ACTA CHEMICA SCANDINAVICA 19 (1965) 135–142

Formation of Folate Enzymes during the Growth Cycle of Bacteria

IV. Formyltetrahydrofolate Synthetase Activity during the Growth of *Streptococcus thermophilus* and *Streptococcus faecalis*

VEIKKO NURMIKKO, JUHANI SOINI, SEIJA TAIMINEN and HERTTA KYYHKYNEN

Department of Biochemistry, University of Turku, Turku, Finland

The changes in formyltetrahydrofolate synthetase (FTFS) activity during the growth of *Streptococcus thermophilus* and *Streptococcus faecalis* have been studied. A rise in FTFS activity was observed in *Str. thermophilus* from the very beginning of the lag phase of growth and the activity reached a maximal level early in the exponential phase. On the basis of this observation it was concluded that FTFS functions as a typical lag phase enzyme in *Str. thermophilus*. During the growth of *Str. faecalis* two maxima appear in the FTFS activity curve, the first in the middle of the exponential phase and the second at the end of this growth phase or at the very beginning of the retardation phase.

The enzyme formyltetrahydrofolate synthetase (FTFS) (systematic name according to the Report of the Commission on Enzymes:[1] formate:tetrahydrofolate ligase (ADP), number 6.3.4.3.) also known as the formate-activating enzyme or tetrahydrofolate formylase, catalyzes a reaction which may be represented as follows: Formate + ATP + tetrahydrofolate $\rightleftharpoons$ N^{10}-formyltetrahydrofolate + ATP + orthophosphate. In previous experiments in this laboratory it was found that marked variations occur in the activities of tetrahydrofolate dehydrogenase [2,3] and serine hydroxymethyltransferase [4] in the cells of lactic acid bacteria during the active growth phases of these organisms. The present paper describes observations on the formation of FTFS (the third folate enzyme in our research project) in growing cells of *Streptococcus thermophilus* and *Streptococcus faecalis*.

EXPERIMENTAL

Reagents. ATP, ADP, and $NADPH_2$ were obtained from C. F. Boehringer & Soehne GmbH, streptomycin from Chemie Grünenthal GmbH, Bacto folic acid assay medium, Bacto-tryptone, and yeast extract from Difco, calciumleucovorin, 2-mercaptoethanol, and L-cysteine from Fluka AG, and β-glycerophosphate and thymine from Sigma. All other reagents were guaranteed reagents from E. Merck AG or AnalaR products from BDH. Tetrahydrofolic acid (FH_4) was prepared by the method of Kisliuk,[5] but modified so that the reaction solution was filtered in a nitrogen atmosphere directly into peroxide-free diethyl ether containing 1 % 2-mercaptoethanol at −40°, in which solution the washed preparate was also suspended. For the determinations the suspension was dissolved in 0.05 M potassium phosphate buffer of pH 7.4 containing 1 % 2-mercaptoethanol. The concentration of FH_4 in the solution was determined spectrophotometrically at 298 mμ ($\varepsilon_{298} = 1.9 \times 10^6$ cm² mole^{-1}). N^{10}-formyl-FH_4 was prepared by the method of Himes and Rabinowitz.[6]

Cultivation of the test organisms. The *Streptococcus thermophilus* strain KQ (Str KQ) was cultivated and stored and *Streptococcus faecalis* R preserved as described in a previous paper.[4] For each experiment 5 ml of the Str KQ cell suspension was poured aseptically into 1500 ml of ScQ medium and incubated at 42° for 17 h. The cells were harvested by centrifugation (Servall SS-3, 4000 *g*, 15 min) and suspended in 3000 ml of ScQ medium at 42°. The growth was followed turbidimetrically with a Klett-Summerson colorimeter employing filter 62. *Str. faecalis* R was transferred from the stock culture to 5 ml of sterilized TSHGA medium and incubated 10 h at 37°. The cells were then poured aseptically into 500 ml of TSHGA medium and again incubated 10 h at 37° before they were harvested by centrifugation (4000 *g*, 15 min), washed twice with 0.9 % NaCl solution and suspended in 6000 ml of growth medium at 37°. Bacto folic acid assay medium containing 0.2 mg of pteroyl-L-glutamic acid per litre was used as growth medium. The growth was followed turbidimetrically with a Klett-Summerson colorimeter employing filter 62.

Preparation of enzyme extract. Samples containing about 25 mg dry weight of cells were withdrawn during the growth and quickly cooled in ice water. The cells were harvested by centrifugation (4000 *g*, 15 min) and washed twice with cold 0.9 % NaCl solution. The Str KQ cells were suspended in 3 ml of 0.05 M potassium maleate buffer, pH 7.4, to which 2-mercaptoethanol had been added just before to make it 0.1 M. The *Str. faecalis* R cells were suspended in 3 ml of distilled water because it was found that the enzyme activity was well preserved in the water. The cells were broken by treating the sample for 5−10 min in a MSA ultrasonic apparatus (18 000−20 000 kc, 1.0−1.5 A). During the treatment the sample was kept in ice water to prevent over-heating. The suspension was centrifuged at 1° (Servall RC−2, 3500 *g*, 15 min) and 250 mg of streptomycin was added to the clear supernatant which was then left to stand for 5 min at room temperature. The precipitate was separated by centrifuging at 1° (23 500 *g*, 15 min) and the supernatant was made to 0.34 saturation with ammonium sulphate and centrifuged clear after 5 min (23 500 *g*, 15 min). The supernatant was again made to 0.47 saturation with ammonium sulphate and left to stand overnight at 4°. The precipitate was centrifuged (23 500 *g*, 15 min) and dissolved in 2 ml of 0.05 M potassium maleate buffer of pH 7.4 and 0.1 M in 2-mercaptoethanol. The protein content of the solution was determined with sulphosalicylic acid.[7] Crystalline bovine serum albumin was used as a standard.

Formyltetrahydrofolate synthetase (FTFS) assay. For the reaction the following substances were pipetted into test tubes (8 × 70 mm): 10 μmoles of $MgCl_2 \cdot 6H_2O$, 40 μmoles of sodium formate, 5 μmoles of ATP (dissolved in distilled water, pH adjusted to 7.0), 1 μmole of FH_4, 0.1 ml of 1 M triethanolamine (pH 8.0), 0.02−0.05 mg of enzyme protein, and distilled water to make up a volume of 1.0 ml.[8] The pipetting was performed with the tubes in ice water. The reaction took place at 37° and the reaction time was 10 min. The reaction was stopped by adding 2.0 ml of 0.3 N HCl. When the protein had precipitated the mixture was centrifuged clear. The absorption of the solution was measured with a Beckman DU spectrophotometer at wavelength 350 mμ after 10−30 min. A mixture lacking the sodium formate was used a control.

Determination of 10-formyltetrahydrofolate deacylase activity. The stability of the 10-formyl-FH_4 formed in the above reaction was determined by the method of Huennekens and Scrimgeour.[9]

Effect of thymine and leucovorin on the activity of FTFS in Str KQ cells. The strain Str KQ was grown in 1000 ml of ScQ medium containing increasing amounts of thymine and leucovorin. After 2 h the cells, then in the early exponential phase, were harvested by centrifugation and washed. The precipitated protein was separated and the enzyme activity determined. The activity was calculated as a percentage of the activity in cells grown in a medium lacking thymine and leucovorin.

RESULTS

Before the activity determinations during bacterial growth were made, the rate of the reaction catalyzed by FTFS was determined with varying reaction times and quantities of certain components of the reaction mixture. The results are presented in Figs. 1—4. On the basis of these results the reaction mixture described under Experimental was chosen. This reaction mixture is the same as that used by Rabinowitz and Pricer, Jr.,[8] except that the quantity of FH_4 is here 1 μmole.

As seen from Figs. 5 and 6, the FTFS activity in the *Str. thermophilus* strain KQ increases from the beginning of the lag phase of growth and reaches maximum at the very beginning of the exponential phase. Fig. 5 shows that the FTFS activity in the inoculum was 1.1 μmoles of N^{10}-formyl-FH_4/mg/10 min, and that the activity increased 2.7-fold during the lag and acceleration phases to the value 3.1 μmoles of N^{10}-formyl-FH_4/mg/10 min. In the experiment to which Fig. 6 refers, the FTFS activity in the inoculum was higher than it was in the preceding experiment, or 3.1 μmoles of N^{10}-formyl-FH_4/mg/10 min, and increased only 1.9-fold to 5.9 μmoles of N^{10}-formyl-FH_4/mg/10 min. After the maximum, the activity decreased during the exponential phase until it was equal to the activity at the beginning of the lag phase.

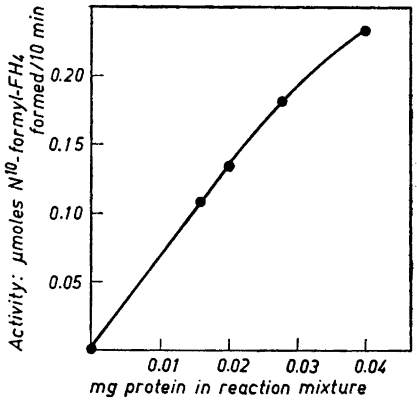

Fig. 1. Effect of protein concentration on the rate of the reaction catalyzed by FTFS in *Str. thermophilus* KQ. The reaction mixtures were composed as described in the text but contained varying amounts of protein. Reaction time 10 min.

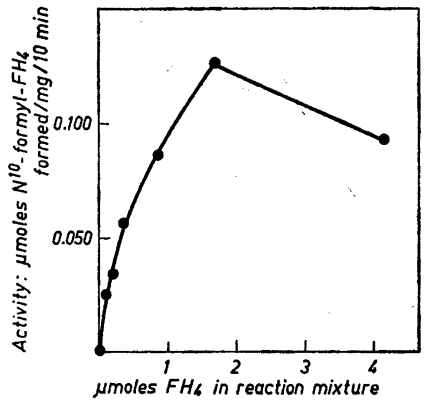

Fig. 2. Effect of FH_4 on the rate of the reaction catalyzed by FTFS in *Str. thermophilus* KQ. The reaction mixture was composed as described in the text but contained 0.05 mg of protein. Reaction time 10 min.

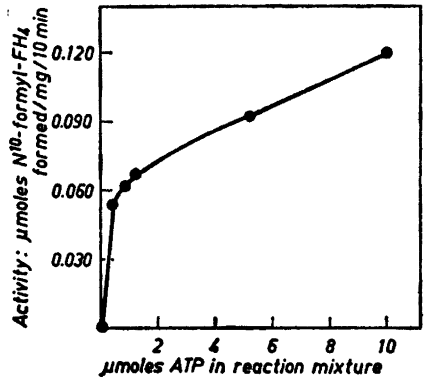

Fig. 3. Effect of ATP on the rate of the reaction catalyzed by FTFS in *Str. thermophilus* KQ. The reaction mixture was composed as described in the text but contained 0.05 mg of protein.

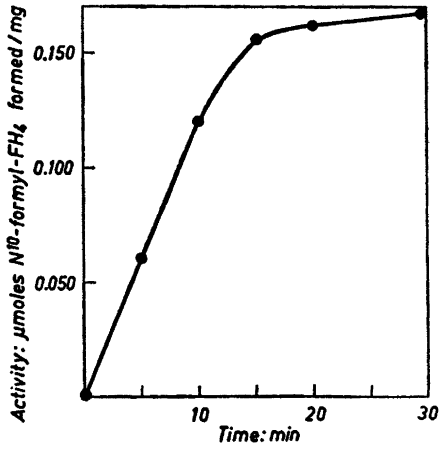

Fig. 4. Effect of reaction time on the formation of N^{10}-formyl-FH_4 in *Str. thermophilus* KQ. The reaction mixture was composed as described in the text but contained 0.05 mg of protein.

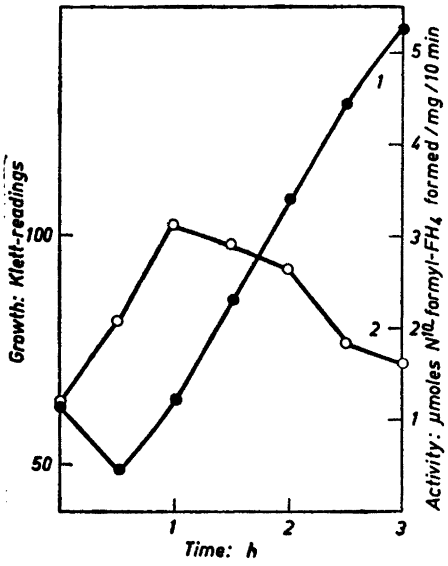

Fig. 5. FTFS activity during the growth of *Str. thermophilus* KQ. 1, Growth; 2, enzyme activity.

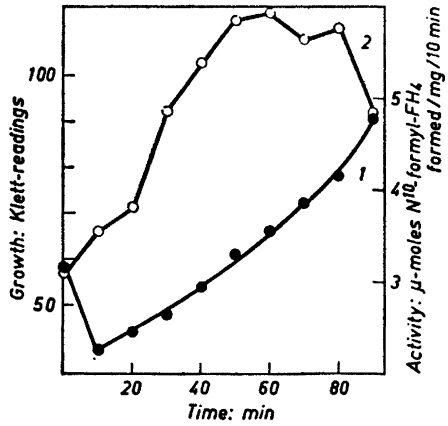

Fig. 6. FTFS activity during the lag and acceleration phases of growth of *Str. thermophilus* KQ. 1, Growth; 2, enzyme activity.

Table 1. Effect of thymine on the FTFS activity in *Str. thermophilus* KQ. The cells used in the experiment were taken from the early exponential phase.

Thymine, μmoles per 1000 ml of medium	Decrease in enzyme activity expressed in per cent of the control value
0.10	0 – 5.0
0.25	24.6 – 25.5
0.50	15.4 – 18.8
1.0	0 – 5.0

Evidently the level of the FTFS activity at the beginning of the lag phase depends on the activity level in the inoculum cells at the end of the pre-cultivation period. Variations occurred in the different experiments even though the time the inoculum was prepared was constant at 17 h. These variations may have been due to a so rapid decrease in enzyme activity at the end of the exponential phase that the FTFS activity of different cultures varied after 17 h of incubation. The decrease in FTFS activity was not caused by the decrease in the pH because a similar activity curve was obtained in experiments in which the pH was kept constant by addition of sodium hydroxide.

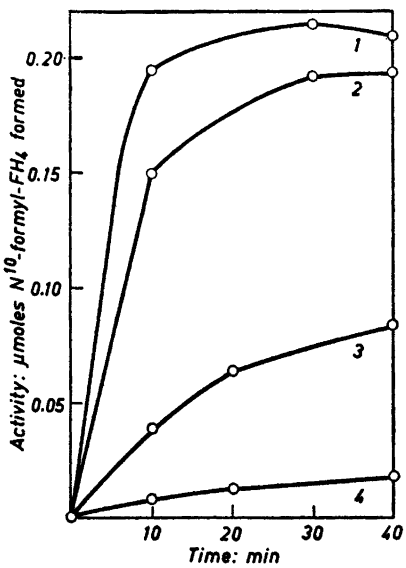

Fig. 7. Effect of reaction time and protein concentration on the FTFS activity in *Str. faecalis* R. The reaction mixture was composed as described in the text but contained protein as follows: curve 1, 3.96 mg; 2, 1.98 mg; 3, 0.99 mg, and curve 4, 0.40 mg.

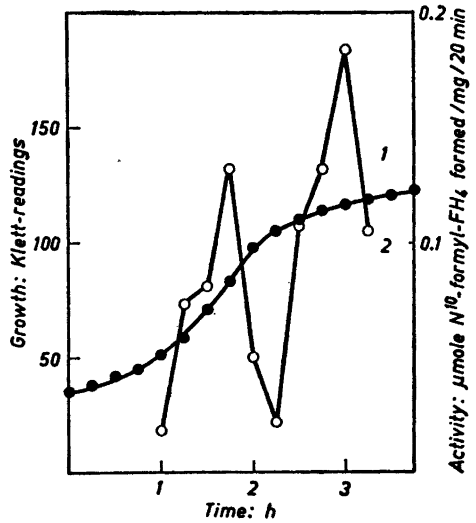

Fig. 8. FTFS activity during the growth of *Str. faecalis* R. 1, Growth; 2, enzyme activity.

The effect of thymine and calcium leucovorin on the FTFS activity in Str KQ cells was studied with the cells in the early exponential phase of growth. It was found that about 0.25 mM thymine caused the greatest fall in enzyme activity (Table 1). Calcium leucovorin also caused a decrease in FTFS activity, but no large variations (using concentrations of 1—200 μM) were observed. The decrease in FTFS activity caused by calcium leucovorin was on an average 24.5 %, independently of the leucovorin concentration.

In connection with the determination of the FTFS activity in Str KQ, the enzyme preparation was also tested for formyltetrahydrofolate deformylase (N^{10}-formyl-FH_4-deacylase), but this enzyme was not detected. Accordingly, there was no need to correct the FTFS activity values.

In the investigation of the FTFS activity in cells of *Str. faecalis* R the same reaction mixture was used as in the experiments with *Str. thermophilus* KQ, except that the amount of protein in the reaction mixture was 0.5—1.0 mg and the reaction time 20 min. The reaction rate is shown as a function of time and protein concentration in Fig. 7. No lag phase was obtained with *Str. faecalis* R: cell division took place immediately after the transfer to the growth medium. Fig. 8 shows the typical FTFS activity curve for *Str. faecalis* R. The enzyme activity curve exhibited two maxima, one in the middle of the exponential phase and another at the end of the same phase or at the beginning of the retardation phase. At the second maximum the FTFS activity was about 64 % higher than at the first. The occurrence of FTFS activity maxima and minima in repeated experiments is shown in Table 2. The FTFS activity values varied slightly in different experiments, probably owing to varying

Table 2. Occurrence of maximum and minimum levels of FTFS activity in repeated experiments. The positions of the maximum and minimum levels are expressed as percentages of the entire growth time with the time between the moment of inoculation and the beginning of the stationary phase taken as 100.

Number of experiments	Per cent of growth time		
	First maximum	Minimum	Second maximum
1	56	62	75
2	60	70	90
3	29	36	50
4	72	78	100
5	43	50	64
6	43	57	78
7	44	56	81
8	63	69	87
9	60	75	80
10	44	56	75
11	44	56	69
12	82	88	94
13	50	55	80
Average	53	62	81

enzyme activities in the inoculum cells. Thus the same phenomenon was observed with *Str. faecalis* R as with *Str. thermophilus* KQ cells. When the pH of the medium was kept constant during the growth, no changes in the FTFS activity curves resulted.

DISCUSSION

When one compares the variations in FTFS activity expressed as a function of the growth time, one notices that the investigated lactic acid bacteria differ in a very interesting way from each other in this respect. The FTFS activity increases from the beginning of the lag phase in the *Str. thermophilus* cells and reaches a maximum level at the end of the acceleration phase or the beginning of the exponential phase. After the maximum the activity decreases during the exponential phase almost to the level at the beginning of the lag period. The changes in FTFS activity in growing *Str. thermophilus* cells thus justify the inclusion of the enzyme among the so-called lag phase enzymes.[2] On the other hand, two maximum levels are clearly observed in the FTFS activity curve of *Str. faecalis* cells, one in the middle of the exponential phase and another at the end of that phase or at the very beginning of the retardation phase. No lag period of growth at all was observed with this organism. Changes in enzyme activity as large as these during the bacterial growth cycle are very interesting. It is difficult to draw any conclusions about the factors causing the large variations at this stage of the work, but the results presented show clearly that the great variations in the activity of a certain enzyme and, evidently, in the formation of that enzyme may depend on the bacterium chosen, and probably also on the growth medium. In this respect the enzyme FTFS is similar to another folate enzyme that we have studied, serine hydroxymethyltransferase. The shapes of the activity curves obtained for the latter enzyme during the growth cycles of three different lactic acid bacteria (*Str. thermophilus*, *Str. faecalis* R, and *Lb. arabinosus* 17—5) differed clearly from each other.[4]

The inhibitory effect of thymine on the FTFS activity is interesting. It might be possible to explain the finding by assuming that N^{10}-formyl-FH_4 (and N^{5-10}-methylene-FH_4) functions as a precursor in the formation of the methyl group of thymine in the *Str. thermophilus* cells. In this connection it may be mentioned that Friedkin[10] and Humphreys and Greenberg[11] have shown that the methyl group of thymine can be formed from N^{5-10}-methylene-FH_4, and Roberts and Nichol[12] that it can be formed from N^{10}-formyl-FH_4.

Acknowledgement. This investigation has been financed by a grant from the *United States Department of Agriculture, Agricultural Research Service.*
The assistance of Mrs. Doris Lindblad and Mrs. Raili Nuolio is gratefully acknowledged.

REFERENCES

1. Report of the Commission on Enzymes of the International Union of Biochemistry 1961, Pergamon Press, New York, p. 140.
2. Soini, J. and Nurmikko, V. *Acta Chem. Scand.* **17** (1963) 947.
3. Nurmikko, V., Soini, J. and Äärimaa, O. *Acta Chem. Scand.* **19** (1965). 129.

4. Nurmikko, V., Soini, J., Terho, T. and Kantonen, U.-M. *Acta Chem. Scand.* **18** (1964) 627.
5. Kisliuk, R. L. *J. Biol. Chem.* **227** (1957) 805.
6. Himes, R. H. and Rabinowitz, J. R. *J. Biol. Chem.* **237** (1962) 2903.
7. Instructions for the use of a Klett-Summerson colorimeter, in the section: The determination of protein in cerebrospinal fluid, Klett Manufacturing Co., New York.
8. Rabinowitz, J. C. and Pricer, Jr., W. E. *J. Biol. Chem.* **237** (1962) 2898.
9. Huennekens, F. M. and Scrimgeour, K. G. In Colowick, S. P. and Kaplan, N. O. *Methods in Enzymology* **6** (1963) 373.
10. Friedkin, M. *Federation Proc.* **16** (1957) 183.
11. Humphreys, G. K. and Greenberg, D. M. *Arch. Biochem. Biophys.* **78** (1958) 275.
12. Roberts, D. and Nichol, C. *J. Biol. Chem.* **237** (1962) 2278.

Received November 9, 1964.