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The influence of the peptide chain length on the activity of peptidyl-tRNA hydrolase from E. coli

Joseph Shiloach, Shabtai Bauer, Nathan de Groot and Yehuda Lapidot

Department of Biological Chemistry, The Hebrew University of Jerusalem, Jerusalem, Israel.

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ABSTRACT

The dependence of the V_{max} and K_m on the length of the peptide moiety in the peptidyl-tRNA series $(Gly)_n$-Val-tRNA, was measured in the system peptidyl-tRNA hydrolase-peptidyl-tRNA. It was found that the K_m value decreases from 7.2×10^{-7} M for Gly-Val-tRNA to 4.6×10^{-7} M for $(Gly)_2$-Val-tRNA and to 1.7×10^{-7} M for $(Gly)_3$-Val-tRNA; further increase of the peptide chain is not followed by decrease of the K_m. The V_{max} values are 5.7 pmole/min/EU for Gly-Val-tRNA and 42 pmole/min/EU for $(Gly)_3$-Val-tRNA. The enzyme activity is inhibited competitively by uncharged tRNA with a K_I value of about 10^{-5} M. The significance of these results described in this paper, in relation to the fact that peptides and peptide esters do not inhibit the enzyme activity, and in relation to the proposed physiological role of the enzyme, is discussed.

INTRODUCTION

Peptidyl-tRNA hydrolase hydrolyses N-acylaminoacyl-tRNA (1-5), and peptidyl-tRNA (6-7). The enzyme cleaves the ester bond between the N-blocked amino acid or the peptide, and the tRNA. As a result of this cleavage free N-blocked amino acid or peptide, and free tRNA are obtained. The hydrolysis rates of different N-acylaminoacyl-tRNA and peptidyl-tRNA were compared (6). It was found that the hydrolysis rate of peptidyl-tRNA containing at least two peptide bonds is faster than that of N-acylaminoacyl-tRNA. No information about K_m and V_{max} values was given. Moreover, the substrates which were used in nearly all these studies contained large excesses of uncharged tRNA, which is known from the literature to inhibit peptidyl-tRNA hydrolase (1). Although the enzyme is found in many biological systems, such as: bacteria, yeast and animals, its biological function is still unknown. It was suggested that it is a correcting enzyme which hydrolyses

peptidyl-tRNA molecules released prematurely from the ribosomes (8). Further information about the catalytic properties of the enzyme may help in solving the problem of its role in the cell. In this paper we report about the dependence of the V_{max} and K_m on the length of the peptide moiety in the series $(Gly)_n$-Val-tRNA. We used peptidyl derivatives of Val-tRNA in these studies, because of their relative resistance to non-enzymatic hydrolysis, compared to peptidyl derivatives of the other aminoacyl-tRNAs (8). We also studied the inhibition of the enzyme activity by tRNA and by free peptides and peptide esters. The results concerning the peptides were described in a previous communication (22); those concerning the tRNA are given here.

MATERIALS AND METHODS

[^{14}C]-Valine was obtained from Radiochemical Centre, Amersham, England. tRNA was prepared from E. coli W by the method of Bauer et al. (9). The molar concentration of the tRNA in the incubation-mixture was estimated spectroscopically by using a value of 19 A_{260} unit/mg and assuming a molecular weight of 25.000.

[^{14}C]Val-tRNA was prepared essentially according to Ziv et al. (10). The reaction mixture in the final volume of 10 ml contained, among others, 125 mg bulk tRNA; 0.5 μmole [^{14}C] Valine (specific activity 260 μc/μmole) and 2.5 ml of a crude preparation of aminoacyl-tRNA synthetases prepared by the method of Muench and Berg (11). The preparation which contained about 3% [^{14}C]Val-tRNA^{val} was precipitated with two volumes of ethanol, washed twice with absolute ethanol and dried in vacuum.

$(Gly)_n$-[^{14}C]Val-tRNA and Ac[^{14}C]Val-tRNA were prepared essentially according to published procedures (12, 13). The peptidation was carried out in 2.0 ml mixture of 80% DMSO and 20% 0.2 M triethanolamine buffer pH 8.0 at 0°C. The amounts of the components in the various peptidation mixtures which summarized in Table 1, are the minimal amounts of activated N-blocked peptide which had to be added to the indicated amount of Val-tRNA in order to assure 100% peptidation reaction.
An aliquot of each peptidyl-tRNA preparation was treated with 0.2 M NaOH

TABLE 1

Synthesis of Various Oligopeptidyl-tRNAs

N-Hydroxysuccinimide ester of N-blocked peptide		Substrate	(A_{260} units)	Oligopeptidyl-tRNA obtained
Peptide	Amount			
Gly	200 mg	Val-tRNA	(28)	Gly-Val-tRNA
$(Gly)_2$	200 mg	Val-tRNA	(40)	$(Gly)_2$-Val-tRNA
$(Gly)_3$	120 mg	Val-tRNA	(160)	$(Gly)_3$-Val-tRNA
$(Gly)_2$	200 mg	$(Gly)_2$-Val-tRNA	(40)	$(Gly)_4$-Val-tRNA
$(Gly)_3$	120 mg	$(Gly)_3$-Val-tRNA	(160)	$(Gly)_6$-Val-tRNA

(final concentration) for 2 hr at 30°C and the hydrolysate was analyzed by high voltage paper electrophoresis at pH 2.5 with the appropriate markers. All the radioactivity (except in the case of $(Gly)_6$-Val-tRNA, which contained 5% of $(Gly)_3$-Val-tRNA) moved as a single peak corresponding to the appropriate peptide marker.

Preparation of Peptidyl-tRNA Hydrolase from 1200 gr of frozen E.coli W cells was essentially according to Kössel (5). The final purified enzyme preparation contained 4 mg protein with a specific activity of 172 000 units per mg. The enzyme was purified about 2000 times compared to the starting material (S-100 supernatant) and the overall yield was about 40 percent. On SDS acrylamide gel electrophoresis only one protein band could be detected.

Assay of the Enzyme Activity. Enzyme activity was determined at 37°C in a final volume of 0.25 ml containing 40 mM triethanolamine buffer pH 8.0; 5 mM magnesium acetate; 5 mM β-mercaptoethanol; 1 to 100 μg uncharged bulk tRNA; peptidyl-tRNA; and 0.05-3 enzyme units. (One enzyme unit was defined as the amount of enzyme which hydrolyses 1 pmole of $(Gly)_3$-Val-tRNA in one minute under the assay conditions in the presence of 25 μg uncharged bulk tRNA), 50 μl samples were transferred in duplicates

after various time intervals into 200 µl cold 6% trichloroacetic acid. The suspensions were centrifuged (2 min, 8000 × g in an Eppendorf centrifuge type 3200). The radioactivity in 200 µl aliquots of the supernatant was determined in a liquid scintillation counter according to Turner (14). Values obtained for incubation of substrate without enzyme were subtracted from the corresponding values found in the presence of the enzyme. Under the experimental conditions used the extent of the enzymatic hydrolysis was linear with time and enzyme concentration.

Data Processing. Experimental data concerning a straight line were fitted to the appropriate equation by the least-square method assuming equal variance for the velocities.

RESULTS

The Dependence of V_{max} and K_m on the Length of the Peptide-Moiety of the Peptidyl-tRNA

According to Chapeville et al. (4) the enzyme activity is inhibited by uncharged tRNA. Our substrate preparations contained only about 3% of the peptidyl-tRNA, the remainder being uncharged tRNA. Therefore, it was impossible to determine the K_m and V_{max} values by simple Lineweaver and Burke plots.

One way to determine the V_{max} and K_m in the presence of inhibitor was proposed by Dixon (15). Two equations were given, one for the case of competitive inhibition (Eq. 1) and the other for non-competitive inhibition (Eq. 2).

$$(1)\quad \frac{1}{v} = \frac{1}{V_m} + \frac{K_m}{V_m[s]} + \frac{K_m}{V_m Ki[s]}\,[I]$$

$$(2)\quad \frac{1}{v} = \frac{1}{V_m} + \frac{K_m}{V_m[s]} + \frac{1}{V_m Ki}\left[1 + \frac{K_m}{[s]}\right][I]$$

In both cases the intercept with the ordinate of a plot of $\frac{1}{v}$ versus [I] gives $\frac{K_m}{V_m[s]} + \frac{1}{V_m}$. Hence the values of K_m and V_{max} may be determined independently from the type of inhibition. This was done by measuring v as

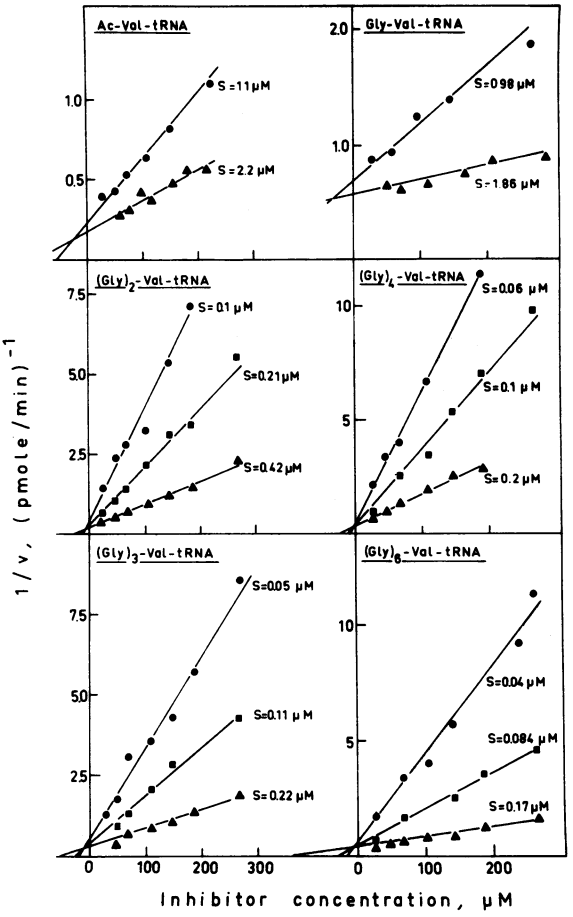

FIG. 1
Dixon plots of the inhibition of peptidyl-tRNA hydrolase activity on various substrates by uncharged tRNA.

Initial velocities were determined as described in Methods. The numbers near the lines are the substrate concentrations. Enzyme concentrations were as follows (EU/ml): 0.8 for Ac-Val-tRNA and Gly-Val-tRNA, 0.12 for $(Gly)_2$-Val-tRNA and 0.08 for $(Gly)_3$-Val-tRNA, $(Gly)_4$-Val-tRNA, and $(Gly)_6$-Val-tRNA.

the function of [I] at two or three substrate concentrations. The results are plotted in Fig. 1. The K_m and V_{max} values, obtained from these plots by solving the Dixon's equations for [I] = 0, are summarized in Fig. 2. The K_m values decrease from 7.2×10^{-7} M for Ac-Val-tRNA and Gly-Val-tRNA to about 1.7×10^{-7} M for $(Gly)_3$-Val-tRNA. Further increase of the peptide chain length does not affect the K_m. The values of V_{max} increase with the length of the peptide moiety from 3.5 pmole/min/EU for Ac-Val-tRNA and

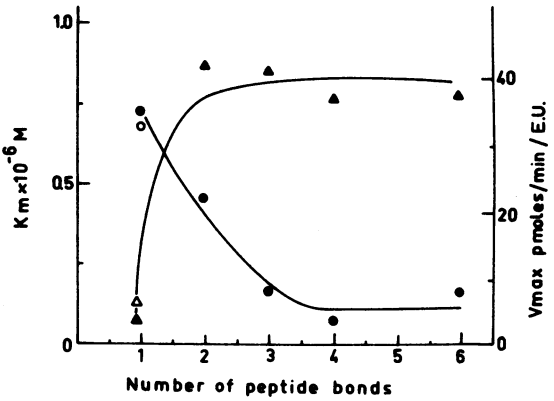

FIG. 2.
The dependence of V_m and K_m on the length of the peptide chain attached to the tRNA.
For details about the calculations — see text.
●——● K_m for $(Gly)_n$-Val-tRNA; ▲——▲ V_{max} for $(Gly)_n$-Val-tRNA; O–K_m for Ac-Val-tRNA; Δ — V_{max} for Ac-Val-tRNA.

5.7 pmole/min/FU for Gly-Val-tRNA to the maximal value of about 42 pmole/min/EU for $(Gly)_n$-Val-tRNA ($n \geq 2$).

Inhibition by tRNA

The value of the inhibition constant, Ki, and the type of inhibition, may be directly determined from the point of the intersection of two lines (two substrate concentrations) in the Dixon plot (15). As may be seen from Fig. 1, in all cases the intersection is above the abscissa and therefore, we may conclude that the inhibition is of the competitive type. The Ki may be estimated to be 10^{-5}M (±50%). Moreover, from the linearity of the plots one may deduce that the inhibition is a linear competitive one (i.e. linear dependence of inhibition on inhibitor concentration) (17), meaning that the enzyme action is inhibited by one molecule of inhibitor per one molecule of enzyme.

In order to compare affinities of the uncharged tRNA and of the peptidyl-tRNAs to the enzyme, one has to determine whether the inhibition is "mixed competitive" or "pure competitive" (16, 18). On the basis of the Dixon plot alone it is impossible to do so (16). Therefore, double reciprocal Lineweaver-Burk plots were drawn from the data of Fig. 1 for $(Gly)_4$-Val-tRNA (Fig. 3). All lines intercept at a single point on the ordinate, indicating a pure competitive inhibition.

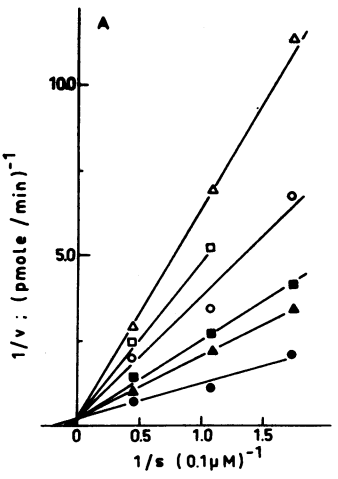

FIG. 3.
Double reciprocal Lineweaver-Burk plot of the enzyme activity on $(Gly)_4$-Val-tRNA in the presence of various concentrations of uncharged tRNA.

●——● 27 μM; ▲——▲ 46 μM; ■——■ 66 μM;
o——o 106 μM; □——□ 146 μM; Δ——Δ 186 μM.

DISCUSSION

It can be calculated that if during the process of protein biosynthesis in the cell, abortive termination would occur only once in 10^4 elongation cycles, the cell can only survive if it is equipped with an effective device to release tRNA from peptidyl tRNA. Therefore we investigated some of the kinetic properties of peptidyl tRNA hydrolase in order to test its potential to fulfill its proposed physiological role, namely that of a salvaging enzyme, regenerating tRNA from peptidyl tRNA.

According to the Michaelis and Menten equation the dissociation constant of the ES complex is equal to or smaller than the K_m value, while in the case of a pure competitive inhibitor the dissociation constant of the EI complex is equal to the Ki value. Therefore, we are able to conclude from the results presented here, that the dissociation constant of the enzyme-peptidyl-tRNA complex is smaller by at least one to two orders of magnitude than the dissociation constant of the enzyme-uncharged tRNA complex. According to Bauer et al. (9) about 0.8% of the dry weight of the E. coli W cell is tRNA. The number of cells in one gram dry material is about 3.8×10^{-12} (19), meaning 3×10^4 molecules of tRNA per cell. As the number of ribosomes per bacterial cell in the logarithmic phase is known to be of the order of one to two thousand (21), only a small portion of the tRNA molecules are possibly bound to the ribosomes. At least a part of the tRNA in the growing cell is in the form of aminoacylated tRNA. We found that the aminoacylated

tRNA inhibits the peptidyl-tRNA hydrolase with inhibition constant the same, or nearly the same as that of uncharged tRNA (results not shown). The concentration of the tRNA uncharged or aminoacylated in the cell sup. (calculated from the cell volume of 1.3×10^{-12} cm^3 (20)) is about 5×10^{-5} M.

From the data presented it can be concluded that even if peptidyl-tRNA constitutes only 1% of the tRNA concentration in the soluble part of the cell, it can be efficiently hydrolysed by the peptidyl tRNA hydrolase. Therefore no accumulation of peptidyl-tRNA which will slow down the rate of protein synthesis by limiting the amount of tRNA available for aminoacylation will occur in the growing cell.

Another conclusion which one can draw from the relative dissociation constants of the enzyme-peptidyl-tRNA complex on one hand, and of the enzyme-uncharged-tRNA complex on the other, is that the peptide moiety of peptidyl-tRNA contributes significantly to the binding of the substrate to the enzyme. As peptides and peptide esters do not inhibit the hydrolase activity, even at the high concentration of 10^{-3} M (22), it is reasonable to assume that the peptides do not interact, or interact very weakly, with the enzyme. It seems unlikely that conformational differences between peptidyl-tRNAs and the corresponding uncharged-tRNA can cause the differences in affinity to the enzyme, because so far there is no solid evidence for conformational differences between uncharged-tRNA and peptidyl-tRNA (23, 24). A possible explanation for the difference between the affinity of peptidyl tRNA and that of uncharged tRNA to the enzyme, is that the binding of the tRNA moiety of peptidyl-tRNA triggers a change in the conformation of the enzyme, allowing the peptide moiety of the peptidyl-tRNA to interact with the enzyme and to contribute significantly to the overall binding affinity of peptidyl-tRNA to the enzyme.

From the results described it is likely that maximum peptide-enzyme interaction occurs when the peptide chain length reaches four amino acid residues.

Reprint requests to: Department of Biological Chemistry, The Hebrew University of Jerusalem, Jerusalem, Israel.

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