

## Formation of Amino Acid-RNA Ligases during Growth Phases of *Streptococcus thermophilus*

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A new method has been developed for the separation of ATP from reaction mixtures in amino acid-RNA ligase assays. Using the solvent ethyl methyl ketone-methanol-water-conc. hydrochloric acid (40:20:20:1 by vol.), ATP was isolated by ascending paper chromatography in 75 min. Proper conditions for disrupting cells of *Streptococcus thermophilus* strain KQ for amino acid-RNA ligase assays were determined.

The activities of amino acid-RNA ligases during various growth phases of *Str. thermophilus* KQ were determined. The most active were leucine-, isoleucine-, valine-, tyrosine-, phenylalanine-, and methionine-sRNA ligases in decreasing order of activity, the activities of other amino acid-RNA ligases being low. The measured activities of all these enzymes changed in a similar way during batch cultivation. The specific activities remained constant in the lag phase, but began to rise in the beginning of the acceleration phase, reached maximal values in the first half of the exponential phase and decreased to the original level at the beginning of the stationary phase. The specific activity rose from the beginning of the cultivation to the maximum, 1.3 to 2-fold, depending on the physiological age of the cell mass used for inoculation. The activity per unit volume of growth medium reached a maximum in the middle of the exponential phase and then remained almost constant.

Amino acid-RNA ligases (E.C. sub-group 6.1.1.) catalyze the binding of amino acids to specific RNA's.<sup>1</sup> This reaction is evidently the first step in protein biosynthesis. It may be important also in the regulation of metabolism, for it has been proposed by some authors that free sRNA inhibits RNA synthesis whereas aminoacyl-sRNA is not inhibitory.<sup>2-4</sup> This proposal has been disputed by others,<sup>5,6,9</sup> and some have claimed that aminoacyl-sRNA is the actual repressor.<sup>7,8</sup> Recently Neidhardt<sup>10</sup> has presented more detailed evidence that shows that amino acid-RNA ligases play some role in the regulation of bacterial metabolism, although the mechanism is not yet known.

In this work changes in the activities of some amino acid-RNA ligases during different growth phases of the *Streptococcus thermophilus* strain KQ were determined. *Str. thermophilus* was chosen as the test organism because

many similar studies concerning other enzymes have been performed in this laboratory using this organism.<sup>12-16</sup> A preliminary report of this work has been published.<sup>17</sup>

### EXPERIMENTAL

*Cultivation of the test organism.* *Streptococcus thermophilus* strain KQ, a strain isolated in this laboratory, was maintained in a 10 ml volume of ST-medium with monthly transfers. At the beginning of an experiment a drop of the culture was transferred to 10 ml of ST-medium, which was then incubated 15 h at 42°. The culture was poured aseptically into 0.5 l litre of ST-medium and incubated again 15 h at 42°. Cells were harvested by centrifugation (Servall GSA-2, 15 min, 3000 g) and suspended in the same volume of new ST-medium at the same temperature. Samples of this suspension were quickly added to 50 ml cultivation bottles, which were immediately placed in a water bath (42°). Every half hour one of the cultivation bottles was transferred to an ice-water bath. After cooling about 10 min, the suspension was mixed by shaking, growth was recorded by measuring the turbidity with a Klett-Summerson colorimeter using filter 62 and a 5 ml sample was pipetted into a centrifuge tube.

*Treatment of samples.* The cells were harvested by centrifugation (Servall SS-1, 10 min, 5000 g) and transferred with 5 ml of cold 0.9 % sodium chloride solution to 10 ml test tubes, where the cells were again separated by centrifugation as above. The supernatant was removed and the tubes were stored in ethanol at -35°.

The cells were transferred with 5 ml of a buffer solution to a 50 ml beaker, which was placed in an ultrasonic disintegrator (Medical & Scientific Equipments Ltd., London, 60 W, 20 kc) so that the tip of the larger probe was about 1 mm above the bottom of the beaker. The disintegrator was allowed to operate at maximal output (about 1.5 A), while the beaker was cooled with a stirred ice-water mixture so that the temperature of the cell suspension never exceeded 10° during the operation. After 8 min the disintegrator was turned off. The broken cell suspension was immediately pipetted into tubes, which were stored in an ethanol bath at -35°.

*Amino acid-RNA ligase assay.* Pyrophosphate solution, 25  $\mu$ l, and 100  $\mu$ l of substrate solution were pipetted into a series of 5 ml test tubes. Samples of the ultrasonically treated cell suspensions were thawed one at a time by shaking gently in a water bath (25°) for 45 sec. Portions (100  $\mu$ l) of the enzyme preparation were pipetted into the test tubes, the contents of which were mixed by shaking. The tubes were placed in a thermostated water bath, where reaction took place at 25° for 60 min. Then 25  $\mu$ l of cold 20 % trichloroacetic acid solution was pipetted into each tube and the tubes were shaken and cooled in an ice-water bath. 20  $\mu$ l of each reaction mixture was pipetted onto Whatman No. 1 chromatographic paper. When the spots had dried at room temperature, ATP was separated by ascending chromatography in a closed glass jar. The solvent was methyl ethyl ketone-methanol-water-conc. hydrochloric acid (min. 36 %) 40:20:20:1 by volume and the ascending time was 75 min.

The chromatograms were allowed to dry at room temperature about 30 min, and ATP spots were marked under UV light. One drop of glycerol was pipetted on a stainless steel planchet, a marked ATP spot was cut out and pressed on the bottom of the planchet. The radioactivity of the spot was measured with an endwindow Geiger tube (Tracerlab TGC-2, Mica window 1.9 mg/cm<sup>2</sup>) and that of the control (no amino acid added to the reaction mixture) was subtracted. By comparing the results with the radioactivity of the standard, the enzymic activity was calculated as  $\mu$ moles of PP bound in ATP in one minute (= microunits/100  $\mu$ l of growth medium). When this quantity was divided by the amount of protein in 100  $\mu$ l, the specific activity was obtained in mU/mg of protein.

*Protein* was estimated by adding to a tube containing 2 ml of enzyme preparation 3 ml of 10 % sulfosalicylic acid solution, shaking the tube, and measuring the turbidity after 10-20 min with the Klett-Summerson colorimeter using filter 42. The amount of protein was read from a standard curve. This method is a modification of the procedure described by Heepe *et al.*<sup>41</sup>

*Free amino acids* were extracted from the cells with boiling water and determined with ninhydrin. The method was that described by Hancock.<sup>18</sup>

*Medium and solutions.* Medium *ST* is a modification of medium *ScQ* originally described by Nurmikko *et al.*<sup>13</sup> It is composed of

Sucrose	5.00 g	CaCl <sub>2</sub> ·6H <sub>2</sub> O	0.05 g
Lactose	5.00 g	L-Glutamic acid	0.15 g
K <sub>2</sub> HPO <sub>4</sub>	3.43 g	L-Cysteine hydrochloride	0.08 g
KH <sub>2</sub> PO <sub>4</sub>	1.17 g	KCl	0.75 g
CH <sub>3</sub> COONa·3H <sub>2</sub> O	3.40 g	MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.30 g
NH <sub>4</sub> Cl	0.83 g	Solution V <sub>6</sub>	35 ml
Ascorbic acid	0.20 g	Distilled water	1000 ml

The medium was autoclaved 10 min at 105°. Biotin, 50 µg/l, was added immediately before use. All the chemicals were of analytical grade from British Drug Houses Ltd., or E. Merck AG.

*Solution V<sub>6</sub>* is composed of thiamine hydrochloride, riboflavin, nicotinic acid, *p*-aminobenzoic acid, pyridoxine hydrochloride, and calcium D-pantothenate, 30 mg/litre of each, in distilled water. Vitamins were from Hoffmann-La Roche & Co., Ltd. and British Drug Houses Ltd.<sup>1</sup>

*Buffer solution for ultrasonic treatment.* 0.25 M Tris-hydrochloric acid buffer of pH 7.5, which contained 750 mg of KCl, 400 mg of MgCl<sub>2</sub>·6H<sub>2</sub>O, and 600 mg of reduced glutathione per 100 ml. Tris was from Sigma Chemical Co., glutathione from Boehringer & Soehne GmbH, and the other chemicals from E. Merck AG.

*Pyrophosphate solution.* Tetrasodium pyrophosphate containing <sup>32</sup>P obtained from the Radiochemical Centre, England, was dissolved in distilled water in such an amount that the radioactivity of the solution was about 80 µC/ml. Tetrasodium pyrophosphate decahydrate (E. Merck AG, G.R.) was added to the solution until the final concentration of pyrophosphate was 1.5 µmole/25 µl. A standard was prepared by diluting 25 µl of this solution to 3 ml with distilled water and by pipetting, after mixing, 25 µl of the resulting solution onto a stainless steel planchet. This standard contained 0.0125 µmole of pyrophosphate and gave about 5000 cpm in the counting conditions used.

*Substrate solution.* 9 mg of ATP (disodium salt, C. F. Boehringer & Soehne GmbH) and 3–5 mg of amino acid (L forms, E. Merck AG, Fluka AG, British Drug Houses Ltd., Nutritional Biochemicals Corporation) were dissolved in 1 ml of Tris-HCl buffer (0.25 M, pH 7.5). The solution contained 1.5 µmole of ATP and 2 µmoles of amino acid in 100 µl.

## RESULTS

*Enzyme assay method.* A new method was developed for the separation of ATP from the reaction mixture. ATP was separated in 75 min by ascending chromatography on Whatman No. 1 chromatographic paper using as solvent methyl ethyl ketone-methanol-water-conc. hydrochloric acid, 40:20:20:1 by volume. The *R<sub>F</sub>* values in this system were *R<sub>F</sub>*(P) = 0.95, *R<sub>F</sub>*(PP) = 0.87, and *R<sub>F</sub>*(ATP) = 0.14. When a 25 µl sample was pipetted from a mixture where no reaction had been allowed to take place, the radioactivity of the ATP spot after chromatography was 20–40 cpm, whereas that of the pipetted sample before separation was about 50 000 cpm. The relative standard deviation of the chromatographic method was 2 % of the mean value (16 spots) when the radioactivity of the spots was about 500 cpm, which is in the middle of the activity range used in this work (100–1000 cpm).

When ATP was separated by adsorption on charcoal as proposed by DeMoss and Novelli,<sup>19</sup> the measured activities of amino acid-RNA ligases were about half of the activities determined using the chromatographic method developed in this work. The difference is evidently due to losses of ATP during adsorption and washing.

In the experiments published in the preliminary report,<sup>17</sup> the cells were broken by alternate freezing and thawing. In the course of this work it became evident, however, that the effect of this treatment depends on the physiological age of the cell mass. This is clearly seen in Fig. 1, where the activities of valine-sRNA ligase during different growth phases of *Str. thermophilus* after four cycles of freezing and thawing and after ultrasonic treatment are plotted.

When similar samples were treated varying lengths of time in the ultrasonic disintegrator, the activities of amino acid-RNA ligases were highest in samples treated 4–5 min and decreased about 3 %/min when the treatment was continued. When reduced glutathione was present in the buffer solution, the activities of amino acid-RNA ligases reached the plateau level after 6–7 min, but did not decrease on prolonged treatment. This suggests that after ultrasonic treatment of cells in buffer solution containing glutathione comparable activities of amino acid-RNA ligases are obtained in different growth phases, because all cells are completely broken without inactivating part of the enzymes.

A linear correlation existed between the measured radioactivity of the ATP spot and the amount of enzyme preparation in the reaction mixture. The relative standard deviation<sup>42</sup> of the whole assay method was 10 % of the mean for 24 samples.

The activities of amino acid-RNA ligases during growth of *Str. thermophilus* KQ. Table 1 shows that most amino acids stimulated ATP–PP exchange only a little (0–30 %) over the endogenic exchange rate, and some amino

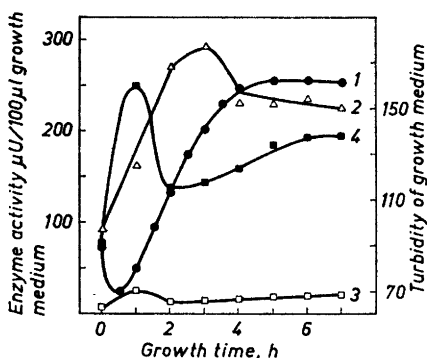


Fig. 1. Comparison of freezing and thawing and ultrasonic treatment in the disruption of *Str. thermophilus* KQ cells. Curve 1: turbidity of the growth medium (Klett colorimeter, filter 62). Curve 2: activity of valine-sRNA ligase in samples treated with ultrasonic disintegrator. Curve 3: activity of valine-sRNA ligase in corresponding samples broken by freezing and thawing (four cycles). Curve 4: values of curve 3 multiplied by 10.

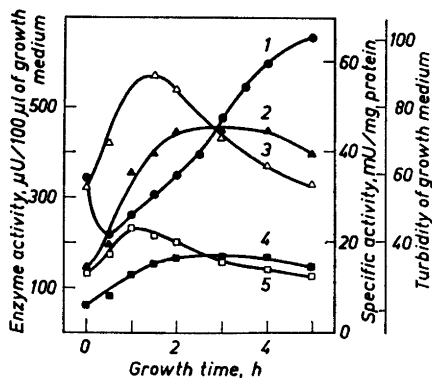


Fig. 2. Activities of valine- and tyrosine-sRNA ligases during batch cultivation of *Str. thermophilus* KQ. Curve 1: turbidity of the growth medium (Klett colorimeter, filter 62). Curve 2: activity of valine-sRNA ligase per unit volume. Curve 3: specific activity of valine-sRNA ligase. Curve 4: activity of tyrosine-sRNA ligase per unit volume. Curve 5: specific activity of tyrosine-sRNA ligase.

Table 1. Stimulation of ATP-PP exchange rate by amino acids in an enzyme preparation from *Streptococcus thermophilus* KQ cells in the middle of the exponential phase.

Added amino acid	ATP-PP $\mu$ U	exchange mU/mg	Added amino acid	ATP-PP $\mu$ U	exchange mU/mg
No amino acid					
acid	51	—	Met	132	11
Gly	58	1.0	Phe	191	19
Ala	67	2.2	Tyr	202	21
Val	448	55	Asp	45	—
Leu	632	80	Asn	64	1.8
Ile	642	81	Gln	50	—
Ser	63	1.6	Glu	48	—
Thr	71	2.7	Trp	56	0.7
Lys	58	1.0	Cys	54	0.4
Pro	51	—	Arg	44	—
His	77	3.6			

acids even seemed to inhibit it. Only valine, leucine, isoleucine, methionine, tyrosine, and phenylalanine increased the rate more than 100 %. Therefore only amino acid-RNA ligases corresponding to these six amino acids were investigated in the subsequent experiments.

Fig. 2 presents the variation of the activities of valine- and tyrosine-sRNA ligases during batch cultivation of *Str. thermophilus* KQ. The specific activities of the enzymes increase about 70 % from the beginning of the cultivation to the midpoint of the exponential phase and decrease thereafter to the original level when the retardation phase begins. If the time from the beginning of the cultivation to the end of the retardation phase is divided into 100 equal parts, the position of the maximum on this scale is 30. The activity per unit volume of growth medium rises from the beginning of the cultivation period to the point 40 and then remains almost constant. The activities of the other amino acid-RNA ligases changed in a similar way; only the activity levels were different. The form of the activity curve was similar in every cultivation, except that the rise in activity varied. The older the culture used for inoculation, the greater were the rises in the activities of amino acid-RNA ligases, but these never exceeded 100 %.

In the experiments described above, no lag phase was clearly observed in the growth of *Str. thermophilus* KQ. Therefore this was induced by separating the cells from a culture in the beginning of the exponential phase by centrifugation in the cold and transferring them immediately to new medium. Fig. 3 shows that the activities of amino acid-RNA ligases remain almost constant during the lag phase and begin to rise in the acceleration phase. Evidently these enzymes are not lag-phase enzymes.<sup>12</sup>

#### DISCUSSION

Already in 1961 von der Decken<sup>20</sup> presented a paper chromatographic method for the separation of ATP from the reaction mixture in amino acid-

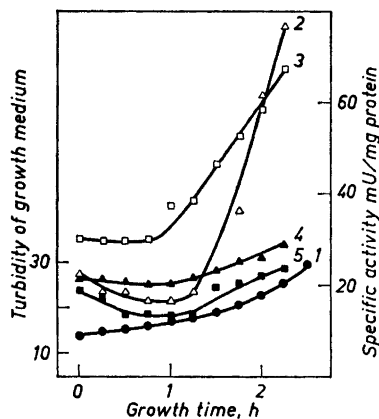


Fig. 3. Activities of amino acid-RNA ligases in the lag phase of *Str. thermophilus* KQ. Curve 1: turbidity of the growth medium (Klett colorimeter, filter 62). Curve 2: specific activity of leucine-sRNA ligase. Curve 3: specific activity of valine-sRNA ligase. Curve 4: specific activity of phenylalanine-sRNA ligase. Curve 5: specific activity of methionine-sRNA ligase.

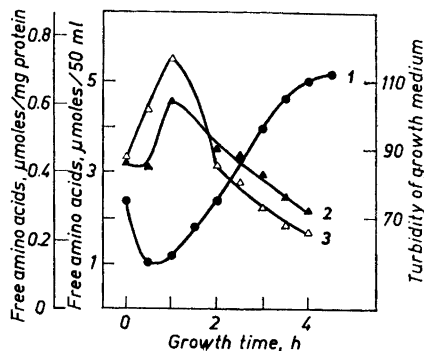


Fig. 4. The content of free amino acids in the cells of *Str. thermophilus* KQ. Curve 1: turbidity of the growth medium (Klett colorimeter, filter 62). Curve 2: the amount of free amino acids in the cells in 50 ml of growth medium. Curve 3: the amount of free amino acids/mg of dry weight.

RNA ligase assays. In this method the time of solvent flow is about three days. Loftfield and Eigner<sup>21</sup> presented another method, in which the separation is complete after one hour. This method involves the use of ion exchange paper, previously treated with pyrophosphate buffer solution and is thus more laborious than the separation method developed in this work.

DeMoss and Novelli<sup>19</sup> determined in 1955 the activities of amino acid-RNA ligases in *Escherichia coli*, *Streptococcus hemolyticus*, and *Neurospora crassa* by measuring the stimulation of ATP-PP exchange in the presence of amino acids. In each organism the most active were isoleucine-, leucine-, valine-, tyrosine-, phenylalanine-, and methionine-sRNA ligases, the activities of other ligases being low. Nisman<sup>22</sup> measured amino acid dependent ATP-PP exchange in ultrasonically treated *E. coli* cells and obtained the same results, but found also that lysine, tryptophan, histidine, and cysteine increased the exchange rate to a level well above the control rate. Maleszewski and Tysarowski<sup>23</sup> found leucine-, isoleucine-, valine-, tyrosine-, and methionine-sRNA ligases to be most active in acetone-treated yeast cells. Thus, including the work presented in this paper, the same amino acid-RNA ligases have been found most active in four different investigations and in five microbes. The results of Nisman, Maleszewski and Tysarowski and those of this work are presented in Table 2 in similar units for comparison. These show that the activities found by Nisman in *E. coli* were of the same magnitude as those found in *Str. thermophilus* KQ in this work, whereas the amino acid-RNA ligase activities in enzyme preparations from yeast were lower. The differences

Table 2. The specific activities of some amino acid-RNA ligases in broken cell preparations of *E. coli*,<sup>22</sup> yeast,<sup>23</sup> and *Str. thermophilus* KQ.

Enzyme	Specific activity mU/mg protein		
	<i>E. coli</i>	Yeast	<i>Str. thermophilus</i>
Ile-sRNA ligase	37	1.0	81
Leu-sRNA ligase	61	4.9	80
Val-sRNA ligase	60	1.0	55
Met-sRNA ligase	19	0.7	12
Phe-sRNA ligase	9	—	19
Tyr-sRNA ligase	26	0.7	21

are actually even greater, because the enzyme assays were performed at different temperatures (*E. coli* at 30°, yeast at 37°, and *Str. thermophilus* KQ at 25°).

Although the same amino acid-RNA ligases have been found most active in most investigations, this does not prove that these enzymes are most active *in vivo*, because the measured activities of different enzymes are not comparable for the following reasons.

a) The stabilities of different amino acid-RNA ligases are not equal.<sup>24,25</sup> Therefore the disruption of the cells may severely inactivate some enzymes, whereas other enzymes may not be inactivated at all. The apparent absence of many amino acid-RNA ligases in ultrasonically treated *Str. thermophilus* cells is possibly due to their inactivation during the treatment.

b) Optimal conditions for activity determinations are not similar for different amino acid-RNA ligases. For example, Moustafa and Lyttleton<sup>27</sup> have shown that the optimal pH of wheat germ amino acid-RNA ligases varies between 6.2 and 8.1. Assays are, however, usually performed in constant conditions, which are nearer the optimum for some enzymes than for others.

c) The rate of ATP-PP exchange is rather high in many broken cell preparations without any added amino acid being present.<sup>19,22,26,28</sup> This is evidently mostly due to the endogenic amino acids present in the preparations. Therefore amino acid-RNA ligases may be partly saturated by amino acids even before any amino acid is added. If the degree of saturation varies with different enzymes, the measured activities are not comparable with the actual activities of the enzymes.

d) Some amino acid-RNA ligases are not absolutely specific in the ATP-PP exchange reaction.<sup>24,29-31</sup> For example, not only valine-sRNA ligase but also isoleucine-sRNA ligase catalyzes this reaction in the presence of valine. Therefore, when valine is added to the reaction mixture, the measured ATP-PP exchange rate is higher than the activity of valine-sRNA ligase.

The activities of the same enzyme in different samples are, however, comparable, because the sources of error mentioned above change activity of the same enzyme in the same proportion in every sample. Only the effect of endogenic amino acids may vary, but this effect is small if the activity of the amino acid-sRNA ligase is clearly higher than the endogenic ATP-PP exchange rate. This requirement was evidently fulfilled in this work in the

case of valine-, leucine-, isoleucine-, tyrosine-, phenylalanine-, and methionine-sRNA ligase (see Table 1).

Shortman and Lehman<sup>32</sup> determined the activity of histidine-sRNA ligase during the growth of *E. coli*. The specific activity was constant during the lag phase, began to rise in the acceleration phase and reached a maximum in the middle of the exponential phase. This is in agreement with the result presented in this paper. The specific activity of histidine-sRNA ligase in *E. coli* did not, however, decrease before the retardation phase, whereas the specific activities of amino acid-RNA ligases in *Str. thermophilus* KQ began to fall already in the latter half of the exponential phase. The difference may depend on the test organism employed.

Pang Ting Chao and Zaitseva<sup>33</sup> estimated the activities of twenty amino acid-RNA ligases in *Azotobacter vinelandii*. Also they found the activities to be maximal in the exponential phase, but the forms of the activity curves of different amino acid-RNA ligases varied greatly in their experiments. They purified their enzyme preparations before assay by precipitation at pH 5 and with streptomycin, and finally by chromatography on a DEAE-cellulose column. Part of the enzymes was evidently lost in these treatments, which decreases the value of their results.

Many investigators have shown<sup>34-38</sup> that the rate of protein synthesis in microbes is highest in the middle of the exponential phase or immediately before. As presented above, the activities of amino acid-RNA ligases are maximal in the same phase. This does not, however, explain why the activities of amino acid-RNA ligases are highest when the need of aminoacyl-sRNA is greatest. Another problem is why the formation of amino acid-RNA ligases is prevented during the lag phase, although many other enzymes are synthesized in this phase as shown by Nurmikko *et al.*<sup>12-16</sup> Nass and Neidhardt<sup>39</sup> have presented evidence which shows that an amino acid (or some metabolic derivative of the acid) may in some cases repress the synthesis of its specific amino acid-RNA ligase. In this work, however, it was found that, like the activities of amino acid-RNA ligases, the amount of free amino acids in the cell is highest in the first half of the exponential phase in *Str. thermophilus* KQ (Fig. 4). This is in agreement with the hypothesis of Henshall and Goodwin,<sup>40</sup> who propose that free amino acids induce the synthesis of amino acid-RNA ligases. Experiments concerning the regulation of synthesis of these enzymes are in progress in this laboratory.

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