated and the steady state turnover number is about 1 s\textsuperscript{-1}, rate constants of four different processes have been determined: steps connected with substrate associations are relatively slow (12 s\textsuperscript{-1} for the entire process); activation of serine is the rate determining step (about 1.2 s\textsuperscript{-1} in presence of tRNA\textsuperscript{Ser}); whereas the transfer of serine onto tRNA\textsuperscript{Ser} (35 s\textsuperscript{-1}) and the dissociation of seryl tRNA\textsuperscript{Ser} (70 s\textsuperscript{-1}) are fast. Similar kinetic parameter seem to hold also for the steady state reactions. This conclusion is based on a detailed study of the substrate, product, and Mg\textsuperscript{2+} concentration dependence of the transfer reaction. The results also indicate that a second serine binding site is operative. Since the transfer of serine from a preformed adenylate complex onto tRNA\textsuperscript{Ser} is fast, seryl adenylate seems to be a kinetically competent intermediate of the aminoacylation reaction although, of course, alternative mechanisms cannot be excluded.

INTRODUCTION

The rates of individual steps of an enzymatic reaction mechanism can be investigated by presteady state kinetics. For such studies the enzyme generally is used in high concentrations and special techniques for analyzing fast reactions are needed. The quenched flow technique which consists of rapid mixing of the reactants and rapid termination of the reaction by addition e.g. of acid has the advantage that reaction products can be directly analyzed. This technique has been introduced into the study of the mechanism of aminoacyl tRNA synthetase action by Fersht et al. (2) who also applied it to a number of synthetases (e.g. 3-5). On the basis of the rates of amino acid activation
and transfer onto tRNA, they concluded that a stepwise mechanism of aminoacylation of tRNA including the aminoacyl adenylate as an obligatory intermediate is valid. Using the same technique Lagerkvist and coworkers (6) qualitatively compared the pre-steady state kinetics of free synthetase, substrate bound enzyme, and activated complex for several systems. They emphasized that the data were only suggestive but not conclusive for a stepwise reaction mechanism.

The mechanism of seryl tRNA synthetase action has been investigated for a number of years in several laboratories including ours (for references see for instance 7,8). Several questions concerning the rates of individual reactions and the localization of the rate limiting step, however, remained open. They now have been attacked by quenched flow experiments which were designed to analyze the early phase of the first turnover of seryl tRNA synthetase.

MATERIALS AND METHODS

Materials and methods are as described elsewhere (7,8) except for the following. Inorganic pyrophosphatase was obtained from Boehringer Mannheim (Mannheim). [γ-32P]ATP was purchased from the Radiochemical Centre (Amersham), [32P]PPi and [8-14C]ATP were from New England Nuclear (Boston). The latter one was further purified on DEAE-Sephacel (9) immediately before use in order to lower the contamination with [8-14C]AMP to less than 0.05 %. Nitrocellulose membrane filters were from Sartorius (Göttingen), DEAE-Sephacel was from Pharmacia (Uppsala), and PEI-cellulose from Merck (Darmstadt).

The buffer system throughoutly used in this study consisted of 50 mM Tris-HCl, pH 7.0, 1 mM reduced glutathione, 1 mM potassium cacodylate, 0.7 mM MgCl2, 0.15 mM EDTA, 1 mg/ml bovine serum albumine, and 2 % glycerol. The MgCl2 concentration was optimized for high steady state rate of aminoacylation at the various ATP and KCl concentrations. The quenched flow experiments were performed at 21° C, all other experiments at 25° C.

For incubation times of 5 to 350 ms a quenched flow device (10) was used which allowed to vary the incubation time by
variation of the flow rate at constant reaction volume. The re-
action was started by rapid mixing of enzyme and substrates
(300 µl from each) and was quenched by an excess of 5 % tri-
chloroacetic acid. The amount of $[^{14}\text{C}]$seryl tRNA$_{\text{Ser}}$ was deter-
mined as described in (11).

Hydrolysis of [$\gamma$-$^{32}\text{P}$]ATP and [$^{32}\text{P}$]PP$_i$-ATP exchange were
measured analogously to (12). In the latter experiments the
MgCl$_2$ concentration was optimized for high exchange rate at the
various PP$_i$ concentrations, e.g. 8 mM at 5 mM ATP and 2 mM PP$_i$.

The amount of synthetase bound seryl AMP was determined
using essentially the nitrocellulose membrane filter test as
described in (12). 20 mM Tris-HCl, pH 7.0, 20 mM EDTA, 1 mM
reduced glutathione, and 0.5 M KCl were found suitable to
stabilize the activated complex and therefore were used to
quench the activation reaction. The significance of the assay
was verified by a number of controls: (a) within the incubation
time activation was finished, the yield of filter bound $[^{14}\text{C}]$
serine did not change for more than $±$ 10 % during 1 h of incu-
bation; (b) the activated complex was stable in the quenching
buffer for at least 2 h at 0° C; (c) the dependence of the
amount of filter bound serine on the amount of synthetase was
linear up to 1 nmole enzyme; 95 $±$ 10 % of the amount of activa-
ted serine as measured by the presteady state transfer reaction
were retained on the filter; only blank values of radioactivity
were detected on a second filter.

Presteady state activation of serine in presence of tRNA$_{\text{Ser}}$
was measured using the quenched flow apparatus and 1 M formic
acid for terminating the reaction. The samples were poured onto
2 ml PEI-cellulose columns. Upon washing with 1 M formic acid
(13) only $[^{14}\text{C}]$AMP together with seryl $[^{14}\text{C}]$AMP, but not $[^{14}\text{C}]$
ATP, was eluted.

RESULTS

In a first set of quenched flow experiments the presteady
state kinetics of tRNA$_{\text{Ser}}$ aminoacylation have been studied by
rapid mixing of seryl tRNA synthetase with a mixture of ATP,
serine, and tRNA$_{\text{Ser}}$. Measurable amounts of seryl tRNA$_{\text{Ser}}$ were
Figure 1. Presteady state kinetics of seryl tRNA synthetase indicate the existence of slow steps within the first reaction cycle prior to the dissociation of seryl tRNA\textsuperscript{Ser}. Using the quenched flow apparatus equal volumes of seryl tRNA synthetase (6 \mu M) were rapidly mixed with a mixture of tRNA\textsuperscript{Ser} (42 \mu M), \[^{14}\text{C}\]serine (1 mM), ATP (10 mM), and MgCl\textsubscript{2} (10 mM) (▲). In the other experiments enzyme-substrate complexes were formed by a 10 min preincubation at 21°C and then combined with the other constituents: synthetase (6 \mu M) preincubated with \[^{14}\text{C}\]serine (0.5 mM) and tRNA\textsuperscript{Ser} (21 \mu M) was mixed with ATP/MgCl\textsubscript{2} (10 mM), \[^{14}\text{C}\]serine (0.5 mM), and tRNA\textsuperscript{Ser} (21 \mu M) (▲); synthetase (6 \mu M) preincubated with ATP/MgCl\textsubscript{2} (5 mM) and tRNA\textsuperscript{Ser} (21 \mu M) (●); synthetase (6 \mu M) preincubated with ATP/MgCl\textsubscript{2} (5 mM) and tRNA\textsuperscript{Ser} (21 \mu M) (□); synthetase (6 \mu M) preincubated with ATP/MgCl\textsubscript{2} (5 mM) and tRNA\textsuperscript{Ser} (21 \mu M) (○).

formed only after a lag phase of about 30 ms. The rate of amidocacylation then increased until, after a total of about 150 ms, the steady state rate was reached (Fig. 1). Preincubation of the synthetase with substrates in all non-productive combinations resulted in a significant shortening of the lag phase (Fig. 1), whereas the general shape of the time curves remained similar. Preincubation of the synthetase with ATP, with ATP and tRNA\textsuperscript{Ser}, and with serine and tRNA\textsuperscript{Ser} resulted in lag phases of 10, 15, and 25 ms, respectively. The time curves observed on preincubation of seryl tRNA synthetase with serine or tRNA\textsuperscript{Ser} alone (not shown) were indistinguishable from that of the experiment with both substrates together. The effect of the preincubation of the enzyme with ATP was not due to the Mg\textsuperscript{2+} content of the ATP solution, since preincubation with MgCl\textsubscript{2} alone did not shorten the lag phase significantly. The occurrence of a lag phase in formation of seryl tRNA\textsuperscript{Ser} indicates the existence of slow pro-
cesses within the reaction sequence up to the state where seryl tRNA\textsuperscript{Ser} is synthesized. Apparently some of them take place during preincubation of the synthetase with substrates and therefore may be related to substrate binding.

The experimental scatter was about ±3 % for values of a single experiment using identical solutions of enzyme and substrates. A certain shape of a time curve or a difference between two curves was considered significant if it was reproduced at least twice in independent complete series of preincubation experiments.

The calculation of presteady state reaction rates had to be based on a model of the reaction cycle. A suitable minimal model (Fig. 2) had to contain three functional states of the synthetase: C\textsubscript{1} for the free enzyme or the preformed enzyme substrate complex; C\textsubscript{2} for the reactive synthetase completely associated with all substrates; C\textsubscript{3} for the complex of enzyme and products immediately after the formation of seryl tRNA\textsuperscript{Ser}. k\textsubscript{1} represents the slow processes related to substrate binding and k\textsubscript{2} refers to the rate limiting step of the productive reaction. The two steps are followed by the steady state reaction (k\textsubscript{8}). From the differential equations describing this model an expression for the time dependent concentration of seryl tRNA\textsuperscript{Ser} was derived (see Appendix). Using this expression for curve fitting, optimized values of the rate constants k\textsubscript{1}, k\textsubscript{2}, and k\textsubscript{8} were obtained (Table 1). The calculated values for

![Figure 2. The reaction model used for calculation of rate constants of the presteady state reactions. Since the lag phase could not be fitted reasonably with a single rate constant, two constants were applied which yielded a satisfactory fit (see table 1).](image)
Table 1. Quantitative evaluation reveals that two slow processes are involved in the pre-steady state reaction, which differ by their dependence on substrate preincubation of the synthetase.

<table>
<thead>
<tr>
<th>Preincubation mixture (C₁)</th>
<th>Amplitude (%)</th>
<th>( k₁ (s^{-1}) )</th>
<th>( k₂ (s^{-1}) )</th>
<th>( k₈ (s^{-1}) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>enzyme (E)</td>
<td>98 ± 5</td>
<td>12 ± 2</td>
<td>1.2 ± 0.15</td>
<td>1.05 ± 0.15</td>
</tr>
<tr>
<td>E, serine</td>
<td>97 ± 4</td>
<td>16 ± 2</td>
<td>1.17 ± 0.1</td>
<td>1.03 ± 0.1</td>
</tr>
<tr>
<td>E, tRNA&lt;sub&gt;Ser&lt;/sub&gt;</td>
<td>96 ± 4</td>
<td>17 ± 2</td>
<td>1.17 ± 0.1</td>
<td>0.96 ± 0.1</td>
</tr>
<tr>
<td>E, serine, tRNA&lt;sub&gt;Ser&lt;/sub&gt;</td>
<td>95 ± 2</td>
<td>17 ± 1</td>
<td>1.19 ± 0.05</td>
<td>0.98 ± 0.15</td>
</tr>
<tr>
<td>E, ATP, tRNA&lt;sub&gt;Ser&lt;/sub&gt;</td>
<td>94 ± 5</td>
<td>45 ± 5</td>
<td>1.2 ± 0.15</td>
<td>0.96 ± 0.15</td>
</tr>
<tr>
<td>E, KCl</td>
<td>98 ± 3</td>
<td>15 ± 3</td>
<td>2.0 ± 0.25</td>
<td>1.65 ± 0.2</td>
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</tbody>
</table>

Mean values of at least two, often three and more independent experiments are given. Experimental conditions are the one of Fig. 1 except for the last experiment, where 150 mM KCl and 1 mM MgCl₂ have been added. The amplitudes of Ser-tRNA formation in the model considerations are given in percent of the synthetase concentration (7). Rate constants \( k₁, k₂, \) and \( k₈ \) see text and Fig. 2.

\( k₁ \) and \( k₂ \) differed by one order of magnitude and by their dependence on substrate preincubation of the synthetase. The value of 12 s\(^{-1}\) for \( k₁ \) obtained with free enzyme increased on preincubation of the synthetase with substrates up to 45 s\(^{-1}\). Therefore this process seems to be composed of several substrate association steps or reactions induced by substrate binding. The latter assumption was favored on the basis of results from quenched flow experiments with free synthetase, in which the ATP concentration was 250 μM at 1.7 mM MgCl₂. Under this condition the population of reactive enzyme substrate complexes was diminished according to the ATP \( K_M \) value of 1.6 mM (8), but the value of \( k₁ \) (12 s\(^{-1}\)) remained unchanged (not shown). \( k₁ \) apparently represents first order structural changes of the synthetase induced by binding of ATP and possibly of the other substrates. The rate constant \( k₂ \) (1.2 s\(^{-1}\)) was independent of substrate preincubations but depended on the salt concentration (Table 1). It probably represents the rate limiting step of tRNA<sub>Ser</sub> aminoacylation. The steady state rate of aminoacylation under these conditions was 1.1 ± 0.1 s\(^{-1}\) as determined by parallel steady state experiments.

In order to localize the rate determining step within the
productive reaction more precisely, the transfer of preactivated serine onto tRNA\textsuperscript{Ser} was studied separately. The synthetase was incubated with ATP and serine prior to rapid mixing with tRNA\textsuperscript{Ser}. Within the first 100 ms about 0.4 mole seryl tRNA\textsuperscript{Ser} per mole synthetase were accumulated; then the rate of aminoacylation declined and reached the steady state value (Fig. 3). The stoichiometry of transferred serine increased to 0.9 mole per mole synthetase in the presence of inorganic pyrophosphatase (Fig. 3). A quantitative evaluation according to the reaction model

\[
E\cdot\text{Ser} \text{ AMP} + tRNA_{\text{Ser}}^{\text{preact}} \xrightarrow{k_{TR}} \text{products}
\]

\[
E\cdot\text{Ser} \text{ tRNA}_{\text{Ser}}^{\text{preact}} \cdot \text{AMP} \xrightarrow{k_S} \text{substrates}
\]

revealed optimized values for the rate constants of the transfer (k\textsubscript{TR}) and the steady state reaction (k\textsubscript{S}) (Table 2). It was concluded that the transfer of serine from a preformed activated complex onto tRNA\textsuperscript{Ser} is about 30 times faster than the steady state aminoacylation. Apparently the transfer reaction is not

Figure 3. Presteady state transfer of activated serine onto tRNA\textsuperscript{Ser} is fast compared to the steady state aminoacylation. Seryl tRNA synthetase (6 μM) was preincubated with [\textsuperscript{14}C]serine (0.5 mM) and ATP/Mg\textsubscript{2}Cl\textsubscript{2} (5 mM) and then mixed in the quenched flow apparatus with an equal volume of tRNA\textsuperscript{Ser} (42 μM), [\textsuperscript{14}C]serine (0.5 mM), and ATP/Mg\textsubscript{2}Cl\textsubscript{2} (5 mM) (•). The same experiment was repeated in the presence of 3 U/ml pyrophosphatase (○). In two chase experiments the synthetase either was preincubated with ATP/Mg\textsubscript{2}Cl\textsubscript{2} (5 mM) and [\textsuperscript{14}C]serine (0.5 mM), but was then mixed with tRNA\textsuperscript{Ser} (42 μM), ATP/Mg\textsubscript{2}Cl\textsubscript{2} (5 mM), and unlabeled serine (50 mM) (●), or the preincubation was carried out with unlabeled serine (0.5 mM) and [\textsuperscript{14}C]serine (0.5 mM) was used for the chase (□).
Table 2. The presteady state transfer reaction proceeds with a rate constant of 35 s\(^{-1}\) as revealed by quantitative evaluation of the time curves of Fig. 3.

<table>
<thead>
<tr>
<th>Transfer experiment</th>
<th>Amplitude (%)</th>
<th>(k_{Tr} (s^{-1}))</th>
<th>(k_b (s^{-1}))</th>
<th>Amplitude (X k_b (s^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>no addition</td>
<td>45 (\pm) 4</td>
<td>35 (\pm) 3</td>
<td>2.4 (\pm) 0.2</td>
<td>1.05 (\pm) 0.15</td>
</tr>
<tr>
<td>chase exp.</td>
<td>46 (\pm) 3</td>
<td>32 (\pm) 3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PPase</td>
<td>90 (\pm) 6</td>
<td>36 (\pm) 1</td>
<td>1.2 (\pm) 0.1</td>
<td>1.04 (\pm) 0.15</td>
</tr>
<tr>
<td>PP(_i)</td>
<td>9 (\pm) 5</td>
<td>35 (\pm) 3</td>
<td>8.7 (\pm) 0.5</td>
<td>0.8 (\pm) 0.3</td>
</tr>
<tr>
<td>KCl</td>
<td>45 (\pm) 3</td>
<td>39 (\pm) 3</td>
<td>3.6 (\pm) 0.4</td>
<td>1.65 (\pm) 0.3</td>
</tr>
</tbody>
</table>

Enzyme and substrate concentrations were as given in Fig. 3 except for the last two experiments where 0.2 mM PP\(_i\) has been added in the one and 150 mM KCl together with 1 mM MgCl\(_2\) in the other experiment.

rate limiting in seryl tRNA\(^{Ser}\) formation.

Additional controls confirmed this conclusion. The preincubated serine was shown to be in an activated state by a number of experiments: enzyme dependent retention of serine on nitrocellulose filters, ATP hydrolysis, isotopic PP\(_i\)-ATP exchange, and seryl AMP or AMP formation (Methods) were observed under the preincubation conditions (data not shown). The preferential usage of the preactivated serine in the transfer reaction was tested by two chase experiments, in which either the serine used for preincubation or the one used for chasing was radioactive. Only serine from the preincubation mixture was seen to be transferred onto tRNA\(^{Ser}\) during the first turnover (Fig. 3). Moreover, the increase in the amount of transferred serine caused by pyrophosphatase indicated that activation of serine is a prerequisite of the transfer reaction. It remained not understood, however, why the presence of pyrophosphatase influenced the transfer reaction to such a great extent. A \(K_i\) value of 0.9 mM for PP\(_i\) was obtained by steady state aminoacylation studies (8), whereas the PP\(_i\) concentration in the present transfer experiments was about 10 \(\mu\)M due to an 0.08 % contamination of ATP and to the PP\(_i\) release during the preactivation step.

In order to clarify this discrepancy the influence of PP\(_i\)
on the activation and the transfer reaction was studied. \[^{14}\text{C}\] serine activation in the absence of tRNA\textsuperscript{Ser} was measured by the nitrocellulose filter binding test (Methods). It was found that the amount of activated serine depends on the PP\textsubscript{i} concentration according to a $K_D$ value of 25 $\mu$M for PP\textsubscript{i}. Practically the same dependence on PP\textsubscript{i} was observed for the amount of transferred serine. Likewise in \[^{32}\text{P}\]PP\textsubscript{i}-ATP exchange experiments in the absence of tRNA a $K_M$ value of 20 $\mu$M for PP\textsubscript{i} was measured, whereas the $K_M$ value was about 0.6 mM in presence of tRNA\textsuperscript{Ser} (all results not shown). Therefore it was concluded that PP\textsubscript{i} binds with high affinity to the activated complex when tRNA\textsuperscript{Ser} is absent. This explains the stimulating effect of pyrophosphatase. The affinity of PP\textsubscript{i} was not changed by addition of tRNA\textsuperscript{Ser}, at least not during the transfer reaction. Only in the following turnover the affinity was diminished to the steady state $K_i$ value as indicated by the optimized $k_i$ values of Table 2. Apparently the presence of either tRNA\textsuperscript{Ser} or seryl tRNA\textsuperscript{Ser} during the activation reaction influences the dissociation of PP\textsubscript{i}.

The finding of a slow process in presteady state kinetics starting with free enzyme and of a fast transfer of serine from the activated complex onto tRNA\textsuperscript{Ser} allow the conclusion that reactions subsequent to the transfer step (e.g. the dissociation of charged tRNA) are not rate limiting. Therefore the slow process must precede the transfer reaction and may be located in the serine activation reaction. A minimum estimation of the rate of serine activation was obtained from \[^{32}\text{P}\]PP\textsubscript{i}-ATP exchange kinetics which at the substrate concentrations used throughout revealed a turnover number of about 5 s\(^{-1}\) in the absence of tRNA\textsuperscript{Ser} and about 0.7 s\(^{-1}\) in the presence of tRNA\textsuperscript{Ser}. More directly the rate of serine activation under aminoacylation conditions was measured by the following presteady state experiments. A straight line was obtained, when \[^{32}\text{P}\]ATP hydrolysis was measured for the first two reaction cycles (Fig. 4A). From the absence of an initial burst of \[^{32}\text{P}\]PP\textsubscript{i} formation it was concluded that activation of serine was not substantially faster than the overall aminoacylation reaction. This was confirmed by quenched flow experiments measuring the hydrolysis of \[^{14}\text{C}\]ATP. A lag phase in the formation of seryl
Figure 4. Serine activation is the rate limiting step in the presteady state formation of seryl tRNA^Ser. Activation of serine under aminoacylation conditions was measured by two experimental techniques. (A) Release of $[^{32}P]PP_i$ from $[^{32}P]ATP$. The synthetase (6 μM) was mixed with an equal volume of tRNA^Ser (42 μM), serine (1 mM), $[^{32}P]ATP$ (0.3 mM), and MgCl$_2$ (2 mM). (B) Formation of seryl $[^{14}C]$AMP and $[^{14}C]$AMP. Using the quenched flow apparatus the synthetase (6 μM) was mixed with tRNA^Ser (50 μM), serine (1 mM), $[^{14}C]$ATP (0.3 mM), and MgCl$_2$ (2 mM) (●). The experiment was repeated, but now the synthetase was preincubated with $[^{14}C]$ATP (0.3 mM) and MgCl$_2$ (1.3 mM) prior to mixing with tRNA^Ser (50 μM), serine (1 mM), $[^{14}C]$ATP (0.3 mM), and MgCl$_2$ (1.3 mM) (○).

AMP or AMP was observed (Fig. 4B). Using the same set of rate constants as in Table 1 and assuming a reaction amplitude according to the $K_M$ value of ATP, the calculated time curves approached the experimental points. These results clearly demonstrate that serine activation or a step preceding it is the rate determining reaction of the presteady state formation of seryl tRNA^Ser.

In order to test the agreement of the presteady state kinetics with the known steady state kinetic data, the presteady state transfer reaction was characterized with respect to its dependence on substrate, product, and salt concentrations. The amount of transferred serine depended on tRNA^Ser concentration.
according to a $K_D$ of 0.59 $\mu$M and a Hill coefficient of 1 in the presence of 5 mM ATP (Fig. 5). ATP binding was antagonistic to tRNA$^{Ser}$ association: at 25 mM ATP the $K_D$ value was enhanced to 3 $\mu$M, whereas at 100 $\mu$M ATP it was below 0.1 $\mu$M in agreement with earlier results obtained from fluorescence titration experiments (14). The transfer rate constant did not depend on tRNA$^{Ser}$ concentration. This result clearly proves that tRNA$^{Ser}$ association is not rate limiting for the transfer reaction.

Surprisingly it was found that the transfer rate depends on serine concentration (Fig. 6). $k_{Tr}$ was $18 \text{ s}^{-1}$ for serine concentrations below 20 $\mu$M, whereas at higher serine concentrations it was enhanced up to $40 \text{ s}^{-1}$ according to a $K_D$ value of 0.2 mM with a Hill coefficient of 1. The amount of activated as well as transferred serine, however, depended on serine concentration according to a $K_D$ value of 40 $\mu$M with a Hill coefficient of 1. These results demonstrate that two serine binding sites are involved in the transfer reaction. At one site serine is activated and transferred onto tRNA$^{Ser}$, whereas the occupation of the second site modulates the rate of the transfer reaction.

A complex relation between the ATP concentration and the amount of transferred serine was observed as to be expected because of the PP$\_i$ contamination of ATP, because of the influence of ATP on tRNA binding (Fig. 5), and because of the various

**Figure 5.** tRNA$^{Ser}$ association is not rate limiting for the transfer reaction. The transfer rate constant ($k_{Tr}$) and the amount of transferred serine as obtained by quantitative evaluation of the pre-steady state transfer experiment were determined at the various tRNA$^{Ser}$ concentrations for three different ATP concentrations: 25 mM ATP/MgCl$_2$ (●), 5 mM ATP/MgCl$_2$ (■), and 0.1 mM ATP with 1 mM MgCl$_2$ (▲). The synthetase concentration was 3 $\mu$M, the one of [$^{14}$C]serine was 0.5 mM. Curve fitting was done using the Hill equation.
Mg$^{2+}$ effects (7). The transfer rate constant did not depend on ATP within the concentration range from 0.02 to 25 mM (not shown).

If the synthetase was preincubated with ATP, serine, and equimolar concentrations of tRNA$^{\text{Ser}}$ which became aminoacylated during the preincubation period, a lag phase prior to the burst in seryl tRNA$^{\text{Ser}}$ formation was observed upon mixing of the preincubation solution with additional tRNA$^{\text{Ser}}$ (Fig. 7). This
effect was not due to the AMP content of the preincubation mixture since the presence of AMP in concentrations up to 1 mM did not influence the transfer reaction (not shown). It could also not be caused by the presence of PP\textsubscript{i} because pyrophosphatase was added to the preincubation mixture. Besides this PP\textsubscript{i} has already been demonstrated to lower the amount of transferred serine, but no influence on the transfer rate constant could be detected for PP\textsubscript{i} concentrations up to 0.2 mM (Table 2). Therefore it is concluded that the altered transfer kinetic is due to the presence of seryl tRNA\textsuperscript{Ser}. Quantitative evaluation using the two-step model (Fig. 2) resulted in rate constants of 35 s\textsuperscript{-1} and 70 s\textsuperscript{-1}. Apparently the seryl tRNA\textsuperscript{Ser} synthesized during the preincubation period remains bound to the synthetase also during the following activation reaction and becomes displaced only upon binding of uncharged tRNA\textsuperscript{Ser}. The rate constant of 70 s\textsuperscript{-1} obviously represents the dissociation of seryl tRNA\textsuperscript{Ser} which has to occur prior to the transfer reaction (35 s\textsuperscript{-1}).

The Mg\textsuperscript{2+} concentration was found to be a limiting factor of both the amplitude and the rate constant of the transfer reaction (Fig. 8). The dependence of the amount of transferred serine on the Mg\textsuperscript{2+} concentration reflects mainly the one of serine activation and indicates a complex interplay of promoting and inhibiting influences of the Mg\textsuperscript{2+} ion. Optimal yields of activated as well as transferred serine were obtained at a Mg\textsuperscript{2+} concentration (3 mM) somewhat lower than the ATP concentration (5 mM). The Mg\textsuperscript{2+} dependence of the transfer rate constant, however, can be interpreted on the basis of a highly cooperative binding of Mg\textsuperscript{2+} to the reactive complex which reaches saturation at about 5 mM free Mg\textsuperscript{2+}. The transfer rate which was obtained by multiplication of the rate constant with the transfer amplitude qualitatively shows the same dependence on the Mg\textsuperscript{2+} concentration as does the steady state aminoacylation rate (Fig. 8B). The various Mg\textsuperscript{2+} effects are not due to the ionic strength variation since the transfer reaction was affected only slightly by addition of KCl (Table 2).

DISCUSSION

The aim of the present study was to gain information on the
Figure 8. The Mg$^{2+}$ concentration influences both the transfer rate constant and the amount of transferred serine. (A) At various MgCl$_2$ concentrations the amounts of activated serine in the absence (o) or in the presence (△) of pyrophosphatase (3 U/ml) were measured by the nitrocellulose filter binding test; the amounts of transferred serine (●) and the transfer rate constants were obtained by quantitative evaluation of the presteady state transfer experiments. The reaction mixture contained: 3 μM synthetase, 21 μM tRNA$^{\text{Ser}}$ (only in the transfer experiments), 5 mM ATP, and 0.5 mM [14C]serine. (B) The V dependence of the transfer rate (obtained by multiplication of the amount of transferred serine with the transfer rate constant) (●) resembled that of the steady state rate of tRNA$^{\text{Ser}}$ aminoacylation (△).

rates of individual steps within the reaction mechanism of seryl tRNA synthetase. From the presteady state kinetic results conclusions were drawn concerning the rates of four distinct parts of the reaction sequence, namely substrate associations, serine activation, transfer of activated serine onto tRNA$^{\text{Ser}}$, and dissociation of seryl tRNA$^{\text{Ser}}$. Apparently all slow processes within the aminoacylation reaction are observed since in all presteady state experiments more than 90% of the steady state
rate of aminoacylation were reached. There is some experimental
evidence, at least for the activation and the transfer reaction,
that the rate constants of the presteady state reactions are
valid also under steady state conditions.

Since under the substrate and Mg\(^{2+}\) concentrations of Fig. 3
only about 40\% of the synthetase molecules catalyzed a rapid
transfer of preactivated serine onto tRNA\(^{\text{Ser}}\), one may suspect
that 60\% of the synthetase molecules were inactive. The doubl-
ing of the amount of transferred serine upon addition of pyro-
phosphatase would then have to be interpreted by activation of
a second serine induced by the pyrophosphatase. However, results
from experiments on the PP\(_1\) and Mg\(^{2+}\) concentration dependence of
the activation and the transfer reaction argued against this
assumption. The high affinity binding of PP\(_1\) to the activated
complex (see above) partially reverts the activation and, with
that, makes the enzyme appear less active than it is. At some-
what lower Mg\(^{2+}\) concentrations (Fig. 8, 3 mM Mg\(^{2+}\) instead of
the 5 mM Mg\(^{2+}\) in Fig. 3) about 80\% of the synthetase molecules
were active in serine activation and transfer onto tRNA\(^{\text{Ser}}\). Un-
der this condition only a 15\% stimulation of the activity of
the synthetase is observed upon addition of pyrophosphatase.
Therefore it is concluded that the seryl tRNA synthetase prepa-
ration contains at least 90\% active enzyme molecules. The action
of pyrophosphatase or the deficiency of Mg\(^{2+}\) increase the amount
of activated serine most likely by favoring the dissociation of
PP\(_1\) according to E·Ser·ATP → E·Ser·AMP·PP\(_1\) → E·Ser·AMP+PP\(_1\).
It cannot be concluded with certainty, however, that the release
of PP\(_1\) is a prerequisite of the transfer reaction, since on the
basis of the present experiments it is not possible to distin-
guish between the two species of activated complexes.

Earlier studies on the kinetics of partial reactions were
restricted to the association of tRNA\(^{\text{Ser}}\). This process was found
to be composed of a diffusion controlled binding of tRNA\(^{\text{Ser}}\) to
the synthetase and a subsequent slow conformational rearrange-
ment of the synthetase tRNA complex with a rate constant of
30 to 50 s\(^{-1}\) depending on the ionic strength (14). Similar va-
ues for the rate constant of tRNA\(^{\text{Ser}}\) association have now been
derived from the presteady state kinetics of either the synthe-
tase ATP complex (45 s\(^{-1}\)) or the preformed activated complex
(35 s\(^{-1}\)). The latter rate constant was shown not to depend on
the tRNA\(^{\text{Ser}}\) concentration. Apparently the rearrangement of the
synthetase tRNA complex, previously termed "identification step",
also determines the rate of the transfer reaction. The results
of the presteady state kinetic experiments indicate slow re-
arrangement steps also for associations of the other substrates
to the enzyme, the one of ATP association being especially slow
(about 17 s\(^{-1}\)). The release of products, on the other hand, is
relatively fast, at least the one of seryl tRNA\(^{\text{Ser}}\) in the presen-
ce of uncharged tRNA\(^{\text{Ser}}\) (70 s\(^{-1}\)). The finding that seryl tRNA\(^{\text{Ser}}\)
is displaced from the synthetase by the binding of uncharged
tRNA\(^{\text{Ser}}\) can be discussed in terms of the anticooperativity in
tRNA binding known for tRNA\(^{\text{Ser}}\) (15) as well as for other tRNA
synthetase systems (e.g.16). However, further experiments are
needed in the seryl tRNA synthetase system to clarify the kine-
tic meaning of anticooperativity in substrate binding.

The rate limiting step of tRNA\(^{\text{Ser}}\) aminoacylation is located
within the activation reaction. It cannot be decided whether
this step is related to a further conformational change of the
reactive enzyme substrate complex or to the process of chemical
bond breakage and formation. Amino acid activation as the rate
determining step occurs also in a number of other synthetases
studied by Lagerkvist et al. (6). The presteady state time cur-
ves for lysine, phenylalanine, and serine specific synthetases
from E. coli, but not the one for valine tRNA synthetase from
E. coli and lysine tRNA synthetase from yeast, may be inter-
preted in this way. On the other hand, A. Fersht et al. showed
that for the synthetases they used the rate limiting step is
located either in the transfer reaction (synthetases specific
for isoleucine, E. coli (3), methionine, B. stearothermophilus
(17), and phenylalanine, yeast (5)), or in the dissociation of
charged tRNA (arginyl tRNA synthetase, yeast (4)); for one en-
zyme it was reported that both, activation and transfer, con-
tribute to the overall rate limitation (tyrosine tRNA synthe-
tase, E. coli (3)). It is unclear whether the differences in the
location of the rate determining step are due to essential
differences in the catalytic mechanisms. It should be mentioned
that A. Fersht has strongly suggested a stepwise mechanism including the aminoacyl adenylate as a stable intermediate. On the basis of the rate constants of the $\text{PP}_i$-ATP exchange and the transfer reaction, seryl adenylate may be a kinetically competent intermediate of the reaction mechanism. On the other hand, the observation of differences between the activation reactions in the presence and in the absence of $\text{tRNA}^{\text{Ser}}$ indicates that alternative mechanisms cannot be excluded.

Serine was found to occupy more than one binding site also at low concentrations which agrees with the earlier finding of non-linear Lineweaver-Burk plots (18). On the other hand, it was shown in the present study that only one serine molecule becomes activated even in the presence of pyrophosphatase (Fig. 8A). Further investigations are needed to decide whether this is due to a half-of-the-sites reactivity of the synthetase.

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APPENDIX

The quantitative evaluation of the presteady state reactions was based on the reaction model of Fig. 2. This model can be described by four differential equations:

\[
\frac{d[C_1]}{dt} + k_1 [C_1] = 0 \quad (1)
\]

\[
\frac{d[C_2]}{dt} - k_1 [C_1] + k_2 [C_2] = 0 \quad (2)
\]

\[
\frac{d[C_3]}{dt} - k_2 [C_2] = 0 \quad (3)
\]

\[
\frac{d[\text{products}]}{dt} - k_s [C_3] = 0 \quad (4)
\]

Equations (1) and (2) were integrated by usual procedures resulting in expressions for the time dependent concentrations of \([C_1]\) and \([C_2]\):

\[
[C_1]_t = [C_0] \left( \frac{k_2 - \lambda_1}{\lambda_2 - \lambda_1} \cdot e^{-\lambda_1 t} - \frac{k_2 - \lambda_2}{\lambda_2 - \lambda_1} \cdot e^{-\lambda_2 t} \right) \quad (5)
\]

\[
[C_2]_t = [C_0] \left( \frac{k_1}{\lambda_2 - \lambda_1} \cdot e^{-\lambda_1 t} - e^{-\lambda_2 t} \right) \quad (6)
\]

with \(\lambda_{1,2} = 0.5 ((k_1 + k_2) \pm ((k_1 + k_2)^2 - 4 k_1 k_2)^{1/2})\)

and \([C_1] = [C_0], [C_2] = [C_3] = 0\) at \(t = 0\).

Equation (6) was used for integration of equation (3):

\[
[C_3]_t = [C_0] \left( \frac{k_1}{\lambda_2 - \lambda_1} \cdot \frac{\lambda_2 - \lambda_1}{\lambda_1 \cdot \lambda_2} \cdot e^{-\lambda_1 t} + \frac{1}{\lambda_2} \cdot e^{-\lambda_2 t} \right) \quad (8)
\]
Equation (8) describes the formation of product during the first reaction cycle of seryl tRNA synthetase. The product concentration at any time in the pre-steady state as well as in the steady state is given by equation (9) which was obtained by integration of equation (4) using equations (6) and (8):

\[
[\text{products}]_t = [C_0] \left( \frac{k_1 \cdot k_2}{\lambda_2 - \lambda_1} \right) \left( \frac{\lambda_2 - \lambda_1}{\lambda_1 \cdot \lambda_2} \cdot (1 + k_s t) \right) + \left( \frac{k_s}{\lambda_1^2} - \frac{1}{\lambda_1} \right) e^{-\lambda_1 t} - \left( \frac{k_s}{\lambda_2^2} - \frac{1}{\lambda_2} \right) e^{-\lambda_2 t} + \left( \frac{\lambda_1^2 - \lambda_2^2}{\lambda_1 \cdot \lambda_2} \cdot k_s \right)
\]

Equation (9) was used for the determination of optimized values of the rate constants \( k_1 \) and \( k_2 \). On account of the symmetry of this equation the rate constants \( k_1 \) and \( k_2 \) are interchangeable. Additional restrictions (e.g. the dependence on pre-incubation of the synthetase with substrates) were necessary to assign the calculated values of the rate constants to the respective pre-steady state reactions.