GENETIC CONTROL OF THE MEMBRANE PROTEIN COMPONENT OF THE LACTOSE TRANSPORT SYSTEM OF ESCHERICHIA COLI*

By C. Fred Fox,† James R. Carter,‡ and Eugene P. Kennedy

DEPARTMENT OF BIOLOGICAL CHEMISTRY, HARVARD MEDICAL SCHOOL

Communicated January 3, 1967

The brilliant contributions of workers at the Institut Pasteur have revealed the existence of a specific, genetically controlled transport system that mediates the uptake of lactose in *Escherichia coli*. Work on this transport system has been the subject of numerous reports and reviews.[1–5]

We have recently reported evidence that a membrane-bound protein distinct from β-galactosidase and thiogalactoside transacetylase (the two previously characterized proteins of the *lac* system) is an essential component of the transport system.[6] Until the exact function of this protein is made clear, the noncommittal designation *membrane protein* (M protein) seems appropriate.

In our working model[6] of the β-galactoside transport system, the M protein is that component in the membrane that specifically binds certain galactosides, forming a complex of the Michaelis-Menten type. The sugar then passes through the membrane as the β-galactoside-M complex. In the presence of sources of metabolic energy, the protein is postulated to undergo transformation to a form designated M_t having little affinity for the sugar, thus leading to accumulation of the sugar within the cell. This working hypothesis is described in greater detail elsewhere.[7]

The function of the M protein in mediating the transport of substrates such as *o*-nitrophenyl-β-galactoside (ONPG)[8] is destroyed when cells are treated with N-ethylmaleimide. However, the protein has a high affinity for thiodigalactoside (TDG) and when the protein is complexed with this sugar, it is protected against N-ethylmaleimide.[6] These properties are the basis of a specific chemical method for the detection of the protein in cell-free fractions of *E. coli*.

In the present publication we wish to report the results of studies on the genetic control of the M protein, which indicate that the protein is coded by the y gene of the *lac* operon. Further evidence that the protein is localized in the spheroplast membrane fraction of the cell has also been obtained. A preliminary estimate of the minimum number of functional subunits of M protein per cell has also been made.

Materials and Methods.—Isopropyl-1-thio-β-D-galactoside (IPTG) and TDG were synthesized using the general procedures described by Černy *et al.*[9] and Helferich and Türk.[10] It is important to establish that these thiogalactosides are free from traces of mercaptan that might react with N-ethylmaleimide in the standard assay for M protein. The sensitive method of Ellman[11] for the detection of thiols was used for this purpose. Other sugars used in this study were obtained commercially.

Radioactive N-ethylmaleimide was synthesized from tritiated N-ethylamine in a microscale procedure to be described elsewhere,[12] based on the methods of Piutti and Giustiniani.[13]

Thiogalactoside transacetylase was measured by the highly sensitive method of Fox and Kennedy.[14]

Strains of E. coli: Cultures of strains ML 30 ($i^+z^+y^+a^+$), originally isolated in Monod's laboratory, were gifts of Dr. A. Koch. ML 308-225 ($i^-z^-y^+a^+$), a derivative of ML 308, was the gift of Drs. Winkler and Wilson. All other strains used in this study were derivatives of *E. coli* K12. Strain 3000 ($i^+z^+y^+a^+$) and YA 470 ($i^+z^+y^-$) were the gift of Dr. François Jacob. YA 470

is a mutant of the *amber* type. Other y^- amber mutants, CA 7047, and CA 8202 and a point mutant CA 8204, all of the $i^+z^+y^-a^+$ genotype, were gifts of Dr. Jonathan Beckwith. Strains designated MS 1054, MS 356, MS 1019, and MS 1068 were the gift of Dr. Michael Malamy. Each of these mutants is a y deletion. Strain A 279A ($i^+z^+y^+a^+$) was the gift of Dr. Salvador Luria.

The ML strains were grown on medium 63 described by Cohen and Rickenberg[15] with succinate as carbon source. The K12 strains, many of which are amino acid-requiring auxotrophs, were grown on medium 63 containing thiamin (1 μg/ml) with 1% casamino acids as the carbon source.

The cells of the i^+ genotype were induced by growth overnight in the presence of 5×10^{-4} *M* isopropylthiogalactoside. Unless otherwise noted, the cells were grown at 37° in a rotary shaker to a cell density corresponding to about 0.3 mg of protein/ml (approximately 10^9 cells/ml). Protein was determined by the method of Lowry *et al.*[16]

Preparation of membrane-containing particulate fraction: The following procedure was carried out with 1-liter batches of cells grown to a density of approximately 10^9 cells/ml as described above. All operations, unless otherwise indicated, were performed at ice-bath temperatures.

The cells were chilled, harvested, and washed with 200 ml of 0.1 *M* potassium phosphate buffer of pH 6.3. The washed cells were suspended in 20 ml of the same buffer containing TDG (1 mM) and subjected to sonic irradiation with a Branson sonifier for 3–5 min. The extract was centrifuged at 3,000 × *g* for 15 min in a refrigerated centrifuge, and the residue containing unbroken cells and debris was discarded. The supernatant fraction was then centrifuged for 1 hr at 40,000 × *g*. The membrane-containing particulate fraction so obtained was taken up and suspended in 20 ml of buffer with the aid of a tissue homogenizer of the Potter-Elvejhem type. The suspension was centrifuged for 30 min at 100,000 × *g* and the washed particulate fraction was suspended in 10 ml of a solution of TDG (20 mM) in 0.1 *M* phosphate buffer of pH 6.3.

Treatment with unlabeled N-ethylmaleimide (NEM) in the presence of TDG: In order to reduce the number of cysteine and other reactive residues on proteins other than the *M* protein, a preliminary incubation of the particulate fraction with unlabeled NEM in the presence of saturating amounts of TDG (to protect the *M* protein) was next carried out. The particulate fraction obtained as described above, suspended in 20 mM TDG, was warmed to 28° and mixed with an equal volume of a solution of unlabeled NEM (1 mM), also at 28°. The final concentration of NEM was thus 0.5 mM, while that of the TDG was 10 mM. The suspension was held at 28° for 60 min after which it was chilled and centrifuged at 100,000 × *g* for 30 min. The residue was washed twice with 20 ml of 0.1 *M* phosphate buffer of pH 6.3 to rid it of unlabeled NEM and TDG. This wash procedure must be careful and thorough. The twice-washed particulate fraction was then taken up in 5 ml of 0.01 *M* phosphate buffer of pH 6.3 and assayed for its content of *M* protein. The protein concentration at this point was usually 5–10 mg/ml.

Assay for M protein: The standard assay mixture contained 0.3 ml of the washed particulate fraction that had been pretreated with unlabeled NEM in the presence of TDG as described above. Triplicate or quadruplicate samples were treated with either water (0.02 ml) or 0.2 *M* thiodigalactoside (0.02 ml). The tubes were warmed to 28° and the reaction was initiated by the addition of 0.1 ml of NEM-H^3 (0.4 mM). The specific activity of the NEM was usually 10^6 to 10^7 cpm/μmole. After 30 min at 28°, the reaction was terminated by the addition of 0.1 ml of β-mercaptoethanol (0.05 *M*). After 10 min, carrier protein (0.3 ml of a sonicate of cells of strain ML 308 containing 5 mg protein/ml) was added. The protein was then precipitated by the addition of 5 ml of ice-cold ethanol. The protein-bound radioactivity was then estimated as previously described.[6]

The results of the assays for *M* protein are expressed as the difference (in $\mu\mu$moles) of radioactive NEM bound per milligram of protein by samples incubated in the presence and in the absence of saturating amounts of TDG. The absolute value of this difference, although reproducible and highly significant within a given set of assays (Table 1), is a function of the conditions used for "protection" during preincubation with unlabeled NEM and during the assay. This results from the fact that the reaction of NEM with cysteine residues on proteins is irreversible. Thus, if the conditions during "protection" are made as severe as possible to reduce the amount of nonspecific labeling of proteins during the subsequent assay, the yield of *M* protein may be greatly reduced.

Isolation of mutants with temperature-sensitive lac transport system: The classical studies of

TABLE 1

M PROTEIN IN PARTICULATE FRACTIONS FROM ML STRAINS

Strain	Protein-Bound NEM ($\mu\mu$moles/mg protein) No TDG	0.01 *M* TDG	*M* protein ($\mu\mu$moles/mg protein)
ML 308	200	86	
($i^-z^+y^+a^+$)	188	87	111 ± 5
Uninduced	204	85	
ML 3	94	99	
($i^+z^+y^-a^-$)	97	99	N.S.
Induced	105	102	
ML 308-225	250	126	
($i^-z^-y^+a^+$)	245	132	119 ± 5
Uninduced	260	129	
ML 35	89	97	
($i^-z^+y^-a^-$)	90	95	N.S.
Uninduced	99	95	
ML 30	235	110	
($i^+z^+y^+a^+$)	239	104	135 ± 3
Induced	246	103	
	246	109	
ML 30	124	123	N.S.
Uninduced	124	127	

The particulate, membrane-containing fraction was prepared and assayed as described in the text. The values given for *M* protein represent the difference (in $\mu\mu$moles per mg of protein) in radioactive NEM bound in the presence and in the absence of saturating levels of TDG. The standard error of the difference of the two means is indicated also. Values marked N.S. indicate no significant difference.

Maas and Davis[17] showed that revertants to prototrophy sometimes exhibit altered temperature labilities of the phenotypic trait. If the *M* protein is coded by the *y* gene of the *lac* operon, spontaneous lac^+ revertants of y^- mutants might include mutants with altered forms of the *M* protein, stable at 25° but labile at 37–40°.

Cells from an isolated clone of the y^- mutant CA 8204 were grown to a density of 10^9 cell/ml on standard medium with 1% glucose as carbon source. The cells were harvested, washed with sterile medium, and spread on minimal thiamine plates with lactose (0.2%) as the sole carbon source, at a cell density of 2 × 10^9 cells/plate. After standing for 2–3 days at 25° the plates were inspected, and colonies present at this time (about 50 per plate) were underscored with an ink mark to distinguish them from revertants which arise upon further incubation. The plates were then incubated at 42–43° for an additional 2–3 days. At this time, the underscored colonies exhibited two distinct types of morphology: (1) colonies with a circular inner core surrounded by a halo of inhomogeneous growth and (2) circular homogeneous colonies. Cells from the second class were suspended in minimal medium, streaked on minimal lactose agar, and the procedure described above was repeated. Those colonies which showed the desired morphology (type 2) were spotted on nutrient agar plates. After sufficient growth at 37°, these were replica-plated on minimal agar-glucose and minimal agar-lactose. Revertant strains which were glu^+ at 25° and at 42° but lac^+ at 25° and not at 42° were then assayed directly for the temperature-sensitive transport phenotype, after reisolation from single clones on lactose-agar plates containing eosin-methylene blue.

Experimental Results.—M protein in ML strains: Cell-free particulate fractions obtained from ML strains grown under various conditions were assayed for their content of *M* protein (Table 1). The accuracy and reproducibility of the assay are indicated by the agreement of triplicate sets of values. It can be seen that the inducible strain ML 30 contains significant amounts of *M* protein in the particulate fraction only if the cells have been induced. The constitutive strain ML 308 contains the *M* protein when grown in the absence as well as in the presence of inducer. ML 308-225, a z^- constitutive strain, also contains the *M* protein, while the y^- strains ML 3 and ML 35 lack detectable amounts of this protein. These results

TABLE 2

M PROTEIN IN MUTANTS DERIVED FROM K12 STRAINS

Strain	Genotype	*M* protein ($\mu\mu$moles/mg protein)
3000	$i^+z^+y^+a^+$	80 ± 15
YA 470	$i^+z^+y^-$	N.S.
CA 7047	$i^+z^+y^-a^+$	"
CA 8202	"	"
CA 8204	"	"
MS 1054	"	"
MS 1068	"	"
MS 1019	"	"
MS 356	"	"

All strains were induced by growth in the presence of 5×10^{-4} *M* IPTG before testing. Values of *M* protein were calculated as in Table 1.

indicate that the *M* protein is a product of the *y* gene, a conclusion which is supported by a more detailed examination of K12 strains.

M protein in K12 strains: When the particulate fractions from a number of mutants of strain K12 were assayed for *M* protein, it was found that no significant amounts could be detected in y^- mutants, whereas fully induced wild-type strains contained amounts of *M* protein comparable to the ML strains (Table 2).

Temperature-sensitive M protein in revertants of y^- mutants: Using the procedures described in the section on *Materials and Methods*, several spontaneous revertants of the y^- mutant CA 8204 were isolated which exhibited the *lac*$^+$ phenotype when grown at 25° but were *lac*$^-$ when grown at 42°. Some properties of one such mutant (8204-*ts* 3) will be described in further detail.

Intact cells of this strain which synthesize thiogalactoside transacetylase and β-galactosidase at the same levels as wild type, induced by growth for three to four generations in the presence of IPTG (0.5 mM) at 25°, were tested for the ability to hydrolyze ONPG after being heated at 42° for various time intervals. Since such cells contain an excess of β-galactosidase which is not heat-labile under these conditions, the entrance of ONPG into the cell, mediated by the *M* protein, is rate-making for the hydrolysis process. Cells of the temperature-sensitive revertant rapidly lose the ability to transport ONPG when heated at 42° (Fig. 1). This is accompanied by a corresponding loss in the ability to accumulate C^{14}-methyl-1-thio-β-D-galactoside (TMG). In control experiments, the parental wild-type strain 3000 was not significantly affected by identical heat treatment.

When the temperature-sensitive strain was heated in the presence of TDG (Fig. 1), a striking protective effect was noted. It appears that the *M* protein is protected by TDG not only against sulfhydryl reagents but also against heat inactivation.

When the cell-free particulate fraction from 8204-*ts* 3 was tested for its content of *M* protein, using the reaction with NEM-H^3, values of about one fourth to one fifth of the wild-type parental strain were found. The *M* protein in such fractions is also temperature-sensitive, being completely inactivated after 15 minutes at 42°. *M* protein in similar fractions from wild-type cells is almost completely resistant to such treatment.

To assure that the temperature-sensitive character of 8204-*ts* 3 is indeed the result of a mutation in a gene of the *lac* operon, this mutant, which is also *HfrH* Sm^s, was mated with strain X-5097 (F^- Sm^r *pro-lac*$_{del}$) kindly provided by Dr.

Jonathan Beckwith. *Pro*⁺ recombinants were selected by plating on minimal glucose-streptomycin agar. Seventy of these were streaked on eosin-methylene blue-lactose-streptomycin agar. After incubation at 25° for two days, only *lac*⁺ colonies were observed. Eight streptomycin-resistant *lac*⁺ recombinants isolated from this cross were also tested for temperature-sensitive transport in an experiment similar to that of Figure 1. All showed the temperature-sensitive property of the parent 8204-*ts* 3. In a control experiment, a cross of wild-type Hfr strain 3000 with X 5097 yielded wild-type *lac*⁺ recombinants.

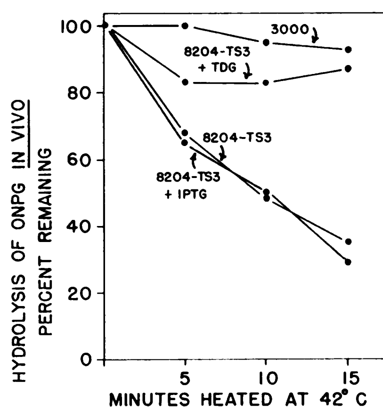

FIG. 1.—Heat inactivation of temperature-sensitive mutant.

Estimation of the minimum number of functional subunits of M protein per cell: On the assumption that a single cysteine residue is protected from attack by NEM when the *M* protein is complexed with TDG, the minimum number per cell of functional subunits containing such a cysteine residue may be calculated. The results of assays such as those in Table 1 and Table 2 represent minimum values of *M* protein, since there is significant loss during the repeated washings of the particulate fraction and during the incubation with unlabeled NEM. The labeling of *M* protein during the assay with tritiated NEM is also incomplete. Experiments in which the washing of the particulate fraction was avoided by the use of Sephadex G-25 to remove small molecules from the suspension, after treatment with unlabeled NEM in the presence of TDG, and in which the time of the incubation with labeled NEM was increased from 30 minutes to 60 minutes, indicated that the content of *M* protein is at least 9000 reactive subunits per cell, assuming that 1 mg of total cell protein represents approximately 3×10^9 cells.

Intracellular localization of M protein: To obtain further information on the localization of the *M* protein in the bacterial cell, spheroplasts of strain ML 308 were prepared by treatment of the cells with lysozyme in 0.3 *M* sucrose containing 1 mM ethylenediaminetetraacetate (EDTA).[18] The cells were lysed by rapid dilution with 9 volumes of distilled water. A few remaining unlysed cells were removed by centrifugation at $3000 \times g$ for ten minutes, and the spheroplast membrane fraction was collected by centrifugation at $10,000 \times g$ for 15 minutes. Preparations from lots of cells grown on different days were tested for their content of *M* protein and compared with particulate fractions derived from sonically disrupted cells, assayed under identical conditions. The spheroplast membranes contained three to four times more *M* protein per milligram of total protein than the total particulate fraction (Table 3).

Discussion.—The results reported here offer strong evidence that the membrane protein component of the *lac* transport system is coded by the *y* gene of the *lac* operon. Thus, the protein appears in inducible cells only after induction, but is present in constitutive strains constitutively. None of a number of strains with amber or deletion mutations in *y* contained the protein. The isolation of mutants with a temperature-sensitive membrane protein as revertants of a mutation in the

y gene strongly supports the view that the protein is coded by the *y* gene. Fox *et al.*[16] have recently described the isolation of y^+a^- strains of *E. coli* completely lacking the enzyme thiogalactoside transacetylase. These strains exhibit unimpaired galactoside transport as measured by ONPG hydrolysis by intact cells and the ability to accumulate TMG. Such strains also contain amounts of *M* protein comparable to wild type, when tested with the NEM method. It is clear that the *M* protein is not coded by the *a* gene, and since these mutants have a lesion in the *a* gene and lack all portions of the operon distal to this lesion,[23] no hypothetical structural gene distal to the *a* gene can be postulated to regulate the *lac* transport system.

TABLE 3

M PROTEIN IN SPHEROPLAST MEMBRANE FRACTIONS AND IN PARTICULATE FRACTION FROM SONICALLY DISRUPTED CELLS

Preparation	*M* protein (μμmoles/mg protein)
Particulate fraction from sonicate of ML 308	131
	86
	93
Spheroplast membrane fraction	372
	408

The spheroplast membrane fraction was prepared as described in the text.

The *M* protein appears to be localized in the spheroplast membrane fraction (Table 3). It is firmly bound, since the content of *M* protein is not reduced by repeated washing with aqueous buffers. The labeled protein, however, can be extracted from the membrane-containing fraction under mild conditions with detergent,[6] suggesting that it is bound to the membrane by lipid-protein bonds. Repeated efforts to demonstrate the *M* protein in the soluble supernatant fraction have yielded negative results, but it must be pointed out that NEM-binding assay is not sufficiently sensitive to detect small amounts of *M* protein in the presence of large amounts of other proteins.

Since the synthesis of membrane protein components of the *lac* system must be directed by a segment of messenger RNA located between those segments coding for β-galactosidase and thiogalactoside acetylase, it is of interest to note that these latter two proteins are recovered almost entirely in the soluble fraction of the cell. The affinity of the *M* protein for the membrane clearly is not the result of some special locus of synthesis, but must be dictated by the specific structure of the protein. The ability of cells to carry out the *M* protein-mediated transport of ONPG appears very soon after induction begins, indicating that extensive changes in the membrane, other than the incorporation of the *M* protein, are not needed for a functional transport system.

Early estimates of the number of functional transport units suggested that this number might be in the range of 200–300/cell,[19, 20] but these estimates were necessarily indirect, and the authors clearly pointed out some of the assumptions needed for their calculations. More direct estimates reported here suggest that the number of functional subunits of *M* protein per cell in strain ML 308 is at least 9000. As pointed out above, we assume in making this calculation that a single cysteine residue is protected from reaction with NEM when the protein is complexed with TDG.

From the point of view of the mechanism of synthesis of proteins of the *lac* operon it is of interest to compare the number of subunits of this protein with those of β-galactosidase and acetylase. Zabin[21] reports that the acetylase constitutes about 0.15 per cent of the total protein of ML 308 when grown at 37° with good aeration. The basic subunit is thought to be of mol wt 35,000. Assuming that 1 mg of total cellular protein represents about 3×10^9 cells, it may be calculated that each cell contains about 8,500 acetylase subunits. A similar calculation can be made for the number of β-galactosidase subunits. Assuming that β-galactosidase makes up 5 per cent of the total protein of ML 308, and that the enzyme is made up of subunits of mol wt 135,000, then each cell contains about 75,000 such units.

Since our estimates of the amount of *M* protein are minimum values, we conclude that the number of subunits of this protein synthesized by the cell is intermediate between the values for acetylase and β-galactosidase, as is consistent with present views of the mechanism of synthesis of the proteins of the *lac* system. On the other hand, it would be difficult to account for values as low as 200–300 per cell.

It is a striking fact that TDG protects the *M* protein against heat inactivation in the temperature-sensitive mutant 8204-*ts* 3, while IPTG does not (Fig. 1). However, this is consistent with our finding[22] that when the *M* protein in wild-type strains is complexed with TDG or mellibiose, a reactive cysteine is prevented from attack by NEM, while other sugars, such as lactose and IPTG, exhibit little or no protective effect. A more detailed study of the interaction of sugars with the *M* protein will be described in another communication.

The work described here provides a direct experimental confirmation of the conclusion reached by Monod and his collaborators that a specific protein coded by the *y* gene is an essential component of the *lac* system. Further studies of the structure and function of this protein are needed, however, if an understanding of the biochemical basis of this transport system is to be achieved.

The authors are indebted to Miss Marilynn Rumley for skilled technical assistance.

* This work has been supported by grants from the U.S. Public Health Service, National Institute of Neurological Diseases and Blindness, NB-02946, General Medical Sciences, GM-13952, and the Life Insurance Medical Research Foundation.

† Fellow of the National Science Foundation. Present address: The Ben May Laboratory for Cancer Research, University of Chicago, Chicago, Illinois.

‡ Special fellow of the U.S. Public Health Service, National Institutes of Health.

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[8] The following abbreviations are used: ONPG = *o*-nitrophenyl-β-galactoside. TDG = β-D-galactosyl-1-thio-β-D-galactoside. IPTG = isopropyl-1-thio-β-D-galactoside. TMG = methyl-1-thio-β-D-galactoside. All galactosides are galactopyranosides. NEM = N-ethylmaleimide.

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