

Changes in Activity Levels of Certain Aminoacid-RNA Ligases during the Growth Cycle of *Streptococcus thermophilus*

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The formation of enzymes which activate the amino acids, aminoacid-RNA ligases, was studied during the active growth periods of *Streptococcus thermophilus* by measuring the rate of the ATP-(³²P) pyrophosphate exchange reaction. The amino acids isoleucine, leucine and valine were activated by the crude enzyme preparations from *Str. thermophilus*; lysine and tryptophan were not activated. Only one maximum level of enzyme activity of the investigated aminoacid-RNA ligases was observed. This maximum occurred before the middle of the exponential phase and was in all cases some 2 or 3 times the activity at the beginning of growth.

The enzymes aminoacid-RNA ligases (E.C. sub-group number 6.1.1.),¹ also known as aminoacyl-sRNA synthetases and amino acid-activating enzymes, are believed to catalyze the first steps in protein biosynthesis. These enzymes catalyze reactions of the type: amino acid + sRNA + ATP \rightleftharpoons aminoacyl-sRNA + AMP + pyrophosphate. Each enzyme is considered to catalyze both the activation of a specific amino acid and its transfer to a specific soluble ribonucleic acid (sRNA).

Aminoacid-RNA ligases are very important enzymes during the growth phases of bacteria. It was therefore of interest to determine the activity levels of some of these enzymes as a function of growth time, especially as the formation of folate enzymes²⁻⁵ and B₆ enzymes⁶⁻⁹ had been studied previously in this laboratory. In the present preliminary paper we are reporting on the activities of five aminoacid-RNA ligases of *Str. thermophilus*.

EXPERIMENTAL

Cultivation of the test organism. The *Streptococcus thermophilus* strain KQ was maintained in 10-ml volumes of ScQ medium (described previously³) with monthly transfers. The inoculum was prepared from this stock culture, by transferring the organism to 10 ml of the ScQ medium. After 17 h of growth at 42° the contents of the tube were poured aseptically into 1.5 litres of the ScQ medium and incubated 15 h at 42°.

The cells were harvested by centrifugation and washed once with cold (+2°) 0.9 % NaCl solution. The washed cells were suspended in 0.9 % NaCl and kept at +2° for 48 h. The cells were then centrifuged and resuspended in a small volume of ScQ medium. The suspension was immediately poured into 1.5 litres of pre-warmed ScQ medium at 42° and the medium was aseptically transferred to vessels holding 50 ml. Incubation took place at 42° in a water bath for 5–6 h. Growth was followed turbidimetrically with a Klett-Summerson colorimeter employing filter 62. Samples were withdrawn at intervals of 15 min beginning immediately after the inoculation and cooled in an ice bath.

Preparation of enzyme solution. The cells were harvested from the growth medium (50 ml) by centrifugation and washed with cold 0.9 % NaCl solution. After centrifugation 1.5 ml of cold distilled water was pipetted into each tube, and the tubes were placed in an ethanol bath at –40°. All these phases of the work were performed at +2°. Immediately before the enzyme reaction was started, the cells were broken up by repeated freezing and thawing. The cells were frozen in an ethanol bath at –40° for 10 min and thawed in a water bath at 42° for 1.5 min; this procedure was repeated 4 times.

Determination of enzyme activity. The reaction mixture consisted of tris-HCl buffer, pH 7.5 (100 μ moles), $MgCl_2 \cdot 6H_2O$ (5 μ moles), KCl (25 μ moles), ATP (2 μ moles), (^{32}P)-pyrophosphate (specific activity 4×10^6 counts/min/ μ mole, 2 μ moles), and amino acid (2 μ moles). The volume of enzyme solution was 0.2 ml and the total volume of the reaction solution 1.1 ml. The (^{32}P)pyrophosphate solution (0.1 ml) and the reaction mixture containing the other reaction components (0.8 ml) were pipetted into reaction tubes which were placed in a water bath at 42° for 10 min before 0.2 ml of enzyme solution was added. The tubes were mixed by shaking lightly. The reaction time was 15 min at 42° in a water bath. The reaction was terminated by adding 2.0 ml of cold 5 % trichloroacetic acid to the tubes, which were then cooled in ice water and carefully shaken. ATP was separated from pyrophosphate by the method of Crane and Lipmann¹⁰ modified as follows. The precipitated protein was removed by centrifugation and a 2.0-ml sample of the supernatant was placed in a centrifuge tube and 4.0 ml of 0.1 M sodium acetate in which 50 mg of Norit A charcoal had been suspended was added. The charcoal had been activated by the method of Pennington.¹¹ The contents of the tubes were mixed by shaking and the charcoal was centrifuged down. The supernatant was poured off and the charcoal washed once with 0.05 M acetate buffer (pH 4.5) which contained disodium-pyrophosphate (0.1 M). The charcoal was washed once with acetate buffer (0.05 M, pH 4.5) and once with distilled water. After the final washing the water was decanted carefully. The charcoal was suspended by shaking in 2.0 ml of 1 N HCl. The tubes were closed with glass spheres and placed in a boiling water bath where hydrolysis took place during 15 min. After the hydrolysis the tubes were immediately cooled in an ice bath, and the charcoal was removed by centrifugation.

For the determination of radioactivity, planchets of stainless steel were employed. Two drops of glycerol was pipetted onto each planchet. A piece of filter paper was pressed tightly against the bottom of the cavity of the planchet and 0.2 ml of the hydrolyzed supernatant and three drops of 1.5 N NaOH, which neutralized the hydrochloric acid, were pipetted onto the paper. After heating the samples at 90° for 30 min, the radioactivity was measured using a Tracerlab counter for a total of 3000 counts. A control in which the enzyme solution was replaced by 0.2 ml of distilled water was included in each experiment. The counts/min obtained for the control were deducted from all other readings in each experiment. Radioactivity determinations were also made on enzyme solutions without adding any amino acid to establish the effect of free amino acids in the extract. The readings obtained in these determinations were deducted from the readings obtained for the same enzyme extract in tests where different amino acids had been added; the difference is reported as the total activity. By dividing the total activity with the corresponding Klett reading on the growth curve, a proportional cell activity was obtained, which is reported as cell activity.

RESULTS and DISCUSSION

First of all optimal experimental conditions were determined because the aminoacid-RNA ligases have not previously been studied using *Streptococcus*

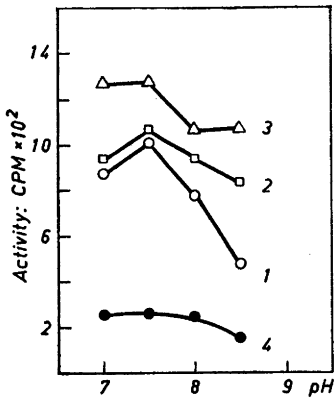


Fig. 1. Effect of pH on the activity of three aminoacid-RNA ligases of *Str. thermophilus*. 1, Leucyl-sRNA synthetase; 2, valyl-sRNA synthetase; 3, isoleucyl-sRNA synthetase; 4, control.

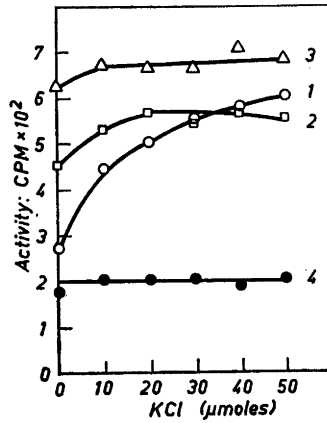


Fig. 2. Effect of potassium ions on the activity of three aminoacid-RNA ligases of *Str. thermophilus*. 1, Leucyl-sRNA synthetase; 2, valyl-sRNA synthetase; 3, isoleucyl-sRNA synthetase; 4, control.

thermophilus. The optimal pH for all three investigated enzymes (isoleucyl-sRNA synthetase, leucyl-sRNA synthetase, and valyl-sRNA synthetase) was about 7.5 (Fig. 1). The activity of leucyl-sRNA synthetase increased nearly

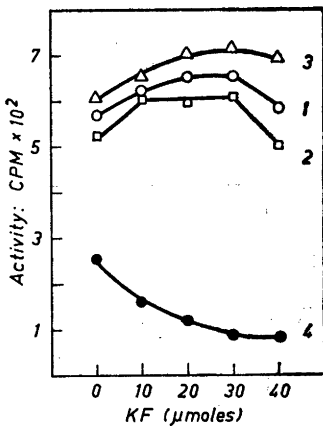


Fig. 3. Effect of fluoride ions on the activity of three aminoacid-RNA ligases of *Str. thermophilus*. The reaction solution (total volume 1.1 ml) was incubated with increasing amounts of KF. Other assay conditions as described under Experimental. 1, Leucyl-sRNA synthetase; 2, valyl-sRNA synthetase; 3, isoleucyl-sRNA synthetase; 4, control.

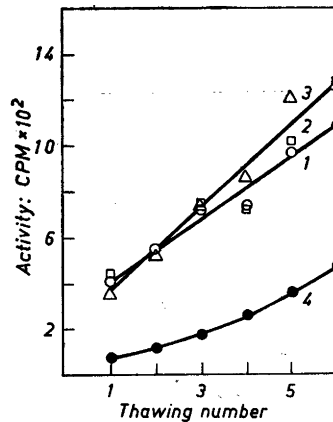


Fig. 4. Effect of the freezing and thawing treatment of the cell preparations on the activity of three aminoacid-sRNA ligases of *Str. thermophilus*. 1, Leucyl-sRNA synthetase; 2, valyl-sRNA synthetase; 3, isoleucyl-sRNA synthetase; 4, control.

two-fold when the concentration of potassium ions was raised from zero to 40 μ moles in 1.1 ml of reaction solution. The increase in activity was obtained with low concentrations of potassium ions in the case of the other two enzymes (Fig. 2). When the effect of fluoride ions was investigated by keeping the concentration of potassium ions constant with potassium chloride, it was found that also the fluoride ions increased the activities of the enzymes (Fig. 3). The cause of this phenomenon cannot be explained without additional experiments. The number of freezing and thawing treatments was found to increase the level of the enzymic activities at least up to the sixth treatment (Fig. 4). However, for practical reasons the number of freezing and thawing cycles was limited to four in all experiments. Unfortunately the effect of magnesium ion concentration on the enzyme activity could not be investigated because larger quantities than 5 μ moles precipitated magnesium fluoride in the presence of potassium fluoride. The curves plotting enzyme activity as a function of time and the amount of the enzyme preparation (Figs. 5 and 6) show that the experimental conditions employed are suitable for determinations of the enzyme activity in question. In control experiments a standard deviation equal to 3.7 % of the mean value was obtained.

Of the five investigated amino acids, lysine and tryptophan did not increase the rate of the ATP-pyrophosphate exchange reaction, but leucine, isoleucine, and valine caused a considerable increase in the rate of the reaction. As can be seen from Figs. 7–9 similar activity curves were obtained for all the enzymes activating these amino acids. After the inoculation the activity remained almost constant for half an hour (during the lag phase of growth); the activity increased rapidly at the beginning of the acceleration phase of growth and reached a maximum, 2–3 times the original activity, about an hour after growth began. As the maximum was reached during the exponential phase,

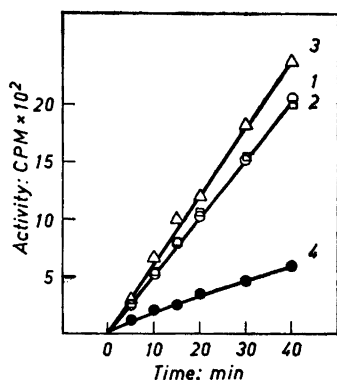


Fig. 5. Progress curves for three aminoacyl-tRNA ligases of *Str. thermophilus*. Reaction solutions and other assay conditions as described under Experimental. 1, Leucyl-sRNA synthetase; 2, valyl-sRNA synthetase; 3, isoleucyl-sRNA synthetase; 4, control.

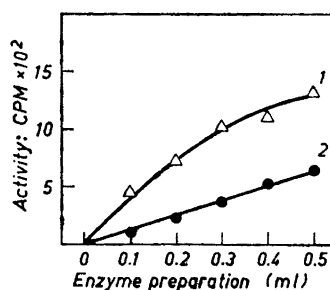


Fig. 6. Dependence of the rate of the ATP-pyrophosphate exchange reaction on the concentration of the enzyme preparation from *Str. thermophilus*. 1, Two μ moles of L-isoleucine per 1.1 ml of reaction solution; 2, control (without amino acid addition).

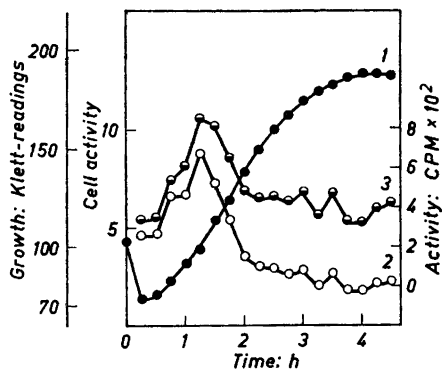


Fig. 7. Isoleucyl-sRNA synthetase activity during the growth of *Str. thermophilus*. 1, Growth; 2, activity per cell unit (scale on left); 3, total activity (scale on right).

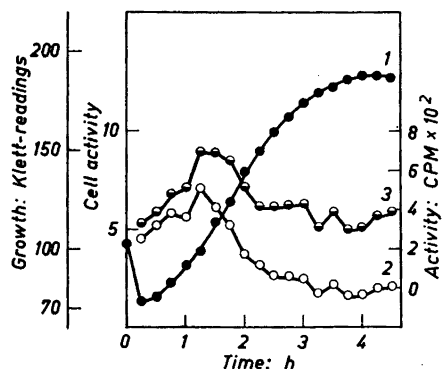


Fig. 8. Leucyl-sRNA synthetase activity during the growth of *Str. thermophilus*. 1, Growth; 2, activity per cell unit (scale on left); 3, total activity (scale on right).

the activity decreased in the later part of the same phase and in the retardation phase it was again almost equal to the activity at the beginning of the growth.

When the inoculum cells had been taken from the stationary phase, the turbidity of the growth medium at the beginning of the experiment decreased 20 to 25 %, as seen in Figs. 7–9. When the inoculum cells were taken in the exponential phase no such decrease was observed after the inoculation (these experiments are not presented in detail in this paper). It is interesting to note that in the last-mentioned case, where no lag phase at all was observed, the activities of leucyl-sRNA synthetase, valyl-sRNA synthetase and isoleucyl-sRNA synthetase began to increase immediately when growth began. These observations indicate that no very remarkable changes are likely to occur in the activities of these three enzymes, or rather in the rate of formation of these enzymes when *Str. thermophilus* cells are in the lag phase of growth. On the other hand, it was found that once the division of cells began, the formation

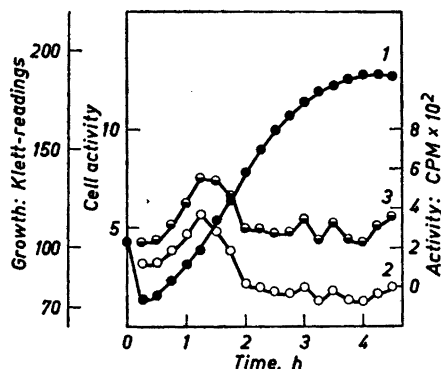


Fig. 9. Valyl-sRNA synthetase activity during the growth of *Str. thermophilus*. 1, Growth; 2, activity per cell unit (scale on left); 3, total activity (scale on right).

of the enzymes also started immediately and reached a maximum level during the exponential phase of growth. As the growth rate decreases during the retardation phase, the formation of enzymes also decreases to a level almost corresponding to that in the lag phase of growth of the organism. According to these results the three above-mentioned *Str. thermophilus* enzymes cannot be grouped among the "lag phase enzymes".²

A detailed report on our work on the formation of aminoacid-RNA ligases in *Str. thermophilus* and in *Escherichia coli* will be published later.

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