Isolation of Labeled Lipoprotein from *Escherichia coli* and *Proteus mirabilis* after Incubation with [^{14}C]Penicillin

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[^{14}C]penicillin binding experiments and membrane analysis were carried out with cell envelope preparations from *Escherichia coli* and *Proteus mirabilis*. After incubation with [^{14}C] penicillin G labeled free lipoprotein could be identified. The analysis of the isolated lipoprotein by SDS polyacrylamide gel electrophoresis indicates that there is only one protein with an apparent molecular weight of 7000. The amino acid composition of isolated labeled free lipoprotein from *E. coli* was identical to the lipoprotein already found in *E. coli*. It is a point of interest that the amino acid composition of the isolated labeled free lipoprotein from *P. mirabilis* D 52 differs from that found in other mutants of this strain. The free form of lipoprotein from *P. mirabilis* D 52 is composed of 61 amino acids and has glycine, phenylalanine and proline as specific components.

Introduction

Penicillin binding proteins of both gram-positive and gram-negative bacteria have received considerable attention in recent years, and many experiments were carried out for characterization of those proteins in order to examine the mode of action of penicillin G [1–3]. In *Escherichia coli*, for example, the role of the individual penicillin binding proteins has been investigated in detail [4–7]. In this organism at least six different penicillin binding proteins are involved in elongation of the cell wall, septum formation during cell division and maintenance of rod shape formation. Some of them have been identified as peptidoglycan transpeptidase and D-alanine-carboxypeptidases [5, 8, 9]. Cytoplasmatic membranes of all other bacteria investigated also contain several penicillin binding proteins [10], which can be detected conveniently by incubation of membrane preparations with [^{14}C]penicillin and by subsequent SDS polyacrylamide gel electrophoresis. In contrast, penicillin binding to outer membrane proteins has not been observed. The outer membrane is composed of lipopolysaccharides, phospholipids and proteins; among them are so-called major outer membrane proteins [11]. One of these proteins is a lipoprotein of low molecular weight, a portion of which is covalently bound to the murein layer [12–14]. In this paper we present [^{14}C]penicillin binding experiments with envelope fractions from *E. coli* and *Proteus mirabilis,* especially studies with outer membrane ghosts [15]. We describe the detection, isolation and characterization of a labeled lipoprotein from these membrane preparations after treatment with [^{14}C]penicillin.

Abbreviations: EDTA, Ethylendiaminetetraacetic acid; SDS, sodium dodecylsulfate; TCA, trichloroacetic acid.

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Materials and Methods

Bacterial strains and growth conditions

Escherichia coli K 12 was derived from the American Type Culture Collection (ATTC 23811), *Proteus mirabilis* D 52 was obtained from Dr. Taubeneck, institute of microbiology and experimental therapy, Jena, GDR. Both strains were cultivated at 37 °C on a shaker either in the minimal medium of Davis and Mingioli [16] or in a complex medium consisting of 1% pepton, 0.5% yeast extract, 0.2% NaCl and 0.5% glucose; the pH was adjusted to 7.2.

Preparations of cell envelope fractions

Cells of the late logarithmic phase were harvested by centrifugation at $6000 \times g$ for 15 min, washed once with 10 mM Tris-HCl buffer pH 7.8, resuspended in 50 mM Tris-HCl buffer pH 7.8 containing 1 mM EDTA (2 ml buffer per g wet weight cells), mixed with two volumes of 0.18 mm glas beads and shaken in a Bühler cell homogenisator for 2 min at 10 °C in the presence of about 0.1 mg/ml DNase. To remove large particles, the filtrate was centrifuged at $3000 \times g$; finally, the supernatant was centrifuged at $48000 \times g$ for 30 min at 4 °C and the pellet was washed two times with 50 mM Tris-HCl buffer pH 7.8. Outer membrane ghosts were prepared as described by Henning *et al.* [15].

Isolation and purification of the lipoprotein

Isolation of the free form of the lipoprotein was carried out according to Hirashima *et al.* [17] with the following modification: envelope fractions or outer membrane ghosts were solubilized with 2% SDS (w/v) for 20 min at 70 °C or 10 min at 100 °C, then the same volume of 20% TCA (w/v) was added, and after incubation for 1 hour in an ice bath the mixture was centrifuged at $48000 \times g$ for 30 min in a Beckman JA 21 rotor. The soluble fraction containing the free form of the lipoprotein was treated 5–8 times with ether to remove TCA and finally extracted with water-saturated n-butanol; the butanol extract was concentrated to dryness, resolubilized in 0.1% SDS (w/v) and filtered through a PSAC-membrane (Millipore) to separate non definite polysaccharides; after that the extract was applied to a Sephadex G 50 columne (3.3×45 cm), equilibrated and eluted with 0.1% SDS (w/v) at room temperature; aliquots were removed from each fraction to test for radioactivity by liquid scintillation counting. For identification of labeled lipoprotein a SDS polyacrylamide gel electrophoresis was carried out.

[^{14}C]Penicillin binding experiments

Membrane preparations from cells of the logarithmic growth phase or outer membrane ghosts were resuspended in 0.05 M cacodylate buffer pH 7.0 and incubated with 1 mM [^{14}C]penicillin for 30–60 min at 37 °C. The reaction was stopped by addition of the same volume of a 1% (w/v) solution of unlabeled penicillin in water. The mixture was centrifuged and washed with a 0.1% penicillin solution until no radioactivity could be detected in the supernatant. For membrane protein analysis the pellets were solubilized in the sample buffer and subjected to a SDS polyacrylamide gel electrophoresis. Additionally, labeled membrane fractions were treated with 2% SDS and 20% TCA according to Hirashima *et al.* [17]; the SDS/TCA soluble fraction and the TCA precipitate were counted for radioactivity.

Analytical methods

Protein was determined by the methods of Lowry *et al.* [18] and Bradford [19] using bovine serum albumine as standard. Amino acid analysis was carried out with a Durrum D-500 amino acid analyser. Samples (proteins 20–30 μg) were hydrolysed at 107 °C ± 1 °C in 200 μl twice distilled 6N HCl for 24 or 72 hours in evacuated sealed tubes. For SDS polyacrylamide gel electrophoresis the procedure of Weber and Osborn [20] was used. Protein samples were preincubated in the sample buffer of Inouye and Guthrie [21] at 100 °C for 5 min. After electrophoresis, gels were either sliced (1 mm slices) and counted for radioactivity, or the stained gels were scanned at 610 nm in a Gilford spectral photometer model 240 equipped with a linear transport system. Staining was done with Coomassie brillant blue G 250. As internal molecular weight standards glucagon (M_r = 3480), insulin (M_r = 5700), lysozyme (M_r = 14000), trypsin (M_r = 23000), pepsin (M_r = 35000) and ovalbumine (M_r = 43000) were used.

Chemicals

Benzyl- [^{14}C]penicillin (specific activity 24–50 mCi/mmol) was obtained from Amersham Buchler/West Germany. Standard proteins and DNase I were from Boehringer/West Germany. Detergents were obtained from Serva/West Germany. All other reagents used were of analytical grade.

Results

Membrane preparations from *E. coli* and outer membrane ghosts from *P. mirabilis* were incubated with [^{14}C]penicillin as described in Materials and Methods. The subsequent analysis by SDS polyacrylamide gel electrophoresis and the results of distribution of radioactivity are shown in Fig. 1. Comigration of the lipoprotein band and the main radioactivity could be observed. Even when incubations were

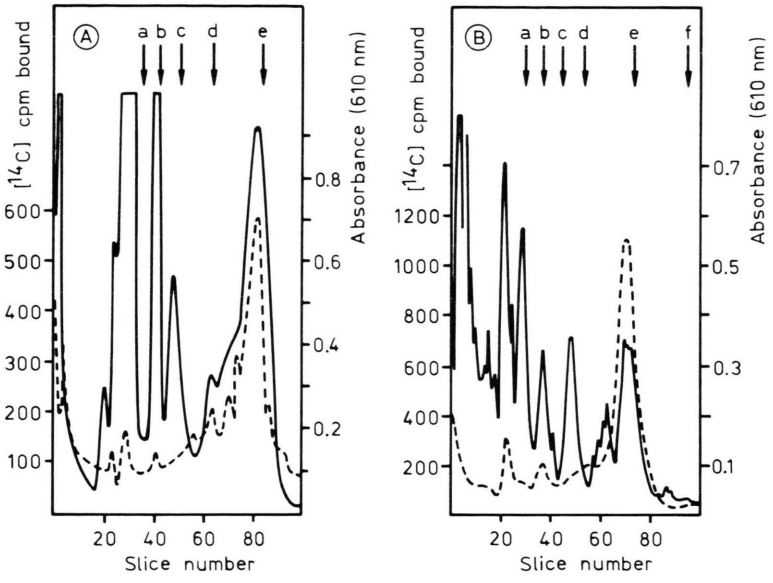

Fig. 1. SDS polyacrylamide gel electrophoresis of labeled membrane preparations from *E. coli* and *P. mirabilis* after treatment with [^{14}C]penicillin. Binding assays and electrophoresis were carried out as described in Materials and Methods. ——, absorbance (260 nm); – – –, radioactivity bound (cpm). Arrows with letters indicate positions of the internal molecular weight standards: a, ovalbumine (M_r = 43000); b, pepsin (M_r = 35000); c, trypsin (M_r = 23000); d, lysozyme (M_r = 14000); e, insulin (M_r = 5700); f, glucagon (M_r = 3500).

(A) Outer membrane ghosts (proteins 1 mg/ml) from *P. mirabilis* were incubated in the presence of 1 mM [^{14}C]penicillin in 0.05 M cacodylate buffer pH 7.0 for 30 min. Aliquots were subjected to SDS gel electrophoresis.

(B) A membrane fraction (proteins 2 mg/ml) from *E. coli* was incubated in the presence of 5 mM [^{14}C]penicillin in 0.05 M cacodylate buffer pH 7.0 for 60 min. Aliquots were subjected to SDS gel electrophoresis.

stopped with a 100-fold excess of unlabeled penicillin there was no significant change in the pattern of labeling. For *P. mirabilis*, the protein pattern of the outer membrane ghosts corresponds to that of *E. coli* as reported by Henning *et al.* [15]. Labeling of the crude envelopes showed good correlation with [^{3}H]penicillin binding experiments with *E. coli* membrane preparations as described by Sukinaka *et al.* [22].

To prove an actual labeling of the lipoprotein these membrane preparations were solubilized with 2% SDS and precipitated with 20% TCA according to the method of Hirashima *et al.* [17]; by this procedure the lipoprotein can selectively be isolated in a single step, for it is soluble even in 10% TCA in the presence of 1% SDS. Both the SDS/TCA soluble fraction and the TCA precipitate are labeled after treatment with [^{14}C]penicillin (Fig. 2 and 3). A con-

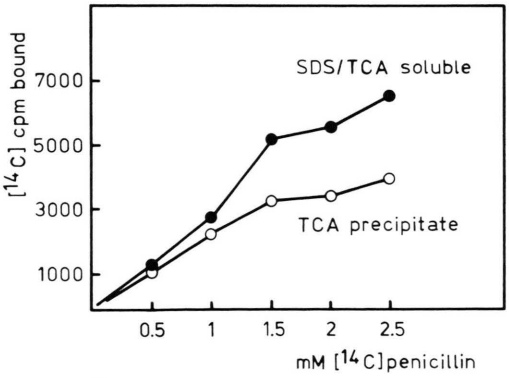

Fig. 2. [^{14}C]penicillin binding experiments with a membrane fraction from *E. coli*. Membranes (400 µg proteins in 50 µl-samples) were incubated at 37 °C for 60 min with 5 different penicillin concentrations and treated as described in Materials and Methods. Finally, both the SDS/TCA soluble material (●) and the TCA precipitate (○) were tested for radioactivity by liquid scintillation counting.

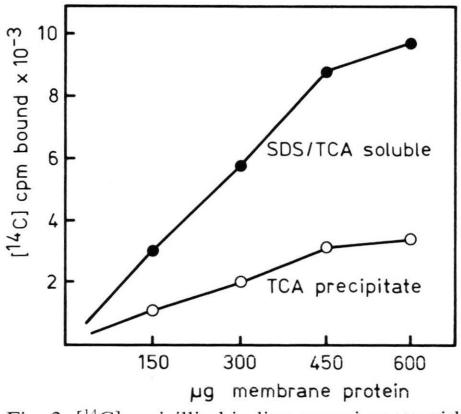

Fig. 3. [^{14}C]penicillin binding experiments with outer membrane ghosts from *E. coli*. Samples with 1 mM [^{14}C]penicillin were incubated at 37 °C for 60 min and treated as described in Materials and Methods. Subsequently both the SDS/TCA soluble fraction (●) and the TCA precipitate (○) were tested for radioactivity.

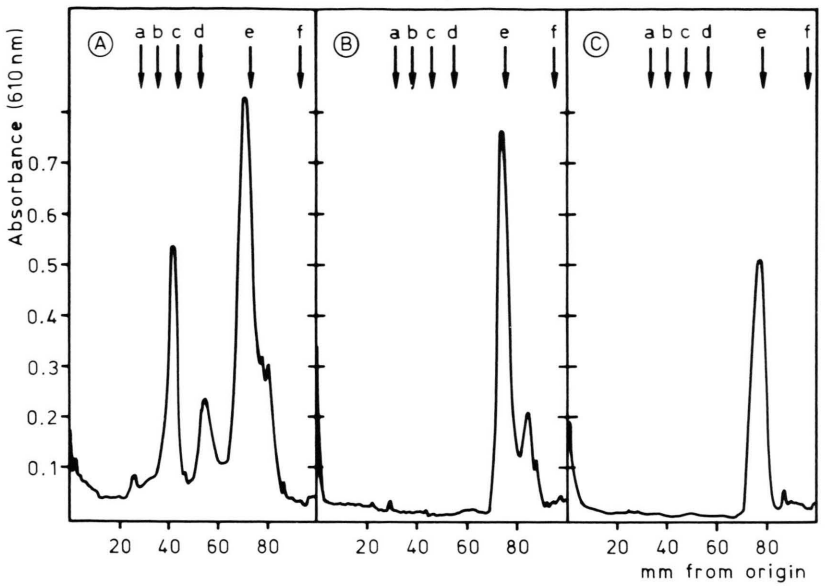

Fig. 4. Isolation of the free form of the lipoprotein from *E. coli* outer membrane ghosts and analysis by SDS polyacrylamide gel electrophoresis. All procedures were carried out as described in Materials and Methods. The internal molecular weight standards are the same as in Fig. 1.

(A), analysis of the SDS/TCA soluble material; (B), analysis of the butanol soluble fraction; (C), analysis of the lipoprotein fraction after chromatography on Sephadex G 50.

siderable higher degree of binding was found in the SDS/TCA soluble material. For further substantiation the SDS/TCA soluble fraction was assayed for protein composition. Additionally, the free lipoprotein was isolated to test for radioactivity and amino acid composition. It could be demonstrated that the outer membrane ghosts, which actually contain the free form of the lipoprotein, are most suitable for isolating this protein. As shown in Fig. 4, however, the lipoprotein was still contaminated to about 20% by higher molecular weight proteins after this step. Those contaminations could be removed by an additional treatment with watersaturated *n*-butanol and chromatography on Sephadex G 50. On the Sephadex G 50 columne the protein was completely eluted within the void volume, indicating an apparent molecular weight of more than 30000; obviously this was in contrast to the electrophoretic mobility in SDS gels from which a molecular weight of about 7000 had been calculated. That discrepancy, however, is due to the aggregation of lipoprotein molecules, a fact which was already reported for *E. coli* lipoprotein [17, 23]. This was confirmed by SDS polyacrylamide gel electrophoresis which resulted in a single protein band with a molecular weight of about 7000 (Fig. 4 and 5, respectively). Upon dialysis using Spectrapor membrane tubing (molecular weight cut-off approximately 3500) no significant loss of radioactivity was observed. No labeling of the lipoprotein could be demonstrated when isolated

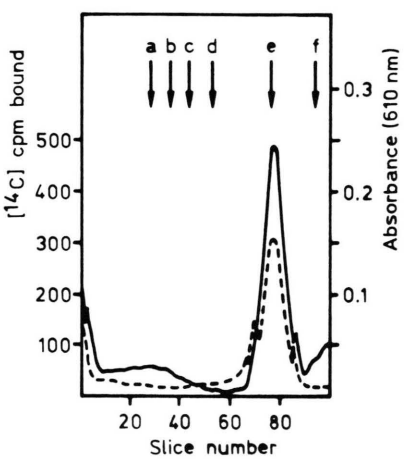

Fig. 5. SDS gel electrophoretic analysis of isolated labeled free lipoprotein from *P. mirabilis*. The internal molecular weight standards are identical with those in Fig. 1.

lipoprotein was incubated with [^{14}C]penicillin under the same conditions as described for the different membrane preparations (see Materials and Methods).

As shown in Tables I and II, respectively, the amino acid composition of the isolated lipoprotein of *E. coli* corresponds to that found by Inouye *et al.* [13]. In contrast, the amino acid composition of the lipoprotein of *P. mirabilis* differs from that isolated by Gmeiner *et al.* [14] and Katz *et al.* [31], especially

Table I. The amino acid composition of the isolated free form of the lipoprotein from *E. coli*.

Amino acid	Molar ratio[a]		Integer	Published values [24, 25]
	24h	72h		
Asp	13.78	14.27	14	14
Thr	2.01	2.11	2	2
Ser	6.27	5.96	6	6
Glu	4.82	4.84	5	5
Pro	0	0	0	0
Gly	0	0	0	0
Ala	9.00	9.00	9	9
Cys	ND	ND	ND	0
Val	3.51	3.56	4	4
Met	1.48	1.57	2	2
Ile	0.86	0.92	1	1
Leu	3.87	4.11	4	4
Tyr	0.98	1.08	1	1
Phe	0	0	0	0
His	0	0	0	0
Lys	4.91	4.92	5	5
Trp	ND	ND	ND	0
Arg	3.96	3.88	4	4
Total			58	58

[a] Assuming 9 alanine residues per molecule lipoprotein.

Table II. The amino acid composition of the isolated free form of the lipoprotein from *Proteus mirabilis*.

Amino acid	Molar ratio[a]		Integer	Published values			
				Katz *et al.* [31]		Gmeiner *et al.* [14]	
	24h	72h		Molar ratio[b]	Integer	Molar ratio[c]	Integer
Asp	6.01	6.28	6	11.0	11	12.90	13
Thr	3.02	3.09	3	2.7	3	3.08	3
Ser	2.84	2.33	3	6.9	7	7.94	8
Glu	7.35	7.42	7	10.0	10	9.68	10
Pro	2.14	2.17	2	0	0	0	0
Gly	4.92	5.04	5	0.8	0–1	0	0
Ala	6.00	6.00	6	8.5	9	9.00	9
Cys	ND	ND	ND	0	0	0	0
Val	3.87	4.47	4–5	3.8	4	3.78	4
Met	1.55	1.39	2	0	0	0	0
Ile	3.13	3.73	4	0.8	1	1.14	1
Leu	5.34	5.13	5	4.2	4	4.35	4
Tyr	1.67	1.66	2	1.6	2	2.14	2
Phe	2.17	2.13	2	0	0	0	0
His	1.87	1.92	2	0	0	0	0
Lys	4.03	4.25	4	2.9	3	3.17	3
Trp	ND	ND	ND	ND	ND	ND	ND
Arg	3.69	3.78	4	2.9	3	3.21	3
Total			61–62		57–58		60

[a] Assuming 6 alanine residues per molecule lipoprotein.
[b] Assuming 11 asparatic acid residues per molecule lipoprotein.
[c] Assuming 9 alanine residues per molecule lipoprotein.
ND, not determined.

concerning glycine, phenylalanine and proline, which have been determined not at all by those authors.

Discussion

In this paper we report on [^{14}C]penicillin binding experiments with envelope fractions or outer membrane ghosts from *P. mirabilis* and *E. coli*, and the amino acid composition of the isolated lipoprotein from both species was compared with data of other authors [14, 24, 25, 31]. We could demonstrate that after [^{14}C]penicillin treatment a labeled free lipoprotein can be identified, isolated and purified.

The question has to be asked why that labeling of the free lipoprotein could not be observed in former penicillin binding experiments with membrane preparations. Firstly, one has to consider that there is only a slight labeling of the lipoprotein, which might have been overlooked in SDS polyacrylamide gel electrophoresis. Both the membrane preparation method and the isolation procedure may also be important to the detection and enrichment of free lipoprotein; in contrast to other authors, who obtained envelope fractions by grinding cells with alumina [22], we prepared membrane fractions by mixing and shaking cells with glass beads in a Bühler homogenisator or we used outer membrane ghosts [15]. Additionally, another explanation might be different methods for fixation and staining the SDS gels. Usually SDS gels are stained and destained with solutions made up from acetic acid, water and methanol or TCA without prior fixation [26]. As reported, lipoprotein is SDS/TCA-soluble, and therefore it might have been extracted from SDS gels during staining and destaining. By fixation of SDS gels according to Jones and McFadden [27] with 20% sulfosalicylic acid only SDS is extracted and the lipoprotein becomes unsoluble. Such a treatment obviously minimized the loss of free lipoprotein in SDS gel analysis.

At present, there is no answer to the question of the nature of the binding site, and the possible mode of action remains a point of speculation; furthermore it is unknown wether the complete penicillin molecule or only a side chain fragment is bound. In consideration of the side chain labeling of the [^{14}C]penicillin used the following reaction might be discussed: Hammerström and Strominger [28] could demonstrate that the membrane bound D-alanine

carboxypeptidase from *Bacillus stearothermophilus* releases phenylacetylglycine after inhibition by covalent interaction with penicillin. Similar results were reported by Frère *et al.* [30, 39]: the penicillin sensitive DD-carboxypeptidase-transpeptidase from *Streptomyces R 61* is reversibly inhibited by penicillin G, but further incubation results in a slow degradation of the penicilloyl-enzyme complex and fragmentation of the penicillin molecule; those fragments are N-acylglycine and N-formyl-D-penicillamine. The formed activated N-acylglycine fragment is transferred either to water to give phenylacetylglycine or to a proper acceptor such as the aminogroup of glycylglycine with formation of phenylacetyl-glycylglycylglycine.

Especially for *E. coli* of which the amino acid sequence of the free lipoprotein is known [24], such an acceptor might be the ε-aminogroup of the C-terminal lysine of a free lipoprotein molecule. Possibly the free form of the lipoprotein may be partly considered as an acceptor of the phenylacetyl-fragment after penicillin degradation.

In this respect it will be of interest to determine the C-terminal amino acid of the free lipoprotein from *P. mirabilis* (work is in progress) to elucidate how far there is any chance for a mode of action as suggested for *E. coli*. Further investigations in detail should be directed towards demonstrating an actual binding site.

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