β-D-Galactoside transport in *Escherichia coli*

M_r determination of the transport protein in organic solvent

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1. INTRODUCTION

The β-D-galactoside transport protein of *Escherichia coli* has been identified as a $M_r \sim 30\,000$ protein using an indirect affinity labeling procedure followed by SDS–polyacrylamide gel electrophoresis as well as gel permeation chromatography in the presence of SDS [1,2]. These results have repeatedly been confirmed using the same methods for M_r determination after genetic amplification and purification of the transport protein [3–5] or after attachment of a direct affinity label [6].

The DNA sequence of the *lac y*-gene was then determined and led to the assignment of a protein M_r 46 500 [7]. The large discrepancy to the previous M_r estimates was not due to N-terminal processing of the primary *y*-gene protein product [8], but the possibility of C-terminal processing was left open [7,8]. Subsequently it was proposed that the transport protein of M_r 30 000 may result from the proteolytic processing of the primary gene product, and evidence for an additional *y*-gene related polypeptide of M_r 15 000 was presented [9]. A mutant transport protein was actually shown to be generated by proteolytic processing of a polyprotein chimera containing both the *lac y*-gene and *lac a*-gene products [10]. Other studies showed that the apparent M_r of the transport protein could be shifted from 30 000–46 000 by increasing the sieving properties of SDS–polyacrylamide gels [11], but conclusions as to an abnormally high amount of bound SDS were difficult to reconcile with the results of column gel permeation chromatography in the presence of SDS where M_r estimates of 30 000–33 000 had been obtained [2,9]. Organic solvents rather than detergents have been successfully used for the isolation of integral membrane proteins [12–16]. Use of the aprotic organic solvent, HMPT, has in fact allowed the first solubilization and reconstitution of the β-D-galactoside transport system [17]. The apparent M_r of the HMPT-dissolved transport protein has now been determined by gel permeation chromatography.

Abbreviations: HMPT, hexamethylphosphoric triamide; SDS, sodium dodecylsulphate; NEM, *N*-ethylmaleimide; TDG, (β-D-galactopyranosyl)-1→1-thio-β-D-galactopyranoside

2. EXPERIMENTAL

2.1. *General methods*

The transport protein overproducing strain, *E. coli* T 206 [4], was kindly provided by Dr P. Overath (Tübingen). Growth of bacteria and membrane preparation were as described [4]. SDS–polyacrylamide gel electrophoresis was performed on 12% (w/v) acrylamide gels as in [5]. Scintillation autography of the gels was performed as in [18]. The amounts of membrane protein were determined in the presence of SDS [12].

2.2. *Indirect affinity labeling procedure*

The procedure in [1] was slightly modified as follows. A membrane suspension from *E. coli* T 206 (40 mg protein, specific TDG binding activity determined by flow dialysis [4], 2.7 nmol/mg protein) was centrifuged (130 000 × *g*, 2 h, 2°C). The

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pellet material was suspended in 10 ml 100 mM potassium phosphate (pH 6.3) containing 20 mM TDG. After incubation for 10 min at 28°C, 10 ml 10 mM NEM was added, and the incubation was continued for 60 min at 28°C. The suspension was then cooled in an icebath and centrifuged (100 000 × *g*, 30 min, 2°C). The pellet material was carefully washed twice with 100 mM potassium phosphate (pH 6.3) and was finally suspended in 5 ml 10 mM potassium phosphate (pH 6.3).

^{3}H-Labeled NEM (220 nmol, spec. act. 1.1 Ci/mmol, obtained from New England Nuclear, Boston MA) was added in 2.5 ml 10 mM potassium phosphate (pH 6.3). After incubation for 30 min at 28°C, 2.5 ml 10 mM NEM was added and incubation was continued for 20 min at 28°C. 2-Mercaptoethanol (50 mM, 10 ml) was added, and incubation was for a further 10 min at 28°C. The labeled membrane material was isolated by centrifugation (100 000 × *g*, 30 min, 2°C) and washed 3 times with 50 mM potassium phosphate (pH 6.6). The final membrane pellet was suspended in 2 ml 50 mM potassium phosphate (pH 6.6) and 50 μl aliquots were stored under liquid nitrogen. Determination of radioactivity indicated that 50 nmol [^{3}H]NEM had been incorporated.

2.3. *Solubilization procedure (cf. [17])*

The membrane suspensions used had been stored under liquid nitrogen, and were thawed within 5 min in a 25°C water-bath. The subsequent steps were carried out at 2°C. The exact procedure used to obtain fig.1 is described: A 50 μl portion of the [^{3}H]NEM-labeled membrane preparation (1 mg protein, ~ 10^6 cpm) was added to a non-labeled membrane preparation (3 mg protein in 0.2 ml 50 mM potassium phosphate (pH 6.6)). The latter had a specific TDG binding activity determined by flow dialysis [4] of 2.9 nmol/mg protein. The combined membrane suspensions were diluted 100-fold with 0.5 M Tris–sulphate (pH 7.5) containing 1 M LiCl and 3 mM dithiothreitol. After 30 min at 0°C, the membranes were isolated by centrifugation (100 000 × *g*, 30 min, 2°C), and dispersed in 6 ml 90% (v/v) HMPT in 50 mM Tris–sulphate (pH 7.5) containing 100 mM LiCl and 0.3 mM dithiothreitol. Dispersion was by means of a Vortex shaker and by a 5 min sonication period in a Branson model 220 sonic bath (0°C). Non-solubilized material was removed by ultracentrifugation (100 000 × *g*, 2 h, 12°C).

3. RESULTS AND DISCUSSION

Membranes from the transport protein overproducing strain *E. coli* T 206 [4] were labeled with [^{3}H]NEM by the indirect affinity labeling procedure in [1]. Treatment of this membrane preparation with 90% (v/v) HMPT resulted in 90–96% solubilization of the radioactive label, and of between 50–90% of the total membrane protein. Gel permeation chromatography of the extract on a calibrated column of Sephacryl S-300 (fig.1A) gave rise to a defined peak of radioactivity corresponding to M_r 47 500 ± 500. The relative width of this peak was similar to that of the marker protein peaks 2 and 3. There was also a peak of radioactivity near the column void volume, corresponding to $M_r \geq 90\,000$. The amount of this dimeric and/or oligomeric material varied between 20–40% in experiments which differed in the exact conditions of solubilization (e.g., in protein or water concentration). The same membrane preparation used to obtain fig.1A was also studied by SDS–polyacrylamide gel electrophoresis (fig.1B). Staining with Coomassie blue showed that a number of polypeptides were present, but only a single relatively broad polypeptide band contained the [^{3}H]NEM label. Calibration of the SDS gels indicated M_r ~ 35 500 for this band. The material of M_r 47 500 and ≥90 000 peaks of fig.1A was also studied by SDS–polyacrylamide gel electrophoresis, after precipitation of the HMPT-dissolved proteins with ethanol [16]. A radioactive band of app. M_r ~ 35 500 was present in both cases, but most of the radioactive label was present in protein aggregates which failed to enter the gels.

In conclusion, gel permeation chromatography in HMPT has led to M_r 47 500 ± 500 which was in agreement with the value derived from the DNA-sequence of the *y*-gene [7]. The same protein sample led to M_r 35 500 upon SDS–polyacrylamide gel electrophoresis. The lower apparent M_r-value could have been due to some special behaviour of the transport protein in SDS-containing buffer. No proteolytic processing of the primary *y*-gene product has to be postulated to explain the discrepancies in the reported M_r-estimates of the β-D-galactoside transport protein.

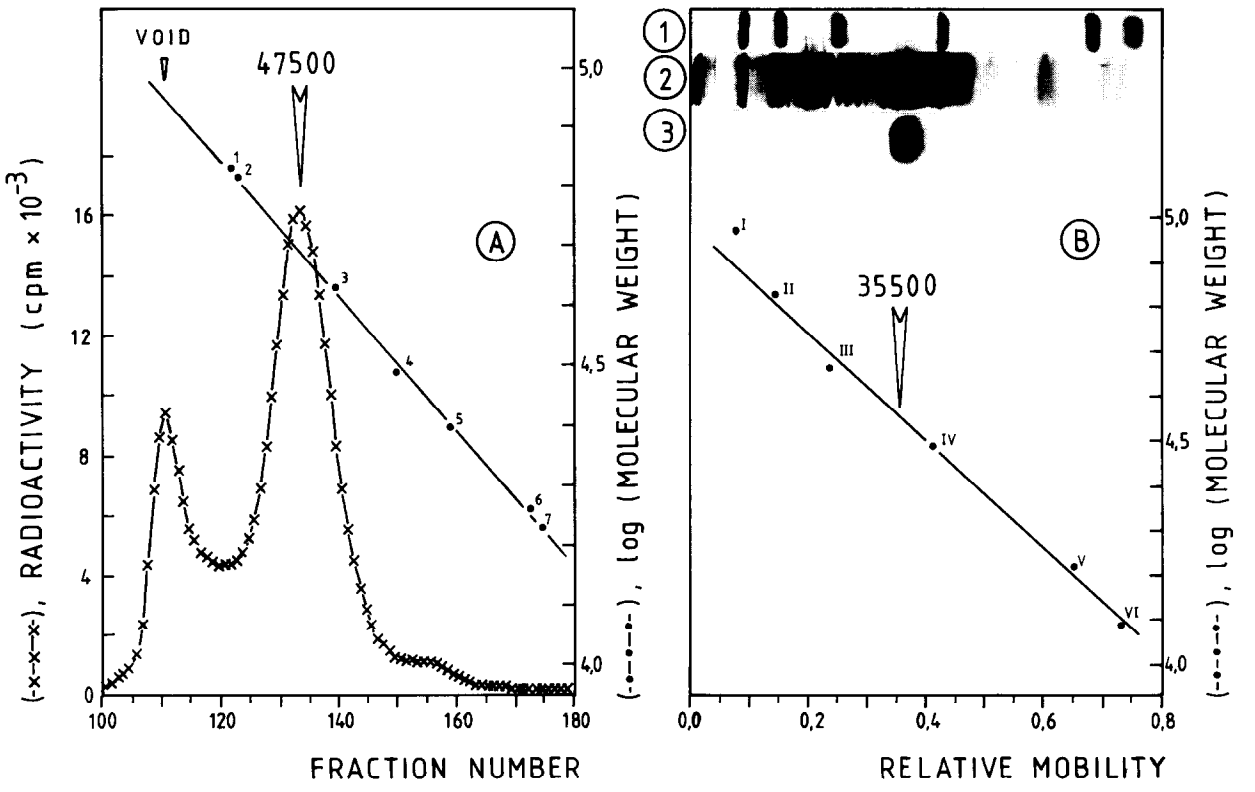

Fig.1. (A) Gel permeation chromatography on Sephacryl S-300. A column (104 × 3 cm) was filled with Sephacryl S-300 (Pharmacia, Freiburg) in a buffer consisting of 9 vol. parts HMPT and 1 part 500 mM Tris–sulphate (pH 7.5), 1 M LiCl, 3 mM dithiothreitol. A portion of 5.7 ml of the HMPT-solubilizate of section 2.3 was degassed under vacuum, and then applied to the column. The column was developed with the column buffer at 8 ml/h and 24°C. Fractions of 2.2 ml were collected. The radioactivity of 1 ml aliquots of each fraction was determined in a Triton X-100 based scintillation fluid. The amount of radioactivity (×—×) was plotted against fraction number. The column was calibrated in independent runs with the following marker proteins (•) whose M_r-values are indicated in brackets; (1) bovine serum albumin (68 000); (2) avidin (66 000); (3) ovalbumin (43 000); (4) carboanhydrase (31 000); (5) chymotrypsinogen (25 000); (6) lactoglobulin (18 400); and (7) myoglobin (17 000). The column void volume was determined with Blue Dextran.

Fig.1. (B) SDS–polyacrylamide gel electrophoresis. In the upper part of the graph the following gel patterns are shown. (1) Mixed M_r marker proteins from whose positions the calibration curve in the lower part of the graph was constructed. The following proteins were used, M_r values being given in brackets: (I) phosphorylase *a* (94 000); (II) bovine serum albumin (68 000); (III) ovalbumin (43 000); (IV) carboanhydrase (31 000); (V) myoglobin (17 000) and (VI) cytochrome *c* (13 000). (2) Affinity-labeled membrane preparation of section 2.2, stained with Coomassie blue. (3) Affinity-labeled membrane preparation of section 2.2, scintillation autograph.

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