Biochimica et Biophysica Acta, 615 (1980) 489—496

BBA 69103

MUREIN TRANSGLYCOSYLASE FROM PHAGE λ LYSATE

PURIFICATION AND PROPERTIES

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(Received March 3rd, 1980)

Key words: Murein transglycosylase; Bacterial lysis; R-gene product; (Phage λ)

Summary

Lysates of induced *E. coli* (λ) lysogens contain two enzymes acting on murein: endopeptidase and murein transglycosylase. The transglycosylase was separated from the endopeptidase and purified to homogeneity. Its bacteriolytic activity was 200-fold higher than that of hen egg lysozyme. The bacteriolytic activity of the lysate depends on the presence of the enzyme. The endopeptidase alone does not lyse the cells, but it enhances the extent of lysis. The properties of the transglycosylase (molecular weight 17 500, pH optimum at 6.6, inactivation by Zn^{2+}), show that it is entirely different from the bacterial enzyme of the same specificity described by others. Data are presented, which suggest that this enzyme is the phage λ R-gene product.

Introduction

Lysates of induced *E. coli* (λ) cells contain two enzymes which might be responsible for murein degradation during the lysis. One of them is endopeptidase, which hydrolyses the crosslinking murein peptide bonds [1]. The other is murein transglycosylase which splits the 1-4-glycosidic bonds in murein with concomitant internal transfer of the reducing group of muramic acid to carbon 6 of the same molecule, resulting in the formation of 1,6-anhydro-*N*-acetylmuramic acid residue [2] present in the main product of *E. coli* murein digestion, muropeptide CA [3]. A bacteriolytic enzyme from the *E. coli* (λ) lysate was purified to homogeneity by Black and Hogness [4], but the purification

Abbreviations: Muropeptide CA, GlcNac-β-1-4-1, 6-anhydro-MurNac-LAla-DGlu-msA$_2$pm-DAla; muropeptide C6, GlcNac-β-1-4-MurNac-LAla-DGlu-msA$_2$pm-DAla; Muropeptide C3, dimer of C6 units connected by D-D peptide bonds between DAla and msA$_2$pm, *meso*-diaminopimelic acid; rivanol, 6,9-diamino-2-ethoxyacridine lactate.

was monitored only by measurement of bacteriolytic activity, hence the specificity of the enzyme remained unknown. The amino-acid sequence of this enzyme was determined by Imada and Tsugita [5] and its molecular weight (17 619—17 622) was calculated from the amino acid composition.

The bacteriolytic activity of phage λ lysates is determined by λ-genes R [6] and Rz [8] one might expect that the two enzymes present in the lysate correspond to these gene-products. It is known, however, that uninfected bacteria also contain enzymes of the same specificities, presumably engaged in the biosynthesis of murein [8]. The bacterial transglycosylase is a protein with a molecular weight of about 65 000 [9]. At least two bacterial endopeptidases, which differ in their sensitivity to penicillin G, are known [8,10].

It is not possible to predict whether each of the two enzymes present in the phage λ lysate would be able to bring about the lysis if acting alone. We undertook the separation of the enzymes and the purification of murein transglycosylase from the lysate, to compare its properties with those described for the bacterial enzyme. We also needed the purified enzymes for the final establishment of the gene-protein relationships.

Materials and Methods

A concentrated solution of partly purified enzymes was prepared as previously described [1,12], by rivanol treatment of induced *E. coli* C600(λcI857) lysate (50 l), concentration of the enzymes on Amberlite CG-50 I and column chromatography on Amberlite CG-50 I with NaCl (0—0, 6 M) gradient elution. The chromatography was repeated twice. The partly purified preparation (300 ml) contained endopeptidase and transglycosylase activities. Its bacteriolytic activity corresponded to 10.6 units/μg protein and the purification factor was 120 in relation to the starting lysate (Table I).

Substrates and standard muropeptides. The previously described methods were used to prepare murein from *E. coli* B, as well as muropeptides C3, C6 [13] and CA [3]. *E. coli* B freeze-dried cells, for the estimation of bacteriolytic activity, were prepared according to the method of Tsugita et al [12] and murein-lipoprotein as described by Braun and Sieglin [14]. [^{3}H]msA_2pm-labelled murein-lipoprotein (0.5 mg murein/ml; $2{,}6 \cdot 10^6$ per mg murein) was a gift from Dr. U. Schwarz.

Chemicals. Lysozyme (EC 3.2.1.17) from egg white, grade I and vancomycin were purchased from Sigma (U.S.A.), penicillin G from Polfa (Poland), moenomycin from Hoechst (F.R.G.), novobiocin from Medexport (U.S.S.R.) and Sephadex G-75, CM Sephadex C-50 and protein molecular weight standards from Pharmacia, (Sweden).

Analytical methods. The bacteriolytic activity was estimated as previously [13], by following the decrease of turbidity of the freeze-dried *E. coli* B cells (test A). for the quantitative determination of the bacteriolytic activity [^{3}H]-msA_2pm-labelled murein-lipoprotein was used and test B was carried out according to Höltje et al. [9]. The standard curves of hen egg lysozyme were prepared; 1 bacteriolytic unit corresponding to the bacteriolytic activity of 1 μg lysozyme. Protein was measured by absorbance readings at 280 nm in a Pye-Unicam SP 8-100 spectrometer. Before measurements all protein samples

were extensively dialysed against distilled water. Hen egg lysozyme was used as the protein standard. TLC was done on cellulose (5578, Merck) in *n*-butan-1-ol/acetic acid/water (4 : 1 : 5), upper phase, and developed twice at 30°C. Thin-layer electrophoresis was performed on cellulose plates (5576, Merck), at pH 1.9, 20 V/cm, for 85 min. The muropeptides were detected by ninhydrin. SDS-polyacrylamide gel electrophoresis was performed according to Laemmli [15] using 14—20% acrylamide gradient gels as suggested by Reeve [16]. The molecular weight was estimated with the aid of protein molecular weight standards of Pharmacia. The buffer used was Tris-HCl/EDTA, 50 mM Tris-HCl buffer pH 7.2, supplemented with EDTA to 2 mM.

Results and Discussion

The purification of transglycosylase is shown in Table I.

CM Sephadex C-50 chromatography

The partly purified preparation of the transglycosylase and endopeptidase (Materials and Methods) was subjected to further purification on a column of CM Sephadex C-50. The results are presented in Fig. 1. To determine the specificity of the enzymes present in the active fractions, 200 µl were taken from each of them and dialysed against 100 mM ammonium acetate, pH 6.6, 50 µl of the solutions were then incubated overnight at 37°C with 100 µg of murein or 25 µg of muropeptide C3. The samples were dried in vacuum and subjected to TLC and electrophoresis together with authentic muropeptides CA and C6 for identification of the reaction products. The presence of transglycosylase was manifested by the appearance of muropeptide CA in murein digestion

TABLE I

PURIFICATION OF MUREIN TRANSGLYCOSYLASE FROM INDUCED *E. COLI* C600(λc1857) LYSATE

Above the dotted line; preparation of starting material, the partly purified enzyme solution (Materials and Methods).

Purification steps	Volume (ml)	Total activity (bacteriolytic units) *	Yield (%)	Specific activity (bacteriolytic units/µg protein) *	Purification factor
Lysate of *E. coli* (λ)	48 000	1 200 000	100		
Rivanol treatment	120 000	1 200 000	100	0.09	1
Concentration on Amberlite CG50 I	3 200	680 000	56	2.1	20
I Chromatography on Amberlite CG50 I	1 400	620 000	51	4.8	50
II Chromatography on Amberlite CG50 I	300	540 000	45	10.6	120
Chromatography on CM Sephadex C-50	20	300 000	25	53.6	600
Gel filtration on Sephadex G-75	18	150 000	12	200.0	2 200

* Measured by test B.

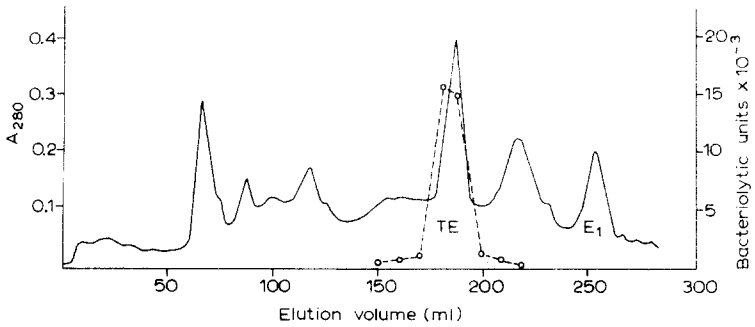

Fig. 1. CM Sephadex C-50 chromatography of transglycosylase and endopeptidase from induced *E. coli* C600 (λcI857) lysate. The partly purified preparation (Materials and Methods) was dialysed against Tris-HCl/EDTA buffer and applied to a CM Sephadex C-50 column (1.6 × 40 cm). The column was eluted with a linear NaCl gradient (0—1 M) in the same buffer (500 ml). Fractions of 10 ml were collected at a flow rate of 10 ml/h. Their protein content was monitored continuously by absorbance readings at 280 nm in a Uvicord III LKB, and bacteriolytic activity estimated by test A. Samples were taken from the active fractions, suitably diluted with Tris-HCl/EDTA buffer and their bacteriolytic activity measured by test B. ———, absorbance at 280 nm; o-o, bacteriolytic activity (test B). Endopeptidase was detected by identification of muropeptide C6 arising from C3. T, transglycosylase; E, E_1, endopeptidase.

products, endopeptidase was detected by identification of muropeptide C6 arising from C3.

The two enzymes were not separated after this step of purification, they appeared together in protein peak TE, but endopeptidase activity was also found in another protein peak (E), devoid of bacteriolytic activity. This attracted our attention to the fact, that endopeptidase is not a bacteriolytic enzyme.

The two fractions with the highest activity (15 000 bacteriolytic units/ml) from peak TE were pooled for further purification, their protein contents and activity then being measured. The purification factor for transglycosylase corresponded to 600 in relation to the lysate.

Gel filtration on Sephadex G-75

The active fractions from peak TE were concentrated 7-fold in Visking dialysing tubing immersed in dry Sephadex G-150 before the gel filtration (Fig. 2). The bacteriolytic activity appeared in a single peak (T). Two fractions with the highest activity (20 000 bacteriolytic units/ml) contained transglycosylase free of endopeptidase activity (Fig. 3). SDS-polyacrylamide gel electrophoresis (Fig. 4) showed the homogeneity of the transglycosylase. All the remaining fractions were also tested for the presence of endopeptidase and it was detected in a small protein peak (E) preceding that of transglycosylase. The combined and concentrated fractions of peak E did not reveal bacteriolytic activity. Occasionally, when the purified transglycosylase was not electrophoreticaly homogeneous, an additional step of purification, molecular sieving on Sephadex G-50 was included. The final purification resulted in a 2200-fold increase of bacteriolytic activity per μg protein in relation to the initial lysate, with an average yield of 12% (Table I).

Properties of the purified enzyme

The molecular weight of the transglycosylase, estimated from a calibration

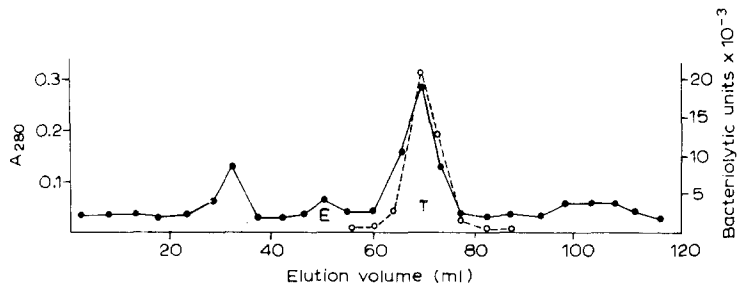

Fig. 2. Gel filtration pattern of transglycosylase and endopeptidase (from CM Sephadex C-50) on Sephadex G-75. 1-ml portions of the solution were successively loaded onto a column of Sephadex G-75 (1.2 X 110 cm) equilibrated with Tris-HCl/EDTA buffer containing 0.4 M NaCl. Fractions of 2 ml were collected at a flow rate of 5 ml/h, then their activity was measured as above and protein content determined. •-•, absorbance at 280 nm; ○-○, bacteriolytic activity (test B). T, Transglycosylase; E, endopeptidase.

curve (not shown) obtained with the aid of protein standards, corresponded to 17 500 (Fig. 4). This value may, however, need correction as molecular weight determinations in polyacrylamide gels are not reliable in this size range.

Bacteriolytic activity of this enzyme toward *E. coli* B freeze-dried cells was 200-times greater than that of hen egg lysozyme. In contrast to this, endopeptidase has no bacteriolytic activity; it lyses neither *E. coli* B cells nor murein-lipoprotein, if present alone. Due to this, a great part of this enzyme might have been lost in the first steps of the purification.

It was found, that the purified transglycosylase was not active against its substrate unless Triton X-100 was present, though in crude preparations such a requirement was not observed. The optimal concentration of Triton was 0.2%. It could not be substituted by deoxycholate.

The optimal pH for the enzyme activity was 6.6 in a 50 mM Tris-maleate buffer as well as in ammonium acetate. There was a sharp drop of activity below pH 5 and above pH 8. At pH 4.5 the enzyme was inactive. Table II shows the effect of cations on the transglycosylase activity. It follows that Zn^{2+} in 10 mM concentration completely abolished the activity. The enzyme in contrast to the bacterial transglycosylase [9] shows a preference for low ionic strength, which is common for phage bacteriolytic enzymes.

The purified transglycosylase is insensitive to vancomycin, moenomycin, penicillin G and novobiocin in concentrations of 0.1—100 μg/ml in the Tris-HCl/EDTA buffer (test B).

The properties of the purified transglycosylase from the lysate of induced *E. coli* C600(λcI857) lysogen should be compared with those described [9] for an enzyme of the same specificity from uninfected bacteria (Table III). It follows from the comparison, that the two enzymes are clearly distinct. We presume that the transglycosylase from induced *E. coli* (λcI857) lysate is a phage gene product. Its strong bacteriolytic activity suggests that it is the R-gene product. It was shown by genetic methods [6], that the R-gene of phage λ is responsible for the presence of bacteriolytic activity in λ lysate. We found, that although the lysate contains two enzymes which cleave bonds in murein, only transglycosylase may lyse bacterial cells, and the bacteriolytic activity of the lysate depends on its presence. The lytic properties of the transglycosylase

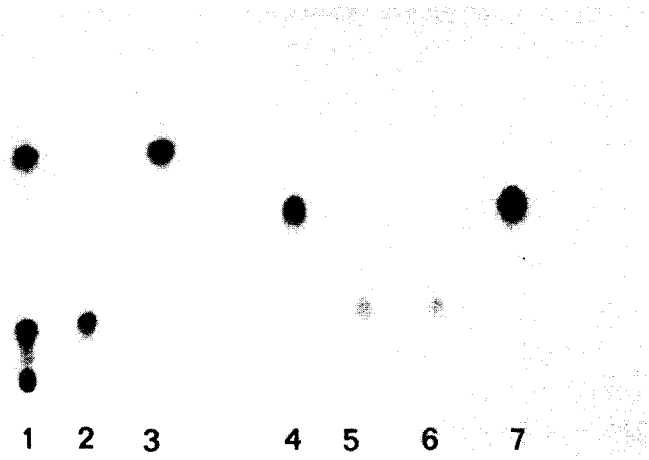

Fig. 3. Specificity of transglycosylase and endopeptidase separated by gel filtration on Sephadex G-75. Murein and muropeptide C3 were digested as described in Results and Discussion. 1. Murein digested by transglycosylase. 2. Muropeptide CB. 3. Muropeptide CA. 4. Muropeptide C3 digested by endopeptidase. 5. Muropeptide C3 digested by transglycosylase. 6. Muropeptide C3. 7. Muropeptide C6.

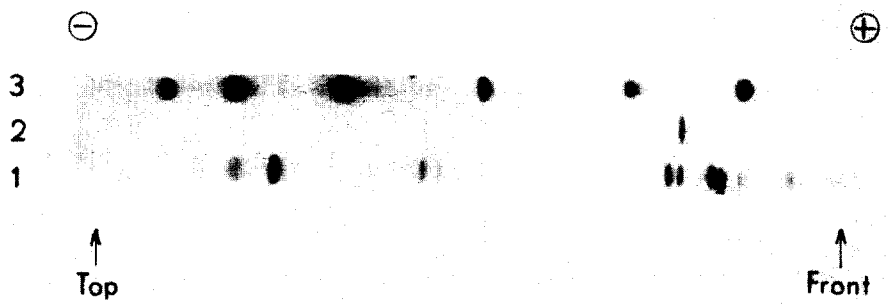

Fig. 4. SDS-polyacrylamide gradient gel electrophoresis of the purified murein transglycosylase. Lane 1, the starting material for the enzyme purification, the partly purified (on Amberlite CG-50) enzyme solution; lane 2, the purified transglycosylase; lane 3, protein molecular weight standards. From left to right: phosphorylase *b* (94 000 daltons) bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), soybean trypsin inhibitor (20 000), lactalbumin (14 000).

TABLE II

EFFECT OF CATIONS ON TRANSGLYCOSYLASE ACTIVITY

10 μl of the enzyme solution (0.06 bacteriolytic units) were incubated with 10 μl murein-lipoprotein in a total vol. 100 μl of a 50 mM Tris-HCl buffer, pH 6.6, supplemented by the cations listed in appropriate concentrations. The reaction was stopped and solubilized radioactivity measured as in test B.

Cation added	Concentration of cation added (mM)	Enzyme activity (cpm)
No addition	0	1500
Na^+	1	1500
	10	1500
	50	1300
	100	800
Mg^{2+}	1	2100
	10	900
Ca^{2+}	1	2000
	10	400
Zn^{2+}	1	400
	10	0

TABLE III

COMPARISON OF SOME PROPERTIES OF MUREIN TRANSGLYCOSYLASE FROM LYSATE OF INDUCED *E. COLI* C600(λc1857) AND FROM UNINFECTED BACTERIA [9]

Property compared	Transglycosylase from *E. coli* (λ) lysate	Transglycosylase from uninfected bacteria [9]
Molecular weight	17 500	65 000
pH optimum	6.6	4.5
Sensitivity to Zn^{2+}	complete inhibition	no inhibition

and endopeptidase from λ lysate conform with the known picture of lysis of *E. coli* infected with λR and λRz mutants. λR mutants are unable to provoke lysis and their progeny may be released only by mechanical disruption of cells or by bacteriolytic enzyme addition from outside. In contrast, λRz mutants provoke lysis (in the absence of Mg^{2+}), which is much less pronounced than that caused by wild λ phage [7]. We presume that lack of lysis in λR infected cells is connected with the absence of transglycosylase, which should be present in λRz-infected cells. We expect that the endopeptidase may turn out to be the Rz-gene product. Its absence in λRz-infected cells would limit the extent of lysis, which would then be effected by transglycosylase alone. Elucidation of this, however, will be complicated by the presence of two forms of endopeptidase in λ lysates (in peaks TE and E1 eluted from CM Sephadex). The most efficient lysis takes place in the case of infections with wild λ phage where the two enzymes act together.

Acknowledgements

The authors wish to thank Dr. U. Schwarz for the generous gift of [^{3}H] labelled murein-lipoprotein. The technical assistance of Miss B. Malankowska is

gratefuly acknowledged. This work was supported by the Polish Academy of Sciences under project No. 09.7.

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