ALTERATION OF STRUCTURE OR LEVEL OF THREONYL-tRNA-SYNTHETASE IN BORRELIDIN RESISTANT MUTANTS OF *E. COLI*

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1. Introduction

Borrelidin inhibits the growth of *E. coli* effectively [1, 2]. Since no other biochemical reaction is known to be attacked directly by Borrelidin than the enzymatic activity of the threonyl-tRNA-synthetase (ThRS) [2, 3], we isolated Borrelidin-resistant mutants of *E. coli* K12 and *E. coli* B with the aim of finding mutants with an altered structure or level of ThRS.

The ThRS of fifteen Borrelidin-resistant mutants of each *E. coli* strain was investigated. The properties of the ThRS of two Borrelidin-resistant mutants of *E. coli* K12 has been analysed extensively [4]. Here now we describe the characterization of the ThRS of the remaining Borrelidin-resistant mutants. By means of determination of enzyme constants and antibody neutralisation curves it is shown that the Borrelidin-resistant mutants can be divided into three groups: one group of mutants exhibits constitutively increased levels of wildtype ThRS, the second group structurally altered ThRS, and in a third group of mutants no alteration of the structure or level of ThRS could be detected. This suggests that Borrelidin-resistance in the latter mutants is due to some other reason than alteration of ThRS activity.

Since it is known that the ThRS participates in the regulation of formation of the threonine biosynthetic enzymes [2, 5], the level of aspartokinase was also determined in the Borrelidin-resistant mutants grown in the presence and absence of Borrelidin.

2. Materials and methods

2.1. *Bacterial organisms and growth conditions*

The *E. coli* wildstrains used were *E. coli* K12B [6, 7] and *E. coli* B [8]. From these strains Borrelidin-resistant mutants were isolated without using any mutagen by plating 4×10^8 cells per minimal medium plate containing 139 μM Borrelidin. The frequency of Borrelidin resistance was 1×10^{-8}. The minimal medium on the plates and the liquid cultures was as described [9, 10]. Cell growth has also been described [10]. In experiments in which cells were grown for determination of aspartokinase the minimal medium was supplemented with 2.5×10^{-3} M l-lysine in order to repress the lysine-sensitive aspartokinase [11]. In experiments in which Borrelidin was added to liquid growing cultures it was performed as previously described [2], the final Borrelidin concentration being 13.9 μM.

2.2. *Determination of the ThRS activity and enzyme constants*

Cell-free extracts of 300 ml samples of cultures were prepared and the activity of ThRS determined as described [4]. One unit of enzyme activity is defined as the attachment of 1 μmole threonine to tRNA threonine to tRNA per hour. K_M-values and V_{max} were determined according to the method of Lineweaver and Burk [12], Hanes [13] and Hofstee [14]; all three methods gave similar results.

2.3. *Immunological procedures*

The antiserum used was a specific anti-ThRS-serum

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Table 1

Growth of *E. coli* strains in minimal medium, preparation of crude extract and determination of the ThRS activity and enzyme constants were performed as described under Materials and methods.

E. coli K12-strain	Threonyl-tRNA-synthetase			Antiserum-neutralisation curve for the ThRS
	Specific activity (Units/mg protein)	K_M for threonine ($M \times 10^{-5}$)	V_{max} – (Units/ 10 μg protein)	
K12B	0.034	10.5	3.3	
I				
Bor Res 3	0.195	10.2	13.2	
Bor Res 5	0.204	11.0	22.0	
Bor Res 9	0.240	13.0	22.0	
Bor Res 10	0.164	14.0	18.2	
Bor Res 15	0.340	12.0	25.0	
II				
Bor Res 2	0.095	0.7	2.4	
Bor Res 4	0.175	2.3	3.6	
Bor Res 6	0.130	2.1	3.0	
Bor Res 7	0.125	0.5	1.5	
III				
Bor Res 1	0.032	8.5	2.5	
Bor Res 11	0.037	13.0	3.6	
Bor Res 12	0.042	14.0	3.8	
Bor Res 13	0.038	9.5	3.9	
Bor Res 14	0.032	10.0	2.4	
IV				
Bor Res 8	0.002	130.0	2.1	

The threonine concentration for determination of the specific activity of the ThRS was limiting with 2×10^{-5} M for the wildtype, whereas the two other substrates were in excess with 2×10^{-3} M ATP and 1×10^{-5} M *E. coli* B $tRNA^{threo}$. The antiserum neutralization curves were prepared as described in the legend to fig. 1. As control in each experiment the antiserum neutralization curve for the ThRS of the wildtype was determined.

[4]. The neutralization curves of the ThRS were prepared according to Cinader [15] and Pollock [16] by adding increasing amounts of antiserum to a constant quantity of enzyme as already described [7].

2.4. *Protein concentration*

Protein concentration was determined by the standard colorimetric method [17], using human albumin as standard protein.

2.5. *Aspartokinase measurements*

These were performed according to the methods of Black and Wright [18] and Stadtman et al. [11] as previously described [2]. Enzyme activities are expressed as OD 540 nm per mg protein per 30 min.

3. Results and discussion

It is known that the level of several aminoacyl-tRNA-synthetases is reactively increased under certain growth conditions, however, in most cases only by a factor of about three [19–23]. Thus for selecting Borrelidin-resistant mutants of *E. coli* B, a Borrelidin concentration was chosen which is ten times the amount inhibiting the growth of these

wildstrains to about fifty percent. By use of this relatively low Borrelidin concentration the chance of finding Borrelidin-resistant mutants in which the resistance is due to constitutively increased levels of wildtype ThRS should be increased.

The ThRS of fifteen Borrelidin-resistant mutants of *E. coli* K12B was investigated (table 1): the specific activity of the ThRS in crude extracts of the mutants was determined using subsaturated concentration of threonine in regard to the K_M-value for threonine of the wildtype ThRS [4]. Thus an increased specific activity of ThRS would resemble either an increased level of ThRS or a lowered K_M-value for threonine. Nine of the fifteen mutants exhibit an increased specific activity of their ThRS by a factor of four to nine (table 1). In a next series of experiments the K_M-value for threonine of the ThRS was determined in crude extract of the mutants (table 1). This enzyme constant had been proven to be similar regardless whether it was determined in crude extract or purified ThRS-preparation [4]. Similar results were obtained with various other aminoacyl-tRNA-synthetases [24–26]. Table 1 shows that the mutants with increased specific activity of their ThRS can be divided into two groups according to their K_M-value for threonine: in five of the mutants the K_M-value of the ThRS is like the one of the wildtype enzyme, the K_M-value of four of the mutants, however, is lowered by a factor of five to twenty. These results demonstrate that the ThRS of the latter four mutants is structurally altered. The ThRS of the five mutants of group I behaves like the wildtype enzyme indicating that in these mutants the increased specific activity is apparently due to an increased level of structurally unaltered ThRS. This indication was further supported by comparison of the antiserum-neutralization curve of their ThRS with the one of the wildtype enzyme. It is known that a different shape of the antiserum-neutralization curve is due to an altered structure of the enzyme [7, 27]. Fig. 1 shows the antiserum-neutralization curve for the ThRS of the wildtype of Bor Res 2 and Bor Res 3. The curve for the ThRS of Bor Res 3 is like the one for the wildtype enzyme whereas the one for Bor Res 2 is shaped differently. These results are in agreement with the wildtype-like K_M-value for threonine of the ThRS of Bor Res 3 and the altered enzyme constant of Bor Res 2. In all other cases the shape of the antiserum-neutralization curve

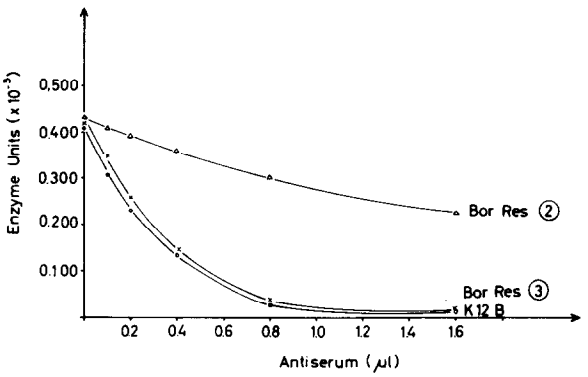

Fig. 1. Growth of *E. coli* strains, preparation of crude extracts and of the antiserum-neutralization curves was performed as described under Materials and methods. The amount of crude extract taken for preparing the curves was chosen so that in the sample without any antiserum a ThRS activity resulted between 0.400 and 0.500 enzyme units; (o–o–o) Shows the neutralization curve for the ThRS of K12B; (Δ–Δ–Δ) for the ThRS of Bor Res 2 and (x–x–x) for the ThRS of Bor Res 3.

of the ThRS is also only altered for those ThRS's which exhibit a different K_M-value (table 1). Our conclusion that the mutants of group I are Borrelidin resistant because of an increased level of their ThRS is further supported by the determination of the V_{max}-value of their ThRS (table 1): their V_{max}-value is increased by a factor similar to the factor by which their specific activity, determined with limiting amounts of threonine, is increased. The V_{max}-value of the ThRS of group II, however, does not show any correlation to the specific activity of the enzyme, as is to be predicted for a structurally altered ThRS.

There was a third group of Borrelidin-resistant mutants the ThRS of which behaved like the wildtype enzyme in all parameters investigated (table 1). Though we did not investigate these mutants further we suggest that Borrelidin resistance in these mutants is due to membrane alteration.

In group IV of the Borrelidin-resistant mutants one mutant is placed with rather puzzling enzyme constants: the specific activity of its ThRS is even lowered by a factor of about eighteen, the K_M-value for threonine is increased by a factor of about thirteen, though its V_{max}-value is only slightly decreased and the shape of the antiserum-neutralization curve is

Table 2
Growth of the *E. coli* strains, preparation of crude extracts and determination of aspartokinase was performed as described under Materials and methods.

E. coli K12 strain	Aspartokinase in cell-free extracts of cells grown	
	Without Borrelidin	In the presence of Borrelidin
K12B	0.104	0.475
I		
Bor Res 3	0.130	0.450
Bor Res 5	0.180	0.420
Bor Res 9	0.093	0.143
Bor Res 10	0.145	0.480
Bor Res 15	0.148	0.267
II		
Bor Res 2	0.095	0.125
Bor Res 4	0.102	0.220
Bor Res 6	0.060	0.130
Bor Res 7	0.090	0.110
III		
Bor Res 1	0.120	0.170
Bor Res 11	0.070	0.140
Bor Res 12	0.068	0.146
Bor Res 13	0.095	0.290
Bor Res 14	0.095	0.093
IV		
Bor Res 8	0.101	0.110

In each experiment cells were grown exponentially for more than three generations in the absence or presence of Borrelidin.

drastically altered. When adding all other nineteen amino acids in a molarity of 5×10^{-4} to the ThRS assay and determining its ThRS activity using 1×10^{-4} M threonine the ThRS activity was not affected, demonstrating that the observed incorporation of cpm was totally due to incorporation of threonine. Furthermore, it was ruled out that there is an inhibitor for the ThRS in the crude extract of Bor Res 8: when mixing crude extract of this mutant with crude extract of the wildtype K12B one to one before determining the ThRS activity, the resulting enzyme activity is the sum of the activity of either extract of all other fourteen Borrelidin-resistant mutants. In no case could an inhibitor or activator of ThRS be detected in any one of the extracts.

In the wildstrain *E. coli* K12B a 5-fold derepression of threonine biosynthetic enzymes occurs during growth limitation by Borrelidin and from these results it was deduced that the ThRS participates in the regulation of the formation of the threonine biosynthetic enzymes [2]. For getting further information about the regulation of the formation of threonine biosynthetic enzymes the level of aspartokinase was determined in the Borrelidin-resistant mutants of *E. coli* K12 grown in the absence and presence of Borrelidin (table 2). The Borrelidin concentration in the growth medium was the one which leads to a fifty percent growth inhibition in the wildstrain [2]. As expected, no reduction of growth rate was observed with the Borrelidin-resistant mutants growing in the presence of this Borrelidin concentration. Table 2 shows that during growth in the presence of Borrelidin in some of the mutants the level of aspartokinase is derepressed. No strict correlation between the properties of the ThRS in the mutants and the degree of derepression of aspartokinase could be observed (table 2). However, the highest aspartokinase levels were found in mutants of group I. These results do not allow a conclusion in which way the ThRS participates in regulation of formation of the threonine biosynthetic enzymes. They are still in agreement with one of the following possibilities: the regulating compound is charged threonyl-tRNA or some other product of the enzymatic action of the ThRS, the ThRS-threonyl-tRNA complex or the ThRS itself.

Fifteen Borrelidin-resistant mutants of *E. coli* B were analyzed in the same way as described for the mutants of *E. coli* K12B. Only mutants of group II (7 mutants) and of group III (8 mutants) were found. This means none of the Borrelidin-resistant mutants of *E. coli* B exhibits an increased level of wildtype ThRS, though five of the fifteen Borrelidin resistant of *E. coli* K12 did. It has to be investigated whether this difference is due to different properties of *E. coli* K12 and *E. coli* B as known for some of their biochemical features [28, 29]. But as the mutants of group II of *E. coli* K12B, the mutants of *E. coli* B with structurally altered ThRS exhibit a lowered K_M-value for threonine by a factor of three to fifteen. Thus we are describing mutants of *E. coli* K12 and *E. coli* B which exhibit an increased affinity for at least one of their substrates. The structural alteration of aminoacyl-tRNA-synthetases reported so far

was such that they showed a lowered affinity for their substrates (cf. [30]). This is probably due to the different selection conditions used so far, i.e. auxotrophy or bradytrophy for an amino acid or temperature sensitivity.

For elucidating the molecular devices regulating the formation rate of ThRS we are now studying the genetical alterations and biochemical responses under various growth conditions in group I and group II of our Borrelidin-resistant mutants.

References

[1] Hütter, R., Poralla, K., Zachau, H.G. and Zähner, H. (1966) Biochem. Ztschr. 344, 190.
[2] Nass, G. Poralla, K. and Zähner, H. (1969) Biochem. Biophys. Res. Commun. 34, 84.
[3] Nass, G., Poralla, K. and Zähner, H. (1971) Naturwiss. 58, 603.
[4] Paetz, W. and Nass, G. (1973) Eur. J. Biochem. 35, 331.
[5] Nass, G. (1969) Zentralbl. f. Bakteriol. I. Orig. 212, 239.
[6] Benzer, S. (1955) Proc. Natl. Acad. Sci. U.S. 41, 344.
[7] Fangman, W.L., Nass, G. and Neidhardt, F.C. (1965) J. Mol. Biol. 13, 202.
[8] Ennis, H.L. and Gorini, L. (1961) J. Mol. Biol. 3, 439.
[9] Fraenkel, D. and Neidhardt, F.C. (1961) Biochim, Biophys. Acta 53, 96.
[10] Nass, G. (1967) Mol. Gen. Genet. 100, 216.
[11] Stadtman, E.R., Cohen, G.N., Lebras, G. and Robichon-Szulmajster, H.D. (1961) J. Biol. Chem. 236, 2033.
[12] Lineweaver, H. and Burk, D. (1934) J. Am. Chem. 56, 658.
[13] Hanes, C.S. (1932) Biochem. J. 26, 1406.
[14] Hofstee, B.H.J. (1959) Nature 184, 1296.
[15] Cinader, B. (1955) Bull. Soc. Chim. Biol. 37, 761.
[16] Pollock, M.R. (1956) J. Gen. Microbiol. 14, 90.
[17] Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265.
[18] Black, S. and Wright, N.G. (1955) J. Biol. Chem. 213, 27.
[19] Nass, G. and Neidhardt, F.C. (1967) Biochim. Biophys. Acta 134, 347.
[20] Williams, L.S. and Neidhardt, F.C. (1969) J. Mol. Biol. 43, 529.
[21] Archibold, E.R. and Williams, L.S. (1972) J. Bacteriol. 109, 1020.
[22] Gahr, M. and Nass, G. (1972) Mol. Gen. Genet. 116, 348.
[23] Parker, J. and Neidhardt, F.C. (1972) Biochem. Biophys. Res. Commun. 49, 495.
[24] Hirshfield, I.N., Dedeken, R., Horn, C.P., Hopwood, D.A. and Maas, W.K. (1968) J. Mol. Biol. 35, 83.
[25] Hoffmann, E.P., Wilhelm, R.C., Konigsberg, W. and Katze, J.R. (1970) J. Mol. Biol. 47, 619.
[26] Folk, W.R. and Berg, P. (1970) J. Bacteriol. 102, 204.
[27] Cinader, B. (1967) in: Antibodies to Biological Active Molecules Cinader, B., ed.), p. 85, (Pergamon Press, Oxford.)
[28] Leavitt, R.J. and Umbarger, H.E. (1962) J. Bacteriol. 83, 624.
[29] Kondo, M. and Woese, C.R. (1969) Biochemistry 8, 4177.
[30] Söll, D. and Schimmel, P.R. (1974) Ann. Rev. Biochem. in press.