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Yeast seryl tRNA synthetase: two sets of substrate sites involved in aminoacylation

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ABSTRACT

Seryl tRNA synthetase from Saccharomyces Carlsbergensis C836 contains two sets of sites for $tRNA^{Ser}$, L-serine, and Mg^{2+}-ATP, both of which are involved in aminoacylation. This is based on the following experimental results: (a) at low serine concentrations, second order kinetics in $tRNA^{Ser}$ are observed; (b) biphasic kinetics result when the amino acid is the varied substrate indicating anticooperative binding of two serine molecules to the synthetase; (c) when two molecules of serine are bound the rate of aminoacylation increases strongly and becomes first order in $tRNA^{Ser}$; (d) the involvement of more than one site for Mg^{2+} and ATP is deduced from systematic variations of the concentrations of Mg^{2+} and ATP. Implications of the anticooperative binding of the substrates for possible reaction mechanisms are discussed. The results indicate that under normal conditions, the activity of seryl tRNA synthetase is regulated mainly by $tRNA^{Ser}$ while at high serine concentrations regulation by the amino acid itself prevails.

INTRODUCTION

The enzymatic aminoacylation of tRNA is a complex process including as essential features the binding of tRNA, amino acid, and Mg^{2+}-ATP to the cognate aminoacyl tRNA synthetase (1,2). The work on seryl tRNA synthetase from yeast has been devoted largely to analyze the binding equilibria and kinetics. It was established that the enzyme contains two binding sites for the tRNA (3,4). The affinities of the two sites differ with respect to their ionic, pH, and ATP dependencies (5,6,7). Two non-equivalent binding sites for $tRNA^{Ser}$ on the synthetase have been found also in ultracentrifugation studies (8). It was concluded from temperature jump relaxation measurements that the first encounter between $tRNA^{Ser}$ and the synthetase is followed by a fast rearrangement of the complex (7,9). An additional slowly

relaxing change of the fluorescence of the enzyme possibly reflects a conformational change of the enzyme-tRNA complex relevant to selection or in the charging of the cognate tRNA (7). The present study contributes to the interpretation of this slow process and to the question of the number of reactive sites on the synthetase.

MATERIALS AND METHODS

Seryl tRNA synthetase was prepared from Saccharomyces Carlsbergensis C836 according to (10) with the alterations described in the following paper (11). No differences were detected between pure fractions from the original and the modified procedure and between one year old and freshly isolated synthetase preparations when they were compared in the experiments of Fig. 3 and 4. Enzyme concentrations were calculated from the absorbance at 280 nm assuming a molecular weight of 120 000 (4,10) and 0.82 mg protein per A_{280} unit (7, 10,12).

$tRNA^{Ser}$ was prepared from brewer's yeast tRNA (Boehringer Mannheim) according to (13). Prior to use the $tRNA^{Ser}$ was incubated for 2 min at 65°C in 5 mM $MgCl_2$, 0.5 mM Tris-HCl, pH 7.0. 1 ml of a solution with an absorption of 1 at 260 nm was assumed to contain 1.4 nmol $tRNA^{Ser}$.

^{14}C-labeled L-serine was used at specific radioactivities between 0.5 and 2.5 Ci/mol. Serine and ATP (Pharma Waldhof, Düsseldorf) solutions were filtered through Millipore filters prior to use.

Unless stated otherwise, assays of the synthetase activity were performed at 25°C in a volume of 100 µl containing 5 µmol Tris-HCl, 2 µmol ATP, 2.5 µmol $MgCl_2$, 0.05 µmol EDTA, 0.5 µmol reduced glutathione, and 2 µg bovine serum albumin, at pH 7.0 ("standard conditions"). Reaction mixtures and enzyme solutions were brought to 25°C prior to the start of the reaction. The reaction was stopped by addition of 1 ml ice-cold trichloroacetic acid followed by 50 µl of bulk RNA (Boehringer Mannheim) containing 100 A_{260} units per ml, in order to ensure complete precipitation of the tRNA. Precipitated radioactivity was determined as described previously (10). Blanks obtained with-

out enzyme were subtracted and only initial rate values were used. When the serine concentration was varied the blanks were measured at the different amino acid concentrations.

For evaluation of the kinetic experiments a non-linear least-squares parameter fitting program was used (14) on a TR440 computer (Leibniz Rechenzentrum, München).

RESULTS

Two reactive sites for Mg^{2+}-ATP. Mg^{2+} stabilizes the tRNA and, at least in some cases, the synthetase conformation. The amino acid activation depends on the presence of Mg^{2+} (1,2,15). Separate binding sites for ATP and the divalent cation exist in a number of synthetases (16). It has been shown that ATP acts with seryl tRNA synthetase as Mg^{2+} complex (17).

In order to discriminate between ATP and Mg^{2+} effects we varied the concentrations of ATP and of Mg^{2+} independently and determined the initial steady state reaction rate employing about 500 different concentrations. The results are mapped in the field of ATP and Mg^{2+} concentrations by connecting conditions of equal activity (see Fig. 1A).

The Mg^{2+} concentration was varied between 1 M and 1 mM, the ATP concentration between 0.1 M and 0.1 µM. The rate of $tRNA^{Ser}$ aminoacylation was between 1.5 and 1.7 mol of product per second per mol of synthetase at Mg^{2+} concentrations between 1 and 15 mM when ATP was present in concentrations between 0.03 and 10 mM and, at higher Mg^{2+} and ATP concentrations when both concentrations were equal. According to the Wilcoxon and the Student tests the activity measured around the border of these two regions is slightly but significantly higher than the activity measurable under the neighbouring conditions.

Regardless of the ATP concentration, the activity decreased steeply when the Mg^{2+} concentration exceeded 150 mM. Starting from the lowest ATP concentrations, appreciable activity was found first at 0.2 µM ATP in the presence of 100 mM $MgCl_2$, and at 20 µM ATP in the presence of 10 mM $MgCl_2$. In the region between 10 µM and 10 mM ATP, and 15 and 150 mM Mg^{2+} the activity stayed at approximately the same level.

The enzyme activity strongly depends on the equilibrium

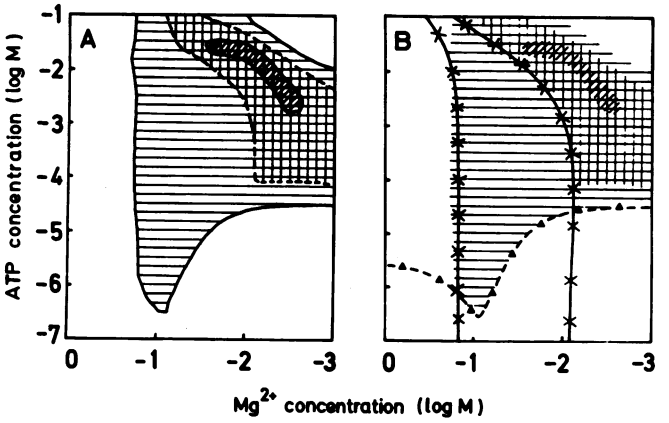

Figure 1. Activity profile of seryl tRNA synthetase in the Mg^{2+}xATP plane. (A) The rate of aminoacylation was measured for 18 $MgCl_2$ concentrations at each of 29 ATP concentrations, under standard conditions. The lines connect coordinate values of average turnover of 0.8 (___), 1.0 (---), and 1.5 s^{-1} (innermost line), respectively. Before constructing the map every experimental value used was converted to the mean value of this point and its immediate neighbours. The different shadings cover the conditions under which the enzyme turnover was between 0.8 and 1.0 (≡), 1.0 and 1.5 (#), and between 1.5 and 1.7 (//) per second, respectively. (B) Comparison of the activity of seryl tRNA synthetase (shaded areas as in (A)) with the concentration of free Mg^{2+} (x-x-). For constructing the lines of Mg^{2+} concentration the affinity constants given in the text are used. The left and right lines (x-x) refer to free Mg^{2+} concentrations of 100 and 7.8 mM, respectively. The curve (▲-▲-) connects conditions of equal reaction rate according to the formula given in the text. The numerical values were $[ATP]=(2.2\times10^{-8}-4.86\times10^{-7}[Mg^{2+}]+3\times10^{-6}[Mg^{2+}]^2)/(7.33\times10^{-4}-8.5\times10^{-3}[Mg^{2+}]+[Mg^{2+}]^2)$.

between different forms of Mg^{2+}-ATP complexes. On the basis of theoretical work (18) we assume that under our conditions the ATP^{4-} and $MgATP^{2-}$ forms prevail. The equilibrium between these forms is characterized by an association constant of about 35 mM^{-1} (19). Using this value we generated in the plane of Mg^{2+} and ATP lines connecting conditions of constant concentrations of free Mg^{2+} (Fig. 1B). For free ATP assuming it to be a competitive inhibitor of $MgATP^{2-}$, an inhibition constant of 0.3 ± 0.15 mM can be derived.

The inhibition by free Mg^{2+} takes place in two stages. Mg^{2+} concentrations of more than about 10 mM limit the molar turnover

to one per second while at concentrations above 100 mM free Mg^{2+} the reaction is strongly or completely inhibited. The Hill plot (Fig. 2) shows that the two concentration ranges of inhibition are well separated; they differ in their Hill coefficients which are 1 for low salt and 2 for the region of high salt concentration. The cooperative inhibition may be caused by nonspecific salt effects of $MgCl_2$ reducing the affinity of the tRNA to the enzyme. In addition, an equilibrium between different conformations of $tRNA^{Ser}$ might be shifted by Mg^{2+}, in analogy to recent observations with $tRNA^{Phe}$ (20).

At a constant rate of aminoacylation the total concentrations of ATP and of Mg^{2+} are related to each other by a function of the type

$$[ATP] = \frac{a_1 + a_2\,[Mg^{2+}] + a_3\,[Mg^{2+}]^2}{a_4 + a_5\,[Mg^{2+}] + [Mg^{2+}]^2}$$

This equation holds with one ATP entering the reaction cycle either as $MgATP^{2-}$ or as Mg_2ATP. The coefficients a_{1-5} are functions of the rate constants in the reaction mechanism and the binding constants for Mg^{2+}, ATP, $MgATP^{2-}$, and Mg_2ATP. By choosing a proper set of values for these coefficients it is possible to obtain a curve following perfectly the observed one (Fig. 1B).

In Lineweaver-Burk plots straight lines were observed when the ATP concentration was varied at Mg^{2+} concentrations between 100 and 150 mM, and below 10 mM. Each of these conditions corresponds to the predominance of one species of Mg^{2+}-complexes because the dissociation constant for Mg_2ATP, and $MgATP^{2-}$ and free Mg^{2+} is near 25 mM under conditions similar to ours (21). As can be deduced from Fig. 1A the presence of 20 mM $MgCl_2$ represents the border of the transition between these two regions. Therefore, the kinetics look complicated at intermediate Mg^{2+} concentrations. The sensitivity of the corresponding kinetic pattern to the tRNA concentration (22) may be caused by competition of the tRNA with ATP for Mg^{2+}.

In conclusion, there exist for Mg^{2+} two (or more) binding sites which influence the aminoacylation of $tRNA^{Ser}$, possibly via different Mg^{2+}-ATP complexes. ATP^{4-}, $HATP^{3-}$, and Mg_2ATP

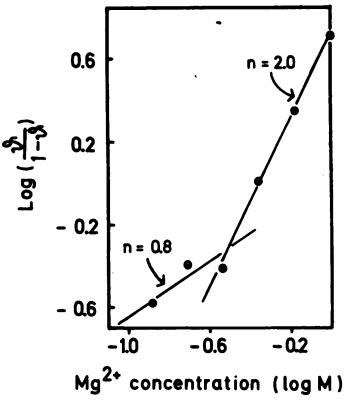

Figure 2. Cooperative inhibition of the activity of seryl tRNA synthetase by high Mg^{2+} concentrations (Hill plot). n is the Hill coefficient, ϑ the normalized extent of inhibition. (⊙) represent mean values for ATP concentrations between 30 mM and 30 µM, as taken from the experiments of Fig. 1.

permit little if any aminoacylation. High enzymatic activity coincides with the presence of $MgATP^{2-}$. Concentrations around 25 mM ATP and 30 mM $MgCl_2$ allow the simultaneous activity of two sites at the enzyme for Mg^{2+}-ATP. This condition, providing maximal activity, was chosen for the experiments with L-serine and $tRNA^{Ser}$ as the varied substrates.

Two sites for $tRNA^{Ser}$. A second order dependence of the steady state reaction rate on the concentration of $tRNA^{Ser}$ was found with seryl tRNA synthetase. Fig. 3A shows a typical result. According to the steady state kinetic rules, one can assume that two $tRNA^{Ser}$ molecules have to be bound at the synthetase in order to allow one seryl-$tRNA^{Ser}$ molecule to dissociate from the enzyme.

In order to test whether or not the dissociation of the charged tRNA could be rate limiting, the reaction kinetics were measured as a function of the salt concentration. It was observed that the second order dependence of the reaction rate on the concentration of $tRNA^{Ser}$ persisted at all concentrations of KCl between 0 and 0.81 M. The "K_m" value (defined analogously to the Michaelis-Menten constant for plots of $^1/v$ vs. $^1/[S]^2$) and the aminoacylation rate constant, k_3, were at a maximum around 0.2 M KCl, as shown in Fig. 3B. A similar result has been obtained earlier (7) when only initial rates of aminoacylation were measured. The decrease at higher and lower KCl concentrations is understandable if there are at least two ionic strength dependent factors determining the enzymatic activity.

Two reactive sites for L-serine. With L-serine as the

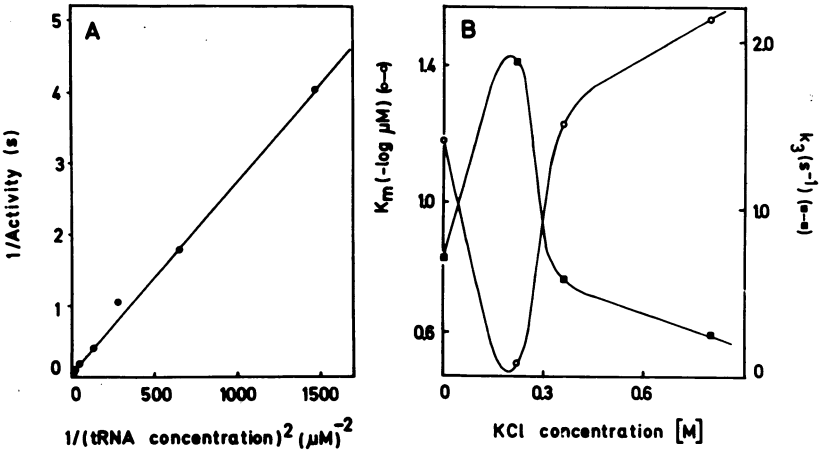

Figure 3. Second order dependence of the aminoacylation rate on the concentration of $tRNA^{Ser}$, in the presence of low concentration of L-serine. Standard conditions, 0.36 M KCl, 0.5 mM L-serine, 2.1 nM synthetase. (A) Second order double reciprocal plot. (B) Dependence of "K_m" (see text) (o) and turnover number, k_3, (■) on the concentration of KCl.

varied substrate it was observed that the aminoacylation rate follows Michaelis-Menten kinetics for amino acid concentrations below 1 mM. When the concentration of L-serine was raised over 10 mM, however, a strong rise of the activity was observed (Fig. 4A) fitting a straight line in a second order Lineweaver-Burk plot (Fig. 4B).

The finding of first as well as second order kinetics in serine clearly indicates that there exist two sites for the amino acid at the enzyme which are differently involved in the reaction. The concentrations of half saturation of the enzyme with the first and the second amino acid differ by more than a factor of 20. This indicates that the two serine molecules bind anticooperatively if the binding sites are intrinsically equal, as was shown for the sites of tRNA in (9).

In the presence of low concentrations of serine, two $tRNA^{Ser}$ molecules must bind to the synthetase for completion of a reaction cycle. The question whether or not this is also true in the presence of high concentrations of the amino acid can be answered negatively because for $tRNA^{Ser}$ normal Michaelis-Menten kinetics were observed under these conditions (Fig. 5A). Here

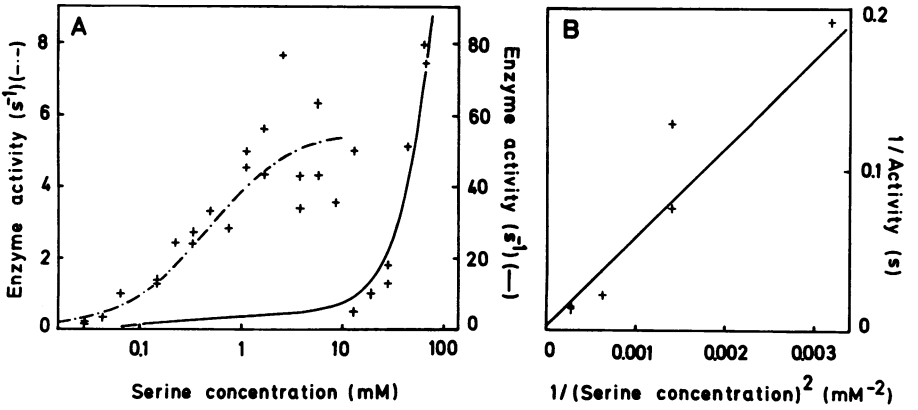

Figure 4. Biphasic dependence of the aminoacylation rate on the concentration of L-serine. Standard aminoacylation conditions were employed with 0.126 M KCl, 37.1 µM $tRNA^{Ser}$ and 2.1 nM synthetase. In (A) the broken line (-·-·-, left ordinate) is calculated for K_m 0.45 mM and k_3 5.7 s^{-1} fitting to the activity values at low serine concentrations. The solid line (——, right ordinate) fitting the values of synthetase activity also at high serine concentrations is generated as follows: it is assumed that the total rate of aminoacylation adds up from the first mode of the enzyme (broken line) and an additional one. For this the values of "K_m" (defined as for Fig. 3, see text) and of the reaction rate, 7 000 mM^2 and 220 s^{-1}, respectively, are derived from the second order double reciprocal plot (B).

the K_m value and the turnover number increased proportionally as the ionic strength increased from 0.1 to 0.45 M (Fig. 5B). Below 0.1 M KCl second order rate dependence in $tRNA^{Ser}$ was observed at all amino acid concentrations.

Whenever non-Michaelis-Menten kinetics are observed the possibility of an inhomogeneity in the enzyme preparation must be considered. We believe this is not the case at hand based upon the following criteria: no preparation has been reported to yield seryl tRNA synthetase with a higher specific activity (4,9,10), the protein seems to be almost pure, the activity is stable, and between different series of experiments absolute values differed by at most a factor of 2. Since perfectly straight lines were observed under appropriate conditions the reported behavior seems to be a property of the enzyme itself.

DISCUSSION

The present study has been undertaken in an effort to

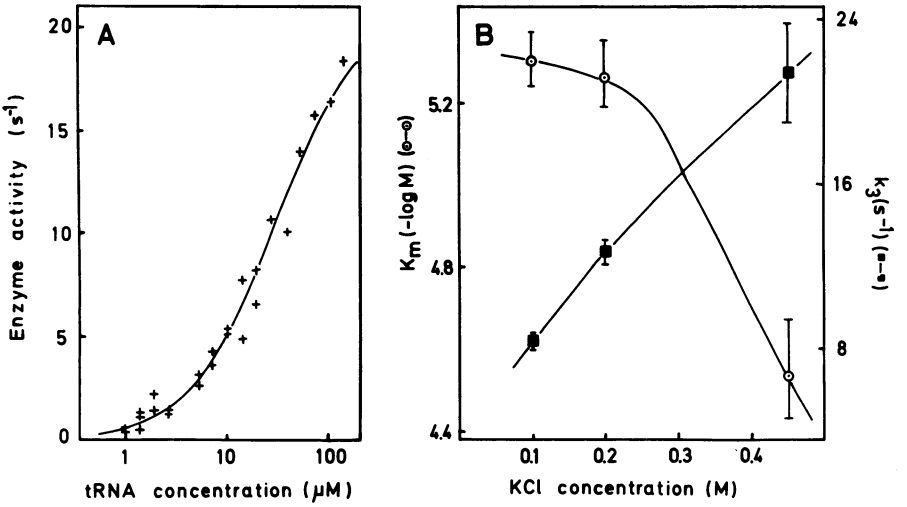

Figure 5. Dependence of the aminoacylation rate on the concentration of $tRNA^{Ser}$ in the presence of high serine concentration, as a function of the KCl concentration. (A) represents a computer fit according to first order rate dependence under standard conditions, 0.45 M KCl, 21 mM serine and 2.1 nM synthetase, using the K_m and k_3 values as indicated in (B) for 0.45 M KCl. (B) Dependence of the values of K_m (O) and k_3 (■) upon the concentration of KCl. The error flags designate ± 2 standard deviations for these determinations.

correlate different states of substrate binding to functional states of seryl tRNA synthetase. Particularly interesting seems to be the anticooperative binding of two tRNA molecules to this enzyme (7,8,9) which has also been observed with $tRNA^{Tyr}$ and tyrosyl tRNA synthetase from E. coli (9). Intrinsic equivalence of the two sites was demonstrated for the tyrosine enzyme and is assumed also for seryl tRNA synthetase (7,9).

Seryl tRNA synthetase seems to share reaction kinetic features with a number of other aminoacyl tRNA synthetases. A second order dependence of the reaction rate on the tRNA concentration as it was previously demonstrated with the serine enzyme (22,23) and is investigated in more detail in the present study, has also been found with the leucine and tryptophan enzymes from E. coli and beef pancreas (23), respectively. Furthermore, one form of glycyl tRNA synthetase from E. coli shows a Hill coefficient of 2 with tRNA as the varied substrate (24). Biphasic kinetics with respect to the amino acid are

exhibited by the tyrosine enzymes from E. coli and B. stearothermophilus (25).

The general scheme of an enzymatic reaction involving two sites for one class of substrates comprises four possible reaction cycles (I-IV in Fig. 6). The finding of second order rate dependence on the tRNA concentration can be due to the prevalence of aminoacylation along cycles II, III or IV. If seryl-$tRNA^{Ser}$ and $tRNA^{Ser}$ are bound anticooperatively to the synthetase under aminoacylation conditions as it was found for uncharged $tRNA^{Ser}$ in the absence of ATP and the amino acid (9), then aminoacylation should predominantly occur along cycle IV. In this case seryl-$tRNA^{Ser}$ is liberated upon association of a second $tRNA^{Ser}$ molecule to the enzyme. The rate limiting step probably is the dissociation of seryl-$tRNA^{Ser}$ under those conditions in which the reaction rates and the K_m values increase with increasing salt concentration because the tRNA dissociates faster from the synthetase at high than at low salt concentrations (7). Under conditions of further increased salt concentrations, however, the reaction rate is lower, perhaps because an increasing percentage of the bound tRNAs dissociates before aminoacylation is completed.

Seryl tRNA synthetase seems to be an intrinsically symmetric enzyme, at least it has an α_2-subunit structure and binding kinetic experiments (7) are in agreement with this assumption. Upon binding of the first tRNA the enzyme changes its affinity for the second tRNA. Also the first and the second serine are not bound identically to the synthetase. This is concluded from the large difference between the two concentrations at half saturation of the enzyme and from the fact that the rate of

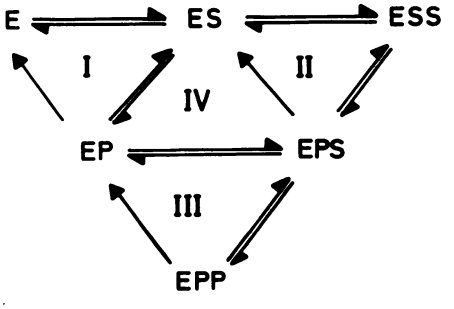

Figure 6. The proposed reaction scheme. The enzyme species are: free enzyme (E) and enzyme with one or two molecules of substrate (S) and/or product (P) bound. The reaction cycles I-IV are discussed in the text.

aminoacylation increases by much more than by a factor of two when the serine concentration is raised from saturation of the enzyme with the first, to saturation with the second serine. These findings are compatible either with the presence of two sterically distinct binding sites for serine and anticooperativity between them, or else with the presence of only one binding site; from this site then the free amino acid would have to displace the esterified one. The recent finding of a second binding mode of methionine to methionyl tRNA synthetase from E. coli (27) would fit either interpretation. On grounds derived from our nuclease digestion studies on serine binding to seryl tRNA synthetase (9) we favor the existence of two sterically distinct binding sites for serine and anticooperativity between them.

In fluorescence stopped flow and temperature jump experiments on the complex of seryl tRNA synthetase with $tRNA^{Ser}$, relaxation times between 1 ms (7,9) and up to 250 ms (7) have been observed. The assumption that these relaxation processes indicate steps relevant to the aminoacylation, is compatible with turnover number-values below 5 s^{-1} which were found in the presence of low serine concentrations. The observation of turnover rates above 20 s^{-1} in the presence of high concentrations of serine, KCl and $tRNA^{Ser}$, however, make further relaxation measurements under those conditions desirable. It might be possible to obtain pertinent results also from transient reaction kinetic experiments.

Due to the second order dependence on the substrate concentrations, the aminoacylation activity of seryl tRNA synthetase is regulated most sensibly by $tRNA^{Ser}$ in the presence of low amino acid concentrations and by serine in the presence of high concentrations of this amino acid. These features of seryl tRNA synthetase might have been a selective advantage in the evolution of the system.

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