COMPARTMENTALIZATION OF MUREIN HYDROLASES IN THE ENVELOPE OF *ESCHERICHIA COLI*

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1. Introduction

Morphological modifications of bacterial shape during cell growth division reflect alterations of the shape-maintaining sacculus [1] in the cell envelope. This envelope is a complex structure in which the sacculus is sandwiched between an outer membrane layer and the cytoplasmic membrane. Since the sacculus is a macromolecule, morphological alterations necessarily require hydrolysis of chemical bonds in the polymer murein [1] from which the sacculus is tailored. Murein hydrolases (review: 2), some of which might also function as transferases, consequently are assumed to be part of the system which produces, shapes and modifies the sacculus. *E. coli* contains a set of at least six different hydrolases [2, 3], some of which are membrane bound [4] and operate under topological and timed control: during cell division, hydrolase action is triggered at a given time in a defined area at the division site [5, 6].

The mechanisms by which topological control of hydrolase action is achieved are completely obscure. However, it is obvious that the arrangement of enzymes and substrate in the envelope is an important parameter in the regulatory system. The location of murein hydrolases in the two substructures of the envelope, the outer and in the cytoplasmic membrane, will be described in this paper. The data have already been reported in part [7].

2. Materials and methods

Escherichia coli W7 [8], requiring meso-2.6-diaminopimelic acid (Dpm) and lysine, was grown under aeration in Penassay broth (Difco) with 4 μg/ml of Dpm at 37°C to a titer of $3-4 \times 10^8$ cells/ml. Radioactive labels: murein-specific G-^{3}H Dpm (3.25 μg/ml final conc.; 0.75 μCi/μg, The Radiochemical Centre, Amersham), or 2-^{3}H glycerol (0.6 μCi/μmol, The Radiochemical Centre, Amersham; 35 μM in medium equivalent to Penassay broth except that glucose was omitted). Radioactivity in aqueous samples was measured in a Triton-Toluene scintillator [4], or on paper in a standard toluene-based scintillator. Enzyme tests were performed as described in the text; protein was determined [9] with cryst. egg white lysozyme as a standard. Enzymes used: egg white lysozyme 3 × cryst. and DNAase from beef pancreas (Merck, Darmstadt); trypsin (Boehringer, Mannheim); trypsin inhibitor from soybean (Worthington, Freehold).

3. Results and discussion

We tried to locate hydrolases in the outer and inner membrane after fractionation of the envelope. The procedure of Osborn et al. [10], which requires lysozyme, could not be used since lysozyme is itself a murein hydrolase. Therefore we developed a procedure using trypsin, based on an earlier observation [11]. Cells were plasmolyzed, opened by sonication,

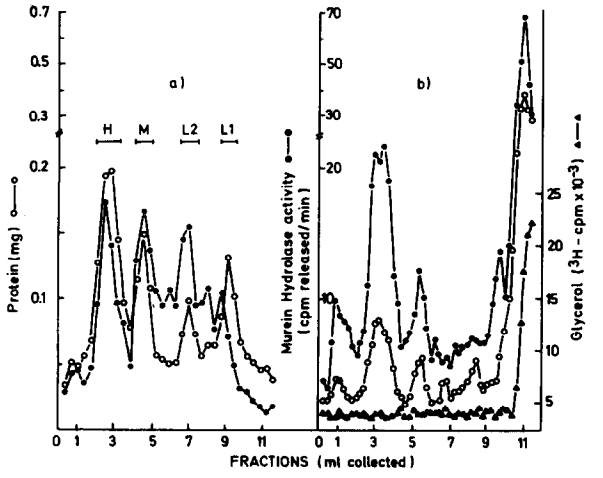

Fig. 1a. Distribution of protein, lipid and murein-hydrolase activity after fractionation of *E. coli* cell walls by sucrose gradient centrifugation. Fractionation: *E. coli* W7 was harvested from a 200 ml culture (Materials and methods) and the sediment resuspended in 5 ml of 0.75 M sucrose, 10 mM Tris–HCl, pH 7.8. Subsequently, 6.5 ml of 1.5 mM EDTA, pH 7.5, were added dropwise and the cells sonicated for 1–2 min (microscopical control). After further dilution with 3.5 ml 1.5 mM EDTA, pH 7.5, intact cells were spun down (1200 *g* for 20 min), and cell walls sedimented at 380 000 g for 2 hr. The pellet was resuspended in 10 mM Tris–maleate, 10 mM EDTA, pH 7.8, adjusted to an absorbance (578 nm; 1 cm light path) of 0.6–0.7 (about 4 ml final volume), rapidly brought to room temperature and trypsin added (per 3 ml suspension 0.1 mg; dissolved in 25 μl H_2O). After two min, the reaction was stopped with trypsin-inhibitor (per 3 ml suspension 0.4 mg, dissolved in 50 μl H_2O). The absorbance (578 nm) usually decreased to 0.45–0.55. After centrifugation at 380 000 *g* as above, the pellet was resuspended in 1–2 ml of 25% sucrose (w/v), 5 mM EDTA, pH 7.5. One ml of the suspension was fractionated on a sucrose gradient [10]. Fractions were obtained by puncturing the tubes and collecting drops. Protein was determined in 25 μl of each fraction (Materials and methods). Lipid was labelled by addition of [^{3}H]glycerol to the growth-medium (Materials and methods); 25 μl of every fraction were counted in 5 ml of Triton-toluene-scintillator and 0.5 ml H_2O. Murein hydrolase activity was measured with ^{3}H Dpm-labelled sacculi [8] as a substrate: 25 μl of every fraction were incubated in 200 μl of 0.01 M Tris-maleate, 0.1 M Mg^{2+}, 1% Triton X-100, pH 6.2, for 1 hr at 37°C. After addition of serum albumin (20 μl; 5% solution) and of trichloroacetic acid (20 μl; 50% w/v), samples were spun for 2 min at 12 000 *g* and radioactivity in 0.1 ml of supernatant was counted (Materials and methods).
Fig. 1b. Fractionation of membranes in a sucrose gradient (see above) containing 2% Triton X-100. Another membrane suspension, prepared as above, was used.

and cell envelopes collected. The envelopes were incubated with trypsin for two minutes and then trypsin inhibitor added. The final isolation of membrane fractions by sucrose gradient centrifugation was performed as described by Osborn et al. [10]. Membranes were also prepared according to the Osborn procedure to allow comparison of the two methods.

Following sucrose gradient centrifugation, four membrane bands were obtained with both procedures (fig. 1, table 1). Osborn et al. [10] have shown that the two upper bands, L1 and L2, are cytoplasmic membrane; the lower band, H, is outer membrane, and the middle band M, is unfractionated envelope. The density of the bands obtained with trypsin treatment (ζ = 1.14; 1.15; 1.19; 1.22) was identical with those prepared by the lysozyme procedure and with the values reported by Osborn et al. [10]. The purity of the bands was determined by measuring succinate dehydrogenase, which is part of the cytoplasmic membrane, and 2-keto-3-deoxyoctonate (KDO), which is part of the lipopolysaccharide complex of the outer membrane [10]. Table 1 shows that the specific activity of succinate dehydrogenase was 10–70-fold higher in the L bands than in the H band; KDO was predominantly found in the H band. On basis of previous calculations [12], the KDO values correspond to 0.07 mg lipopolysaccharide per mg protein in the L1 band and to 0.4 mg lipopolysaccharide per mg protein in H band; corresponding figures in *Salmonella* are 0.09 in L1 and 0.98 in the H band [10]. Murein, which remains intact in our procedure, is part of the H band (table 1).

Total murein hydrolase activity, measured with isolated sacculi as a substrate [8], shows peaks in the band areas. This coincidence is very probably not accidental. After recentrifugation of the isolated and washed (10 mM Tris–maleate, pH 7.5; 5 mM EDTA) bands on a sucrose gradient, hydrolase activity is exclusively found together with the membrane bands. After membrane fractionation on a gradient containing 2% Triton X-100, L band [13] and murein hydrolases in the L band area are no longer found. They appear in the top fractions of the gradient together with the lipid. Interestingly, bands and hydrolase activity are still present in the area of the H and M bands. This, in addition to the centrifugation experiment, suggests that some hydrolases are not a contamination but an integral part of the o 'er membrane.

Table 1
Composition of sucrose gradient fractions and of isolated bands

I. Bands in sucrose gradient after trypsin treatment		H	M	L2	L1
Protein; % of total found in bands		49	20	12	19
^{3}H glycerol label; % of total found in bands		43	18	16	23
Murein; % of total found in bands		80	15	3	2
Murein hydrolases; cpm released from [^{3}H]sacculi/min/mg protein × 10^{-3}		7.7	11.7	16.0	7.0
II. Comparison of washed bands obtained with trypsin (A) or lysozyme (B)					
Protein; % of total found in bands	A	60	19	6	15
	B	52	1	27	20
KDO; nmoles per mg protein	A	164	132	106	24
	B	189	–	42	34
Succinate dehydrogenase; nmoles product formed/min/mg protein	A	32	91	155	233
	B	4	–	290	275
Murein hydrolases; cpm released from ^{3}Hsacculi/min/mg protein × 10^{-3}	A	4.1	8.1	16.7	7.0
Murein hydrolases; % inhibition by 2×10^{-4} M penicillin G	A	2	20	29	48
Endopeptidase; nmoles product formed/min/mg protein	A	0.2	–	11.7	9.1
	B	0.13	–	13.0	17.8
Carboxypeptidase I; nmoles product formed/min/mg protein	A	0.23	0.51	6.4	10.4
	B	0.98	–	13.1	15.1

Envelopes were fractionated, and the fractions analyzed (fig. 1); total amounts found in the band fractions were taken as 100%. Bands were collected from gradients, diluted with 5 ml of 5 mM EDTA, pH 7.5, centrifuged (2 hr at 350000 *g*), washed once under the above conditions with 10 mM Tris–maleate; 10 mM Mg^{2+}, pH 6.2, resuspended in 0.2 ml of the same buffer and used for the tests. Hydrolase activity with sacculi as a substrate was determined as described (fig. 1a). Endopeptidase activity was measured with muropeptide C3 [1] as a substrate (50 µl enzyme suspension in 10 mM Tris–maleate, pH 7.9, 10 mM Mg^{2+}, 1% Triton X-100 containing 7 nmol ^{3}H-labeled C3; 1.2×10^7 cpm, isolated [1] from sacculi after lysozyme digestion). The mixture was separated into substrate and reaction product by paper chromatography [22] and radioactivity on the chromatogram counted (Materials and methods). Carboxypeptidase I was measured with UDP-N-acetyl-muramyl-L-Ala-D-Glu-meso-Dpm-D-Ala-D-^{14}C-Ala as a substrate essentially as described [14]. Ten µl of membrane suspension were incubated in a final volume of 50 µl in 10 mM Tris–HCl, pH 9; 10 mM Mg^{2+}; 1% Triton; with 5.56 nmoles of substrate (4.4×10^4 cpm). Substrate was kindly given to us by Dr. D. Mirelman. The reaction mixture was separated into substrate and ^{14}C-D-Ala as a reaction product by paper chromatography in butanol–acetic acid–H_2O (4:1:5, v/v). All enzyme reactions were carried out at 37°C for 30–60 min. KDO and succinate dehydrogenase were measured according to Osborn et al. [10]; a sample of KDO was kindly supplied by Dr. O. Lüderitz.

The membrane bands contain different murein hydrolases. Endopeptidase, which splits peptide bridges in murein [3], was found specifically in the cytoplasmic membrane fractions. Only about 5% of total membranebound endopeptidase activity is present in H (table 1). The specific activity of the enzyme is 60 times higher in the L2 band than in H. Endopeptidase is inhibited by penicillin [8]. As expected from the distribution of endopeptidase in the bands, total murein hydrolase activity was inhibited by penicillin much more strongly in the L bands than in the H bands (table 1; II). When specifically tested with muropeptide C3 as a substrate (legend table 1), endopeptidase activity in the bands is inhibited by more than 95%. In addition to endopeptidase, carboxypeptidase I [14] was measured; we found a distribution similar to that of endopeptidase (table 1). This supports the suggestion [15] that endopeptidase and carboxypeptidase I reactions are performed by one and the same enzyme or enzyme complex. Carboxypeptidase II activity [14] was not detectable in any of the bands from the gradient.

It remains completely open why trypsin action detaches cytoplasmic from outer membrane [16]. Because murein and lipoprotein are found together with the outer membrane band, we assume that it is not the hydrolysis of the covalent bonds [11] between the murein and the lipoprotein which causes separation of the outer membrane and the cytoplasmic membrane. There are sites within the cell enve-

lope where the cytoplasmic membrane is attached to the outer membrane [18] and/or the sacculus; it is conceivable that under trypsin treatment these connections are loosened or destroyed.

Intimate contact between cytoplasmic membrane with the strongly bound endopeptidase [4, 8], and the hydrolase substrate murein, is probably needed to permit enzyme action. How else could the reaction between a fixed enzyme and its macromolecular, nondiffusible substrate be achieved. This substrate is probably asymmetric; for structural reasons the sugar chains and peptide bridges presumably lie in different planes of the murein network, one outside the other [19]. If so, one might speculate that the peptide bridges, substrate of endopeptidase, face the enzyme in the cytoplasmic membrane and are placed at the inner side of the sacculus.

The cell confines murein hydrolysis during cell division to a narrow zone in the sacculus [5]. Such topological control of hydrolase action is correlated with other macromolecular processes like DNA replication. Since some steps of the DNA replication sequence may take place in or at the cytoplasmic membrane [20] and since additional intracellular signals to which the hydrolase system responds have much better access to the cytoplasmic membrane than to the outer membrane, we assume that topological control of hydrolase action occurs within the cytoplasmic membrane. Since endopeptidase is exclusively found in this substructure of the envelope, it is a good candidate for an enzyme under topological control as we have assumed previously [8]. Other hydrolases may not be subject to strict topological control. Such enzymes which e.g. effect the randomization of murein in the growing cell [21] could be confined to the outer membrane where, as our data show, hydrolases of still unidentified specificity are indeed found.

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