

Role of lysine-195 in the KMSKS sequence of *E. coli* tryptophanyl-tRNA synthetase

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Abstract Lysine 195 in the K¹⁹⁵MSKS sequence of *E. coli* tryptophanyl-tRNA synthetase (TrpRS) was replaced with alanine. The resulting K195A mutant TrpRS had essentially unchanged K_m values for ATP and Trp, but a 1500-fold decreased k_{cat} in a pyrophosphate-ATP exchange reaction. This large decrease in k_{cat} reduces the rate of aminoacyladenylate formation (step 1) to a rate comparable to the rate of aminoacylation of tRNA^{Trp} (step 2) by the K195A mutant enzyme. Both the TIGN and KMSKS sequences are important for step 1 of class I aminoacyl-tRNA synthetase reactions.

Key words: Aminoacyl-tRNA synthetase; Mononucleotide binding fold; KMSKS sequence; Kinetics; Overexpression; Mutagenesis

1. Introduction

Class I aminoacyl-tRNA synthetases (aaRS) are distinguished from class II by having two short sequence motifs: the HIGH sequence and the KMSKS sequence [1–3]. *E. coli* TrpRS is a class I enzyme with the two sequence motifs represented as T¹⁷IGN and K¹⁹⁵MSKS, respectively. Thr¹⁷ of the T¹⁷IGN sequence in *E. coli* TrpRS, just as the His⁴⁵ of the H⁴⁵IGH sequence in *B. stearothermophilus* TyrRS, is involved in stabilizing ATP in the transition state in the first step of the reaction [4,5]. In addition, Thr¹⁷ interacts with the substrate in the transition state in the second step of the reaction [5]. In the HIGH (or TIGN) sequence, only the Gly at the third position is invariant [5].

The KMSKS sequence has been discovered in different aaRS by three different approaches [6–13]. In *B. stearothermophilus* TyrRS, the KMSKS sequence is represented as K²³⁰FGKT (Table 1). The two Lys residues were found to be involved in stabilizing both the transition state and the E·Tyr-AMP·PP_i complex [13]. Yet, the ϵ -amino groups of Lys²³⁰ and Lys²³³ in the crystal structure of the enzyme are at least 8 Å too far away to interact with the pyrophosphate moiety. An induced fit mechanism was proposed in which the binding of the substrates induced a conformational change in the enzyme. Consistent with this proposal is that the two Lys residues are in flexible regions of the protein.

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Abbreviations: aaRS, aminoacyl-tRNA synthetase(s); TrpRS, tryptophanyl-tRNA synthetase; TyrRS, tyrosyl-tRNA synthetase; HIGH, His-Ile-Gly-His sequence; TIGN, Thr-Ile-Gly-Asn sequence; KMSKS, Lys-Met-Ser-Lys-Ser sequence; KFGKT, Lys-Phe-Gly-Lys-Thr sequence.

The first and second Lys in the KMSK³³⁵S sequence in *E. coli* MetRS have also been investigated kinetically [32,33]. Both Lys were found to interact with the pyrophosphate part of ATP in the transition state. In the crystal structure of the MetRS·ATP complex, the second Lys is located 3.4 Å away from the γ -phosphate of ATP. This also suggests a conformational change in the KMSKS region after substrate binding. Indeed, a 'mobile loop' mechanism has been proposed for the KMSKS sequence [33,34].

E. coli TrpRS contains exactly the KMSKS sequence, which is highly conserved among known prokaryotic and eukaryotic TrpRS (Table 1). The first Lys residue in this motif is more thoroughly conserved than is the second Lys residue. In bovine and human TrpRS, the second Lys residue is substituted by Ala. In this paper, we report the effect of changing the first Lys in the K¹⁹⁵MSKS sequence to Ala, in *E. coli* TrpRS. Kinetic results support the hypothesis that Lys¹⁹⁵ may interact with ATP in the transition state. The results also suggest that Lys¹⁹⁵ of the K¹⁹⁵MSKS sequence has a larger effect on the k_{cat} of the first step of the reaction than does Thr¹⁷ of the T¹⁷IGN sequence.

2. Materials and methods

pET-5-EF and pET-5-EF(T17A) were constructed as previously described [5]. *E. coli* BMH 71-18 mutS and JM109 were from Promega (Madison, WI). Single stranded pSelect-EF was prepared according to the protocol provided by Promega. dGTP Reagent Kit for DNA sequencing and Sequenase Version 2.0 DNA polymerase were from United States Biochemical (Cleveland, OH). Other materials were as in [5].

Site-directed mutagenesis was performed as previously described [5], using single stranded DNA derived from pET-5-EF [5] and the oligonucleotide 5'-CTTGGACATCGCCTTGGTCGG-3', which is complementary to codons 198–192 of the *E. coli trpS* gene [24], but with mismatches at the underlined bases in codon 195. The mutation was confirmed by DNA sequence analysis. The overexpression and purification of the TrpS enzyme, and the pyrophosphate exchange and Trp charging assays [35] for the wild-type and mutant enzymes, were performed as previously described [5].

3. Results

In a crude extract from 20 ml of culture, the overexpressed K195A mutant TrpRS showed about 8% of the charging activity of the wild-type enzyme (with saturating substrate concentrations). SDS gels showed an overexpressed protein of M_r 37,000 Da in strains producing the K195A mutant (data not shown) compared to strains that overproduced the wild-type and the T17A mutant enzymes (see Fig. 3 of [5]). The K195A mutant enzyme was purified from a 1 l culture; the final yield of purified enzyme was about 2 mg, as measured by the absorbance at 280 nm [35].

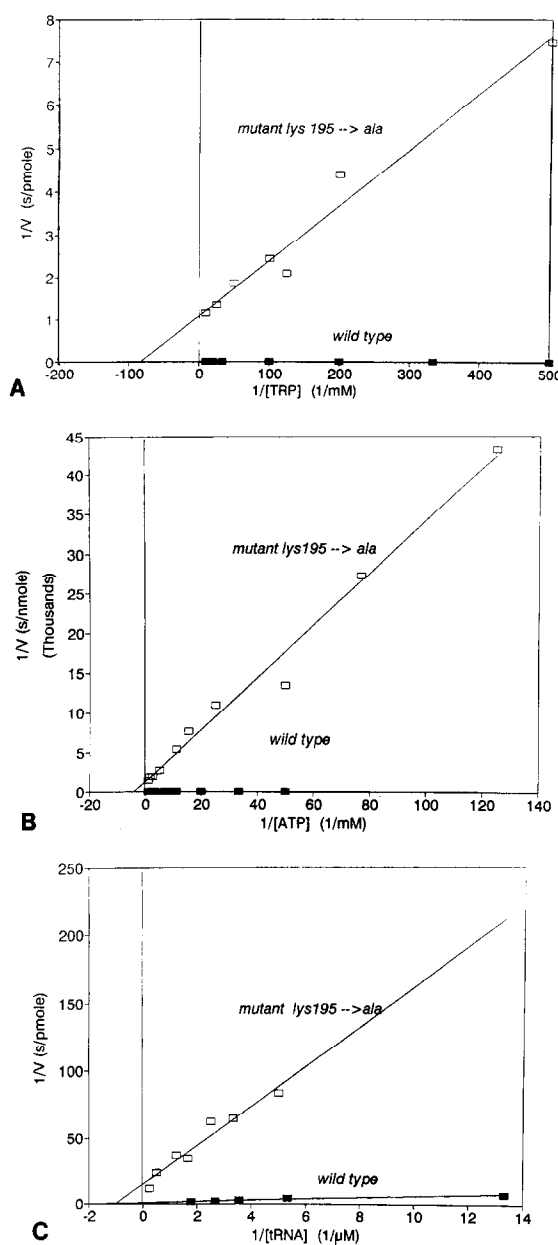


Fig. 1. Comparison of the catalytic activities of wild-type *E. coli* TrpRS and K195A mutant TrpRS. Kinetic data are presented as double reciprocal plots of initial rates vs. initial substrate concentrations. (A) Rate of ATP- 32 P $_i$ exchange reaction as a function of tryptophan concentration, in the presence of 2 mM ATP and 4 nM wild-type or mutant TrpRS. (B) Rate of ATP- 32 P $_i$ exchange reaction as a function of ATP concentration, in the presence of 2 mM Trp and 4 nM wild-type or mutant TrpRS. (C) Initial rate (over the first 3 min) of the overall aminoacylation reaction as a function of the tRNA^{Trp} concentration, with saturating amounts of the other substrates.

3.1. Enzyme kinetics

To assess the functional importance of lysine at position 195, the apparent kinetic parameters of both the mutant and native enzymes were measured by steady state kinetic analysis. The results are shown in Fig. 1 and Table 2. Fig. 1A and B show the double reciprocal plots for the first step of the acylation reaction [5], measured by the pyrophosphate exchange assay, with ATP and Trp as the variable substrate, respectively. The

rate for the pyrophosphate exchange decreases dramatically for the K195A mutant, but the K_m values for both substrates remain more or less unchanged, as indicated by the nearly equal x-intercepts. Whether Trp or ATP is the substrate whose concentration is varied, k_{cat} is decreased by a factor of about 1500 (Table 2).

Fig. 1C shows the double reciprocal plots for the aminoacylation assay, with the concentration of tRNA^{Trp} being varied. The k_{cat} for the K195A mutant is 0.06 s⁻¹ compared to 1.3 s⁻¹ for the wild-type, i.e. reduced by a factor of 23. The K_m (tRNA) for the mutant enzyme is only two times that of the native enzyme. The apparent kinetic parameters for the wild-type and K195A mutants are summarized in Table 2, which also includes the parameters for the T17A mutant from [5].

4. Discussion

The objective of these experiments has been to investigate the importance of the first residue in the KMSKS sequence of *E. coli* TrpRS. The functional importance of the second Lys in this motif was demonstrated by kinetic studies of mutants of *B. stearotherophilus* TyrRS and *E. coli* MetRS [13,32–34]. In a similar fashion, the first Lys in the KMSKS sequence was implicated initially in *B. stearotherophilus* TyrRS [13], and more recently also in *E. coli* MetRS [33]. To clarify the general importance of this Lys among class I aaRS, we have characterized the purified A¹⁹⁵MSKS mutant of *E. coli* TrpRS.

The results of the kinetic analysis show that the K195A mutant has similar K_m values to those of the wild-type for all three substrates. K_m (Trp) and K_m (ATP) for the mutant are unchanged. K_m (tRNA) is increased by only 2 fold, from 0.53 μ M in the wild-type to 1.11 μ M in the mutant (Table 2). So the K195A mutation does not have a significant effect on the affinity of the enzyme for any of the substrates.

The k_{cat} measured by the pyrophosphate exchange assay is a measure of the first step of the reaction. The k_{cat} measured by the aminoacylation assay is a measure of the second step of the reaction, if the first step is much faster than the second step. For wild-type *E. coli* TrpRS, the first step is at least 1,000 fold faster than the second step of the reaction. For the K195A mutation, the ~1,000 fold decrease in the rate of the first step of the reaction is dramatic (Table 2). In comparison, for a T17A mutant, previous experiments showed a decrease of only 30 fold in the k_{cat} for the first step [5]. The drastic effect on the first step caused by the K195A mutation reduces the first step to a rate that is comparable to that of the second step. In the K195A mutant enzyme, therefore, the second step is probably not rate limiting. The 23 fold decrease in the overall rate of aminoacylation is then likely to be due to the large decrease in the rate of the first step of the reaction and not necessarily to a direct effect of the K195A mutation on the second step.

Unlike Thr¹⁷, which directly affects both the first and second steps [5], Lys¹⁹⁵ probably only interacts with the substrate during the first step of the reaction. The lack of effect of position 195 on the second step of the reaction rules out the possibility that the kinetics here could be caused by contamination with a small percentage of wild-type enzyme. Because the expression and purification systems were identical, this observation also strongly suggests that the earlier results with the T17A mutant TrpRS [5] also should not be attributed to contamination by the native enzyme, thus laying to rest an earlier concern [5].

Table 1
Alignment of sequences of class I aminoacyl-tRNA synthetases that contain a KMSKS sequence

Enzyme	Source	Sequence	Size	Reference
Arg-tRNA synthetase	<i>E. coli</i>	³⁷⁸ KPFKT	1 × 577	[14]
Cys-tRNA synthetase	<i>E. coli</i>	²⁶⁶ KMSKS	1 × 461	[15]
Gln-tRNA synthetase	<i>E. coli</i>	²⁶⁷ VMSKR	1 × 551	[16]
Glu-tRNA synthetase	<i>E. coli</i>	²⁶⁵ KLSKR	1 × 471	[17]
Ile-tRNA synthetase	<i>E. coli</i>	⁶⁰³ KMSKS	1 × 939	[18]
	<i>S. cerevisiae</i> (cytoplasmic)	⁶⁰³ KMSKS	1 × 1073	[19]
Leu-tRNA synthetase	<i>E. coli</i>	⁶¹⁹ KMSKS	1 × 860	[20]
Met-tRNA synthetase	<i>E. coli</i>	³³² KMSKS	2 × 677	[21]
Trp-tRNA synthetase	<i>B. stearothermophilus</i>	¹⁹⁷ KMSKS	2 × 327	[22]
	<i>B. subtilis</i>	¹⁹³ KMSKS	2 × 330	[23]
	<i>E. coli</i>	¹⁹⁵ KMSKS	2 × 334	[24]
	<i>S. cerevisiae</i> (mitochondrial)	²⁴⁴ KMSKS	2 × 374	[25]
	Bovine	³⁵³ KMSAS	2 × 475	[26]
	Human	³⁴⁹ KMSAS	2 × 471	[27]
Tyr-tRNA synthetase	<i>B. caldotenax</i>	²³⁰ KFGKT	2 × 419	[28]
	<i>B. stearothermophilus</i>	²³⁰ KFGKT	2 × 419	[29]
	<i>E. coli</i>	²³⁴ KFGKT	2 × 424	[30]
Val-tRNA synthetase	<i>E. coli</i>	⁵⁵⁴ KMSKS	1 × 951	[31]

The kinetic results demonstrate that the K195A mutation affects the k_{cat} of the first step of the reaction without changing the K_m for the substrates. The results support the hypothesis that Lys¹⁹⁵ interacts with the substrate in the transition step. However, the data presented here are not sufficient to demonstrate whether ATP or Trp is involved in interacting with Lys¹⁹⁵. From the crystal structure of *B. stearothermophilus* TrpRS and three other class I aaRS [36–39], it is clear that the KMSKS sequence interacts with ATP. This suggests that in *E. coli* TrpRS, ATP may also be the substrate which interacts with Lys¹⁹⁵.

The contribution of Lys¹⁹⁵ of *E. coli* TrpRS to the binding of the transition state can be calculated using the equation:

$$\Delta G = -RT \ln[(k_{\text{cat}}/K_m)_{\text{mut}}/(k_{\text{cat}}/K_m)_{\text{wt}}] \quad [40]$$

The ΔG value calculated in two ways, using the $(k_{\text{cat}}/K_m)_{\text{Trp}}$ and $(k_{\text{cat}}/K_m)_{\text{ATP}}$, is 18.2 kJ/mol or 19.6 kJ/mol, respectively. Although large, this energy is reasonable because a similar energy cost for mutating the second Lys to Ala in the KMSKS sequence has been obtained in *E. coli* MetRS (19.3 kJ/mol) [32] and in *B. stearothermophilus* TyrRS (11.4 kJ/mol) [13].

When Lys²³⁰ in *B. stearothermophilus* TyrRS is changed to Ala (K²³⁰FGKT → A²³⁰FGKT), there is a 97-fold decrease in

the k_{cat} of the first step of the reaction [13]. When His⁴⁵ in *B. stearothermophilus* TyrRS is mutated to Gly (H⁴⁵IGH → G⁴⁵IGH), the k_{cat} for the first step of the reaction is decreased by 240-fold [41]. Apparently, in *B. stearothermophilus* TyrRS, the first Lys in the KMSKS sequence has a smaller effect on the k_{cat} of the first step of the reaction than the first His in the HIGH sequence.

In *E. coli* TrpRS, the T17A mutation (T¹⁷IGN → A¹⁷IGN) causes a 30-fold decrease in the k_{cat} of the first step of the reaction [5], while the K195A (K¹⁹⁵MSKS → A¹⁹⁵MSKS) mutation causes at least a 1000-fold decrease in the first. Therefore, unlike the situation in *B. stearothermophilus* TyrRS, the first residue in the KMSKS in *E. coli* TrpRS has a larger effect on the k_{cat} of the first step of the reaction than does the first residue in the HIGH sequence. This finding should be tempered by the possibility that traces of wild-type subunits (in heterodimers) could differently influence our A¹⁷IGN and A¹⁹⁵MSKS mutants. Nevertheless, there appears to be a difference in the relative importance of the two sequence motifs in affecting the k_{cat} of the first step of the reaction among different class I aaRS.

If the two mutations in *B. stearothermophilus* TyrRS are independent, the double mutations (K230A, H45G) should

Table 2
Apparent kinetic parameters for wild-type and mutant TrpRS enzymes

	K_{cat}	K_m	K_{cat}/K_m
Tryptophan			
Wild-type	1500 ± 250 s ⁻¹	0.010 ± 0.002 mM	150,000 s ⁻¹ ·mM ⁻¹
Mutant (K195A)	1 ± 0.2 s ⁻¹	0.011 ± 0.003 mM	95 s ⁻¹ ·mM ⁻¹
Mutant (T17A)*	44 ± 3 s ⁻¹	0.011 ± 0.002 mM	4,000 s ⁻¹ ·mM ⁻¹
ATP			
Wild-type	2800 ± 280 s ⁻¹	0.37 ± 0.004 mM	7,600 s ⁻¹ ·mM ⁻¹
Mutant (K195A)	0.9 ± 0.25 s ⁻¹	0.32 ± 0.11 mM	2.8 s ⁻¹ ·mM ⁻¹
Mutant (T17A)*	100 ± 7 s ⁻¹	0.64 ± 0.06 mM	160 s ⁻¹ ·mM ⁻¹
tRNA			
Wild-type	1.34 ± 0.026 s ⁻¹	0.53 ± 0.08 μM	2.5 s ⁻¹ ·μM ⁻¹
Mutant (K195A)	0.06 ± 0.009 s ⁻¹	1.11 ± 0.17 μM	0.05 s ⁻¹ ·μM ⁻¹
Mutant (T17A)*	0.04 ± 0.007 s ⁻¹	0.56 ± 0.003 μM	0.08 s ⁻¹ ·μM ⁻¹

*From [5].

cause an approximately 23,000-fold (97×240) decrease in the k_{cat} . Similarly, in *E. coli* TrpRS, the double mutations (K195A, T17A) should cause about a 30,000-fold (1000×30) decrease in the k_{cat} . Therefore, the combined effects of the initial residues in the two sequence motifs on k_{cat} are expected to be more or less the same in these two class I aaRS, but the relative importance of the two residues is reversed. Functionally, the HIGH and KMSKS motifs may compensate for each other.

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