Properties of a mutant lactose carrier of *Escherichia coli* with a $Cys^{148} \rightarrow Ser^{148}$ substitution

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The cysteine residue at position 148 in the lactose carrier protein of *Escherichia coli* has been replaced by serine using oligonucleotide-directed, site-specific mutagenesis of the *lac*Y gene. The mutant carrier is incorporated into the cytoplasmic membrane to the same extent as the wild-type carrier, confers a lactose-positive phenotype on cells, and actively transports lactose and other galactosides. However, the maximum rate of transport for several substrates is reduced by a factor of 6–10 while the apparent affinity is reduced by a factor of 2–4. Carrier activity in the mutant is much less sensitive to sulfhydryl reagents ($HgCl_2$, *p*-(chloromercuri)benzenesulfonate and *N*-ethylmaleimide) than in the wild type, and β-D-galactosyl 1-thio-β-D-galactoside does not protect the mutant carrier against slow inactivation by *N*-ethylmaleimide. It is concluded that the Cys^{148} residue is not essential for carrier-catalyzed galactoside: proton symport and that its alkylation presumbly prohibits access of the substrate to the binding site by steric hindrance. A serine residue at position 148 in the amino acid sequence appears to alter the protein structure in such a way that one or more sulfhydryl groups elsewhere in the protein become accessible to alkylating agents thereby inhibiting transport. Recently, Trumble et al. [(1984) Biochem. Biophys. Res. Commun. 119, 860–867] arrived at similar conclusions by investigating a mutant carrier with a $Cys^{148} \rightarrow Gly^{148}$ replacement.

Lactose carrier *Site-directed mutagenesis* *Galactoside transport* *Sulfhydryl reagent*

1. INTRODUCTION

In their classic experiments, Fox and Kennedy [1] identified the lactose:H^+ carrier as an integral protein of the cytoplasmic membrane of *Escherichia coli* by differential labeling of a sulfhydryl group by *N*-ethylmaleimide (see [2–5] for recent reviews on the structure and function of this protein). This critical sulfhydryl group was assigned to the cysteine residue 148 in the amino acid sequence [6]. Because modification of this thiol abolishes substrate binding [7–9], Cys^{148} is considered to be at or near the sugar binding site. Studies on the effect of collisional quenchers on carrier-bound dansyl-galactosides or carrier alkylated at Cys^{148} by *N*-(1-pyrenyl)maleimide suggest that the binding site is buried within the protein and not on the surface and that the cysteine residue is located at a position corresponding to the depth of C-atom 5 of the phospholipid acyl chains [10]. However, it remains unknown whether this thiol is essential for substrate recognition at the single binding site in the protein or whether its modification prohibits access of the substrate by steric hindrance. Exchange of amino acids via site-directed mutagenesis of the cloned and sequenced *lac*Y gene [11,12] coding for the carrier protein can distinguish between these alternatives. This communication describes the properties of a mutant lactose carrier in which Cys^{148} is replaced by a serine residue. While this work was in progress, Trumble et al. [13] using

Abbreviations: GalSGal, β-D-galactosyl 1-thio-β-D-galactoside; NpαGal, *p*-nitrophenyl-α-galactoside; MeSGal, methyl 1-thio-β-D-galactoside; NpβGal, *o*-nitrophenyl-β-D-galactoside

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the same approach reported experiments with a mutant carrier with a $Cys^{148} \rightarrow Gly^{148}$ substitution. Both studies come to the conclusion that the thiol at Cys^{148} is not essential for substrate recognition or galactoside:H^+ symport.

2. MATERIALS AND METHODS

Oligonucleotide synthesis was carried out as described [14,15]. Cells were grown and induced with isopropyl-β-D-thiogalactoside (0.2 mM for strains carrying the mutated *lac*Y gene, 0.1 mM for *lac*Y^+ cells) as detailed in [11]. Cells were treated with EDTA (1 mM) for 2 min in 0.2 M Tris, pH 8.0, at 25°C before the transport measurements [9]. Uptake was terminated by addition of 0.15 M LiCl plus 2 mM $HgCl_2$. Carrier levels in membranes were quantitated by substrate binding [9,16] or by an immunological method [17].

3. RESULTS

3.1. *Oligonucleotide-directed site-specific mutagenesis*

The strategy for changing codon 148 in the *lac*Y gene is outlined in fig.1. The 2.3 kb *lac*Y-containing fragment of plasmid pGM21 [11] was ligated into the single *Eco*RI site of the vector pEMBL8(+) [18] yielding plasmid pTJN2. Upon superinfection of a strain carrying this plasmid with phage IR1, single strands of the plasmid pTJN2 containing the sense strand of the insert are packaged into viral particles. Single-stranded plasmid pTJN2 was annealed to a 19-meric oligodesoxyribonucleotide with the indicated mismatches in codons 147 and 148. The mutant gene having these base changes codes for Gly^{147}-Ser^{148}. Furthermore, a new recognition site for the restriction enzyme *Xho*II (recognition sequence Pu↓GATCPy) is created which simplifies the identification of mutant clones. As described in the legend to fig.1, a clone having the expected properties was found. The mutated gene was re-inserted into the vector pACYC184 yielding plasmid pJAS3. pJAS3 codes for a serine residue at codon 148 of the *lac*Y gene, carries a conservative base substitution in codon 147, but is otherwise identical to the previously described *lac*Y^+-containing plasmid pGM21 [11]. The properties of the mutant and wild-type carrier are compared in a Lac Z^+Y^- (β-galactosidase posi-

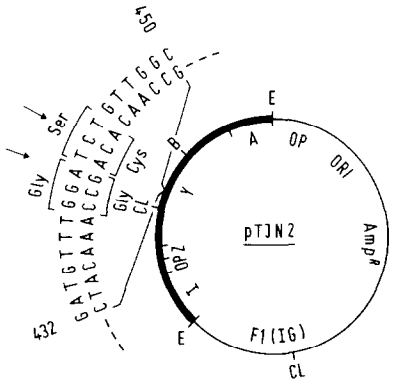

Fig.1. Localized mutagenesis of the *lac*Y gene. The 2320 bp-*Eco*RI restriction fragment of plasmid pGM21 [11] which carries the *lac* $\Delta(I)P^+O^+\Delta(Z)Y^+\Delta(A)$ region was inserted into plasmid pEMBL8(+) [18] yielding plasmid pTJN2. Upon superinfection with phage IR1 (a variant of phage F1) cells harboring plasmid pTJN2 produce phage particles containing single-stranded pTJN2 with the sense strand of the *lac*Y gene. Localized mutagenesis was carried out [19] using single-stranded phage DNA and the synthetic oligonucleotide primer indicated in the figure. Transformants containing the mutated sequence were identified by colony hybridization [20] using ^{32}P-end-labeled primer: prehybridization in 6 × SSC, 10 × Denhardt's solution, 2.5 mM Na-EDTA, 0.5% SDS (2 h at 68°C); hybridization in the same solution containing ^{32}P-labeled primer (16 h at 37°C); post-hybridization washes in 6 × SSC (30 min, room temperature; 30 min, 52°C; 30 min, room temperature). A hybridizing clone (pTJN2-5) was found that contained a new *Xho*II restriction site at the expected position corresponding to Ser^{148} in the mutated Y gene and a second undesired *Xho*II site towards the 3′-end of the Y gene. This latter *Xho*II site was eliminated by combining the 0.5 kb *Cla*I-*Bcl*I fragment of plasmid pTJN2-5 with the large *Cla*I-*Bcl*I-fragment of plasmid pTJN4 (a derivative of pTJN2 lacking the *Cla*I-site in the vector) yielding plasmid pJAS2. The 0.5 kb *Cla*I-*Bcl*I fragment of plasmid pTJN2-5 was sequenced and shown to have the two expected nucleotide changes (lacY $C^{441} \rightarrow A^{441}$ and $G^{443} \rightarrow C^{443}$). The *lac*Y-containing *Eco*RI fragment of pJAS2 was transferred to the vector pACYC184 yielding plasmid pJAS3. Transformation of strains T215 (*lac* $I^+O^+Z^+Y^-/F'I^qO^+Z^+\Delta(Y)$) and T184 ($I^+O^+Z^-/F'I^qO^+Z^{U118}(Y^+)$, cf. [11]) with pJAS3 resulted in strains T2JP and T1JP, respectively. The corresponding isogenous wild-type strains carrying plasmid pGM21 are T217 and T206. E, *Eco*RI; B, *Bcl*I; Cl, *Cla*I; I, repressor gene of the *lac* operon; PO, *lac* promoter-operator; Y, lactose carrier gene; ORI, origin of replication; Amp^R, ampicillin resistance; F1(IG), intragenic region of phage F1.

tive, lactose carrier negative) background (strain T2JP carrying pJAS3 and strain T217 carrying pGM21) or a Lac Z^-Y^- (β-galactosidase negative, lactose carrier negative) background (strain T1JP carrying pJAS3 and strain T206 carrying pGM21).

3.2. *Expression of the mutant* lac*Y gene*

A recently developed immunological method allows the quantitation of the lactose carrier in cytoplasmic membranes irrespective of the functional properties of the protein [17]. Membrane proteins are separated by SDS-polyacrylamide gel electrophoresis and electrotransferred to a nitrocellulose filter. The filter is treated with specific rabbit antibodies against the synthetic C-terminal decapeptide of the carrier [21] and goat anti-(rabbit immunoglobulin) antibody conjugated to alkaline phosphatase. Using suitable chromogenic substrates for alkaline phosphatase, the location of the carrier is revealed on the filter, and the amount is quantitated by comparison with known amounts of wild-type carrier as a standard. This analysis shows that the mutant carrier has the same electrophoretic mobility as the wild-type protein and that it is incorporated into the membrane to the same extent (fig.2 and table 1).

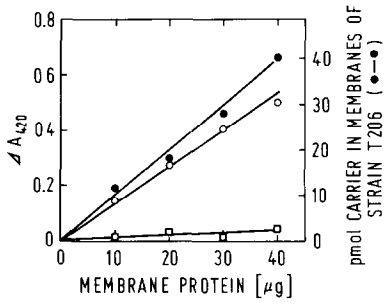

Fig.2. Immunochemical quantitation of mutant carrier. Total cell envelopes were prepared from induced (○—○) or uninduced strain T1JP (mutant, □—□) and from induced strain T206 (wild type, ●—●) by sonication. Equal amounts of protein were subjected to electrophoresis in the presence of SDS, and the proteins were transferred to a nitrocellulose filter. The filter was treated with rabbit antibody directed against the synthetic carboxy terminus of the carrier and subsequently with goat anti-(rabbit immunoglobulin) conjugated to alkaline phosphatase. Carrier bands were visualized as described in [17], the bands were cut from the filter, and the phosphatase activity measured by the hydrolysis of 1 mg/ml *p*-nitrophenyl phosphate (ΔA_{420}). The slope for the samples from strain T1JP is 79.7% of that for those from strain T206, which contains 1.0 nmol NpαGal binding sites/mg protein (cf. [9]). Membranes from strain T1JP contain 0.8 nmol carrier/mg protein when induced and only 0.05 nmol/mg when inducer is not present.

3.3. *Galactoside active transport*

The mutant carrier confers a Lac^+ phenotype upon a strain tested on lactose-containing indicator plates or mineral salts lactose plates. Transport data for several substrates appear in table 1. Lactose is transported at a maximum rate, V_{max}, 10-times smaller than in the wild type while the half-saturation constant, K_T, is increased by a factor of two. A decrease in V_{max} and an increase in K_T are also observed for GalSGal, NpαGal and MeSGal. This increase in K_T explains the inability to measure substrate binding to cytoplasmic membranes of the mutant using either NpαGal or GalSGal (not shown). On the other hand, a change in apparent affinity is not evident for NpβGal or melibiose.

Sugar:H^+ stoichiometries, n_H, are computed in mutant cells by comparison of galactoside accumulation in the steady state ($\Delta\mu_{lactose}$ = 102 mV, $\Delta\mu_{GalSGal}$ = 114 mV) with the estimated driving force $\Delta\tilde{\mu}H^+ = -131$ mV ($\Delta\psi = -88$ mV; ΔpH = -43 mV) yielding values for lactose, $n_H = 0.78$, and for GalSGal, $n_H = 0.87$. Thus, both substrates are actively transported with a symport stoichiometry close to 1.

3.4. *Sensitivity against sulfhydryl reagents*

The activity of the wild-type carrier is inhibited by 0.5 mM *N*-ethylmaleimide (half-time 1.5 min, fig.3, ●—●). The substrate GalSGal provides essentially complete protection against this inhibitor (○—○). In contrast, the mutant carrier is inactivated with a half-time of ≈5 min (▲—▲), but GalSGal affords no protection (△—△). Also, the mutant carrier is much less sensitive to inactivation by mercurials. When assayed by rate measurements of NpβGal hydrolysis in vivo both 2 mM $HgCl_2$ and 0.2 mM *p*-(chloromercuri)benzenesulfonate inactivate the wild-type protein in less than 5 s while the mutant is inhibited with a half-time of 80–90 s.

Table 1

Comparison of kinetic constants for mutant and wild-type lactose carrier

Substrate	Strain	V_{max} (nmol/min per mg protein)[a]	K_I (mM)	Concentration (nmol/mg protein)[b]
Lactose	wild type	480	0.27	
	mutant	49	0.56	1.1[c]
GalSGal	wild type	264	0.063	1.1[d]
	mutant	43	0.123	0.8[c]
NpαGal	wild type	241	0.048	0.9[d]
	mutant	43	0.192	0.8[c]
NpβGal	wild type	1030	0.97	
	mutant	120	1.1	1.0[c]
MeSGal	wild type	–	0.87[c]	
	mutant	–	5.1	
Melibiose	wild type	–	0.5	
	mutant	–	0.5	

[a] Measurements refer to cells. V_{max} was estimated either from the rate of uptake of radioactively labeled substrates (lactose, GalSGal, NpαGal) or from the rate of NpβGal hydrolysis in vivo. K_T values for MeSGal and melibiose were obtained from the inhibition of NpβGal hydrolysis in vivo

[b] Data refer to total cell envelope protein

[c] For estimation cf. fig.2

[d] Determined by substrate binding

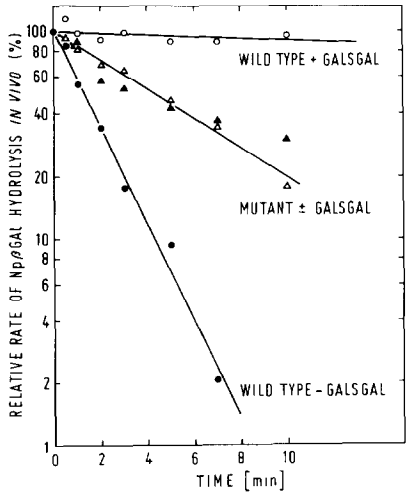

Fig.3. Inactivation of wild-type and mutant carrier by *N*-ethylmaleimide. Induced cells (400 μl, A_{420} = 12) in Cohen-Rickenberg buffer [22] were treated at room temperature with *N*-ethylmaleimide (50 μl, 5 mM) in the presence or absence of GalSGal (50 μl, 0.2 M) in a final volume of 0.5 ml. After the indicated times reactions were terminated by addition of 25 μl of 80 mM dithiothreitol. Cells were washed twice by centrifugation, resuspended in buffer, and the rate of NpβGal hydrolysis in vivo was determined [23]. Strain T217 (wild type, without GalSGal, ●—●), same plus GalSGal (○—○); strain T2JP (mutant with, ▲—▲, or without, △—△, GalSGal). For absolute rates cf. table 1.

4. DISCUSSION

Because site-specific mutagenesis using oligonucleotides frequently leads to undesired mutations elsewhere in the template, it is important to point out that restriction mapping and DNA sequencing ensure that the reconstituted *lac*Y gene in the mutant plasmid pJAS3 contains only the expected base changes in codons 147 and 148 (cf. legend to fig.1). The mutated *lac*Y gene is translated in the correct reading frame because the product contains the correct C-terminus. Moreover, the mutated protein is incorporated into the cytoplasmic membrane of *E. coli* to the same extent as the wild-type carrier (fig.2).

The mutant carrier actively transports β-galactosides with a galactoside:H^+ symport stoichiometry close to one and confers a lactose-positive phenotype on cells. Therefore, a role of this sulfhydryl group in the binding of the translocated proton is excluded. However, the maximum rate of influx for several substrates is reduced by a factor of 6–10 (table 1). Within the framework of the translocation cycle [4] this observation indicates that the translocation rate constants of the loaded or unloaded carrier or both are reduced. Also, for several (lactose, GalSGal, Npα Gal, MeSGal) but not all substrates (Npβ Gal, melibiose) the apparent affinity is reduced by a factor of 2–4. Finally, the K_T for active lactose transport in the mutant is still much smaller (K_T = 0.56 mM) than the equilibrium binding constant (K_D = 20 mM) of the wild-type protein suggesting that the mutant protein still exhibits the $\Delta\tilde{\mu}H^+$-induced change in the apparent affinity observed for this and certain other substrates [9].

As expected, mutant and wild-type carrier differ markedly in their reactivity against sulfhydryl reagents. Because the mutant carrier reacts much more slowly with $HgCl_2$ or *p*-(chloromercuri)benzenesulfonate than the wild type, it is clear that Cys^{148} is a preferred target for these inhibitors in the wild-type protein. The rate of inactivation by *N*-ethylmaleimide is also much slower in the mutant (fig.3) whether or not saturating substrate is present. Unexpectedly, this rate is nevertheless faster than the rate of inactivation of the wild-type protein in the presence of the protective substrate GalSGal. Thus, it appears that a change in conformation caused by the mutation and reflected in altered values of V_{max} and K_T in the mutant makes a cysteinyl residue elsewhere in the protein more accessible to *N*-ethylmaleimide. This residue can now be identified by the methods developed in Beyreuther's laboratory [5,6].

The mutant described by Trumble et al. [13] which carries a $Cys^{148} \rightarrow Gly^{148}$ substitution has properties very similar to those described for the mutant in this study. This mutant carrier also actively transport lactose albeit at a reduced rate, and it is slowly inactivated by *N*-ethylmaleimide in the absence or presence of GalSGal.

In summary, the data suggest that the replacement of an SH group at position 148 by an OH group leaves the overall structure and function of the carrier unaltered but causes a subtle change in conformation which quantitatively alters the substrate affinity and the rate of translocation. The situation may be analogous to the behavior of a cysteinyl residue near the sugar binding site of the L-arabinose-binding protein [24,25]. In this protein the thiol group is not directly involved in sugar binding but its modification by sulfhydryl reagents inhibits access of the substrate to the binding site by steric hindrance.

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