KINETIC STUDIES ON THE INTERACTION OF SERYL-tRNA SYNTHETASE WITH $tRNA^{Ser}$ AND $Ser\text{-}tRNA^{Ser}$ FROM YEAST

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1. Introduction

The fluorescent properties of aminoacyl-tRNA synthetases have been widely used to study the binding equilibria between the synthetases and their substrates [1–8]. There are only few reports, however, dealing with rapid kinetic investigations of these interactions: Blanquet et al. [7] and Holler et al. [9] have examined the methionine and the isoleucine activation, respectively. So far no systematic kinetic examination of the interaction between aminoacyl-tRNA synthetase and tRNA has been published. In this paper equilibrium and kinetic data of the interaction of $tRNA^{Ser}$ and seryl-$tRNA^{Ser}$ with the seryl-tRNA synthetase from yeast are presented. The influence of serine and ATP on the binding of tRNA to the synthetase is reported. Experimental evidence for the rate-determining step in the aminoacylation reaction is discussed.

Abbreviations:
SRS, seryl-tRNA synthetase; DTE, dithioerythrol; EDTA, ethylenediaminetetraacetic acid; K_{ass}, association constant; k_R, rate constant for association; k_D, rate constant for dissociation.

2. Materials and methods

2.1. *Synthetase and tRNA*

SRS was prepared from baker's yeast [10]. The purity of the crystallized enzyme according to polyacrylamide gel electrophoresis under various conditions was better than 95%. It had a specific activity of 365 units/mg protein (cf. [11]).

$tRNA_I^{Ser}$ was isolated from brewer's yeast tRNA enriched in $tRNA^{Ser}$ (Boehringer, Mannheim) according to a procedure modified from [12]. It had an amino acid acceptor activity of 1350 pmoles serine/A_{1cm}^{260nm}.

All measurements were done in standard buffer (30 mM K-phosphate pH 7.2, 0.5 mM EDTA, 0.5 mM DTE); the concentrations of $MgCl_2$, KCl and glycerol are given in the text.

2.2. *Ultracentrifugation*

Sedimentation equilibrium and velocity runs were carried out at 5° in a Spinco model E ultracentrifuge equipped with a photoelectric scanning device. Equilibrium centrifugation runs for the determination of the molecular weight of the SRS were performed at 12,000 rpm. Velocity runs for the evaluation of the stoichiometry of the complex between SRS and $tRNA^{Ser}$ were carried out at 48,000 rpm.

2.3. *Fluorescence titrations*

The fluorescence titrations were performed at 20° in a Schoffel RRS 1000 spectrofluorometer. The tryptophane fluorescence of the SRS was excited between 302 and 310 nm depending on enzyme concentration, emission was observed at 350 nm. In order to correct for the inner filter effect due to the tRNA added during the titration two titrations were carried out simultaneously: one in the presence of 5 mM $MgCl_2$ and the other in the absence of $MgCl_2$ at 5 mM EDTA. $tRNA^{Ser}$ does not bind to the enzyme in the absence of $MgCl_2$ (cf. sect. 3. 2). Thus, the fluorescence intensity measured in the absence of $MgCl_2$ can be used to correct the value obtained in the presence of $MgCl_2$ for the inner filter effect (leading to F_{corr}). The experimental data were analysed using a computerized curve fitting procedure, in which the best values for the binding constant (K_{ass}) and the relative fluorescence yield of the complex (F_∞) were evaluated for various numbers of binding sites (n).

2.4. *Kinetic experiments*

Rapid mixing experiments were performed at 20° in a stopped-flow spectrophotometer adapted to fluorescence detection. The observation cell was a 15 mm quartz tube of a quadratic cross section (2 × 2 mm inside dimension) which was mounted perpendicular to the incident light beam in a stainless steel thermostated block*. A highly stabilized Xenon lamp (Osram, 75 W) was used for illuminating the sample; the slit width of the high-intensity monochromator (Bausch & Lomb, 33-86-01) was 1.2 mm. Tryptophane fluorescence was excited at 285 nm, the emission was observed with an aperature of 90° after passing a WG 320 and a UG 11 filter (Schott & Gen.). The mixing chamber and the exchangeable driving syringes were thermostated as described elsewhere [13]. The dead-time was determined to be 1.5 msec under optimal conditions.

The time course of the reaction has been fitted to the following reaction scheme:

$$A + B \underset{k_D}{\overset{k_R}{\rightleftharpoons}} AB$$

The following integrated rate expression was obtained

$$C_{AB}(t) - \overline{C_{AB}} = \frac{(C_{AB_0} - \overline{C_{AB}}) \cdot \sqrt{X}}{C_{AB_0} - \overline{C_{AB}} - (C_{AB_0} - \overline{C_{AB}} - \sqrt{X}) \cdot \exp(k_R \sqrt{X}\, t)}$$

where C_A^0 and C_B^0 are the total concentrations of A and B, respectively, and $C_{AB}(t)$, C_{AB_0}, $\overline{C_{AB}}$ are the complex concentrations at time t, time zero and infinite time, respectively. X is defined as

$$X = (C_A^0 + C_B^0 + 1/K_{ass})^2 - 4\, C_A^0 \times C_B^0$$

where $K_{ass} (= k_R/k_D)$ was taken from equilibrium experiments. In association experiments comparable molarities of SRS and $tRNA^{Ser}$ were mixed together; C_{AB_0} was zero. Since the binding constant decreases with increasing ionic strength, dissociation could be initiated by mixing a solution of SRS, $tRNA^{Ser}$ and the complex in standard buffer containing 5 mM $MgCl_2$ with a buffer of high salt concentration (salt jump); due to dilution, C_{AB_0} was half of the complex concentration in the driving syringe containing both SRS and $tRNA^{Ser}$.

The curves were corrected for the dead-time [10].

3. Results and discussion

3.1. *Molecular weight of SRS*

The molecular weight of the dimeric SRS has been determined by the sedimentation equilibrium method to be 95,000 under various conditions (protein conc. 0.1 to 0.5 mg/ml, ionic strength: 0.05 to 1.0, pH 6.0 to 7.2, absence and presence of $MgCl_2$). There was no evidence for a dissociation of the dimeric enzyme at low protein concentration in contrast to the work of Heider et al. [11]. In all instances a linear log absorbance versus r^2 plot was observed.

3.2. *Determination of the stoichiometry of the complex by ultracentrifugation*

The stoichiometry of the complex formed between synthetase and its specific tRNA can be easily determined by sedimentation velocity runs according to the method described in the accompanying paper [14]. For the serine system the formation of a complex

* This cell was constructed by Dr. K. Kirschner.

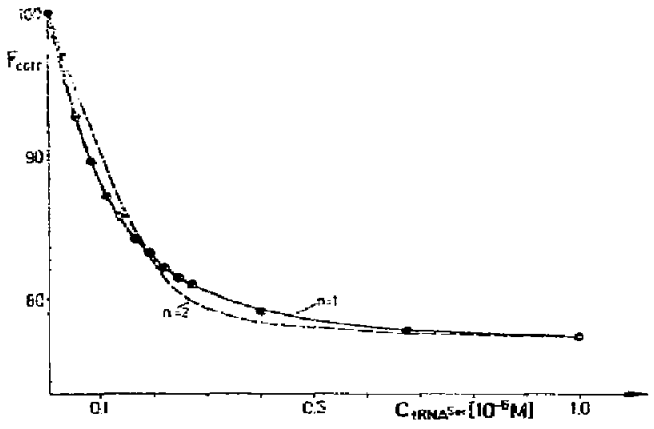

Fig. 1. Fluorescence titration of 0.11 μM SRS at 5 mM $MgCl_2$ and 8.5% glycerol with $tRNA^{Ser}$ at 20°. For definition of F_{corr} see text. Shown are the measured points and the optimally fitted binding curves for $n = 1$ and $n = 2$.

between one $tRNA^{Ser}$ molecule and one SRS molecule (dimer) was established. It could be shown by ultracentrifugation studies that there is no complex formation in the absence of Mg^{2+} ions or at high salt concentrations.

3.3. *Equilibrium and rate constants*

Fluorescence titrations of varying amounts of SRS with $tRNA^{Ser}$ gave identical binding constants within the limits of experimental error. The binding constant showed a strong dependence on ionic strength (table 1). As seen in fig. 1 the binding of $tRNA^{Ser}$ to the enzyme caused a 22% decrease of the fluorescence intensity (F_∞). A better fit is obtained for $n = 1$ than for $n = 2$ in agreement with the ultracentrifugation studies. The binding constants determined by Rigler et al. [6] and

Table 1

Equilibrium and rate constants for the interaction of SRS with $tRNA^{Ser}$ at 20° at 5 mM $MgCl_2$ and various concentrations of KCl.

KCl [M]	K_{ass} [M^{-1}]	k_R [M^{-1} sec^{-1}]	k_D [sec^{-1}]
0	2×10^7	2.1×10^8	(10.5)*
0.05	1×10^7	1.5×10^8	19
0.1	4×10^6	7×10^7	33
0.2	1.4×10^5	$(1.7 \times 10^7)^{x)}$	122

* Calculated, not measured independently.

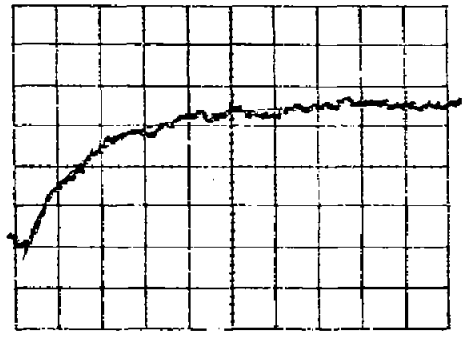

Fig. 2. Stopped-flow experiment showing association of SRS and $tRNA^{Ser}$ at 0.05 M KCl and 5 mM $MgCl_2$. Total concentrations after mixing: 0.86 μM $tRNA^{Ser}$, 0.86 μM SRS. Oscilloscope settings: 50 mV/unit; 5 msec/unit; 1 msec rise-time; final signal: 2.3 V. Increasing fluorescence corresponds to negative deflection. The fitted curve is superimposed on the oscilloscope trace.

Engel et al. [8] show similar magnitudes. There are discrepancies, however, regarding the number of binding sites. Whereas Rigler et al.'s data did not allow a definite conclusion as to the stoichiometry of the complex, Engel et al. reported a 1:1 stoichiometry already for the monomeric enzyme. It cannot be decided yet, whether these discrepancies are due to different enzyme preparations (cf. 3. 1.) or experimental conditions.

Fig. 2 and 3 show typical oscilloscope traces of stopped-flow experiments, where association of SRS and $tRNA^{Ser}$ and dissociation of the SRS–$tRNA^{Ser}$ complex have been examined, respectively. The validity of the simple reaction scheme (cf. 2. 4) was

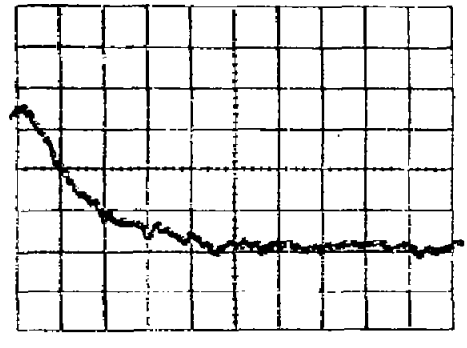

Fig. 3. Stopped-flow experiments showing dissociation of the SRS–$tRNA^{Ser}$ complex due to a salt jump. Total concentration after mixing: 0.86 μM SRS, 0.86 μM $tRNA^{Ser}$ in standard buffer, 5 mM $MgCl_2$ and 0.2 M KCl. Oscilloscope settings: 50 mV/unit; 5 msec/unit; 1 msec rise-time; final signal: 2.65 V.

Table 2
Equilibrium and recombination rate constants for the interaction of SRS with $tRNA^{Ser}$ and Ser-$tRNA^{Ser}$ in the presence of other substrates at 20°.

Substrate present	$MgCl_2$ [mM]	K_{ass} [M^{-1}]	k_R [M^{-1} sec^{-1}]
$tRNA^{Ser}$ 5 mM Ser	5	2×10^7	1.8×10^8
$tRNA^{Ser}$ 5 mM ATP	12.5	3.3×10^6	7.7×10^6
$tRNA^{Ser}$ {5 mM Ser, 5 mM ATP	12.5	--	5.2×10^7
Ser-$tRNA^{Ser}$ {5 mM Ser, 5 mM ATP	12.5	3×10^6	7×10^7

substantiated by the excellent agreement between oscilloscope trace and theotical curve (fig. 2) as well as by the independence of rate constants upon initial concentrations of protein and tRNA, which was verified over a concentration range of 0.43 μM to 3.4 μM SRS and tRNA, respectively. The rate constants k_R and k_D for several concentrations of KCl have been determined independently from each other in association and dissociation experiments, respectively (table 1).

Serine concentrations up to 5 mM had no significant effect on K_{ass} and the rate constants. In the presence of ATP, however, the binding and rate constants were changed considerably (table 2).

The equilibrium constant for the interaction between acylated $tRNA^{Ser}$ and SRS was determined in the presence of 5 mM ATP, 5 mM serine and 12.5 mM $MgCl_2$. For the kinetic experiments the tRNA was acylated in the driving syringe by preincubation with 5 mM ATP, 5 mM serine and catalytic amounts of SRS for 15 min at 20°. Afterwards it was mixed rapidly with a SRS solution of higher concentration containing also ATP and serine. K_{ass} and k_R are given in table 2.

3.4. *Single turnover experiments*

The aminoacylation reaction was analyzed in stopped-flow experiments by mixing together a solution of SRS, ATP and serine with a $tRNA^{Ser}$ solution (fig. 4). The rapid initial reaction corresponds to fluorescence quenching as a consequence of tRNA binding to the enzyme in the presence of ATP and serine. The subsequent increase in fluorescence corresponds to a slow reaction that is monitored by the previously determined rapid dissociation step, which had a k_D of 23 sec^{-1} (calculated from K_{ass} and k_R). The slow process has a characteristic life time of 0.4 sec and is therefore rate determining for the turnover reaction. It may represent either a rearrangement of the quarternary complex or the esterification of $tRNA^{Ser}$ with serine. Since a similarly slow reaction was found in experiments, where a solution of SRS and $tRNA^{Ser}$ was mixed with an ATP solution, the first alternative is favored; this will be clarified in further investigations.

4. Conclusions

The association of SRS and $tRNA^{Ser}$ is nearly diffusion controlled (cf. accompanying paper [14]). This implies that there is little specificity through this

Fig. 4. Stopped-flow experiment showing a single turnover. Total concentrations after mixing: 0.43 μM SRS, 0.43 μM $tRNA^{Ser}$, 5 mM ATP, 5 mM serine, 12.5 mM $MgCl_2$. Oscilloscope settings: 20 mV/unit; 200 msec/unit; 10 msec rise-time; final signal: 2.1 V.

process. Obviously, the correct tRNA is recognized after association, possibly through a favorable mutual charge distribution. The electrostatic nature of this interaction was demonstrated by the decrease of K_{ass} and k_R and the increase of k_D with increasing ionic strength. The kinetic experiments and the equilibrium data exclude slow rearrangements of the binary complex as can be seen from the agreement of the measured K_{ass} with the quotient of the independently determined k_R and k_D. Thus, the high specificity required for the recognition process may be determined entirely by the dissociation rate.

Yarus and Berg [15] and Hélène et al. [4] have compared the rate of release of aminoacylated tRNA's from the synthetase with the turnover rate; they conclude that release of aminoacyl-tRNA is the rate limiting step in the aminoacylation reaction. Since their experiments were performed at pH 5.5 a comparison of their results with ours is questionable. In the serine system, at least at neutral pH, Ser-tRNA^{Ser} is not limiting. A reaction closely preceding it represents the rate determining step. A similar result is reported by Krauss et al. [14], indicating that the results given here are not unique to the serine system of yeast.

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