Galactoside–proton symport in a *lacY^{UN}* mutant of *Escherichia coli* investigated by analysis of transport progress curves

Malcolm G. P. PAGE

Department of Microbiology, Biozentrum, Klingelbergstrasse 70, CH-4056 Basel, Switzerland

The kinetics of galactoside–proton symport catalysed by a wild-type strain and one carrying a mutation, previously reported to cause uncoupling of the symport reaction, have been examined. The mutation does not affect the stoichiometry during the initial period of uptake, when the internal concentration of galactoside is low, but it does result in much greater competition from the galactoside as it is accumulated. Simple methods for the analysis of the uptake progress curves have been developed and used to estimate the initial rate of uptake and affinity for internal galactoside. The maximum rate of uptake is decreased by a factor of 2 at most whereas the affinity for internal galactoside is increased up to 50-fold by the mutation. The pH-dependence of the galactoside efflux reaction is changed in a manner which suggests that the defect is in the interaction between proton-binding and galactoside-binding sites rather than in the structure of either site.

INTRODUCTION

The mechanisms by which polar solutes not only penetrate the diffusion barrier constituted by the cytoplasmic membrane but also accumulate on one side of it in response to the input of energy from metabolism have been the subject of considerable investigation. The lactose permease from *Escherichia coli* is a much-studied example of an active transport system where the energy driving the transport of the sugar substrate against its chemical potential gradient is obtained by the movement of the symported proton down its electrochemical potential gradient (West, 1970; West & Mitchell, 1973). Transport in this system can be successfully treated as a two-substrate reaction in which the galactoside and the symported proton bind to the permease by a random mechanism (Page & West, 1981, 1982) to form a ternary complex which then re-orientates and releases the products (internal galactoside and proton). Steady-state kinetics cannot, however, give much information about the first-order steps that occur after substrate-binding (that is to say, those involved in the re-orientation of the ternary complex or unloaded permease) and hence the nature of the coupling between galactoside and proton fluxes, which is the key to understanding the mechanism of active transport, remains obscure. One way to collect the necessary information would be through the study of mutants that are defective in various aspects of the transport process. The *lacY^{UN}* mutants obtained by Wilson and colleagues (Wilson *et al.*, 1970; Wong *et al.*, 1970; Wilson & Kusch, 1972) are particularly pertinent in this respect for they have a phenotype that suggests that the coupling of the symport reaction is defective (West & Wilson, 1973). The mutants accumulate galactosides to much lower levels and take up protons more slowly in response to addition of galactoside than do the parents. This, in itself, is consistent with either a low amount of permease or a low rate of uptake, both of which have been shown to affect the level of accumulation that can be attained (Wilson & Maloney,

1973). However, the *Y^{UN}* mutants are remarkable in that the amount of permease is about the same as in the parent (Wong *et al.*, 1970; Herzlinger *et al.*, 1985; Wright & Seckler, 1985) and that the rate of unidirectional flux in galactoside exchange reactions is at least as great as in the parent (Wong *et al.*, 1970; Wilson *et al.*, 1970). It was suggested that the coupling process was defective, either because of a defective proton-binding reaction (Seto-Young *et al.*, 1984; Herzlinger *et al.*, 1985) or because the proton–permease binary complex could translocate at an increased rate (West, 1980). Wright & Seckler (1985) showed that, although the apparent stoichiometry of the permease (determined thermodynamically) is less in the mutant than it is in the parent, it is not sufficiently different to account for the apparent uncoupling between flux of proton and galactoside reported by West & Wilson (1973) and they suggested that the decreased proton flux was due to a decrease in the rate of turnover. However, Wilson & Kusch (1972) had already shown that, at least under some circumstances, the initial rate of net uptake into the mutant is at least as fast as uptake into the parent, which argues against this explanation. Page & West (1982) discussed another way whereby the rate of net turnover is decreased, namely through product inhibition from the accumulated galactoside and internal protons. As the internal concentration of galactoside increases the rate of influx is expected to decrease until a steady-state is reached where no net flux occurs. It is shown here that the range of internal galactoside concentrations over which this effect operates in the mutant is much lower than the corresponding range in the parent. This results in a relatively rapid collapse of the initial rate of net uptake into the mutant compared with the parent and, ultimately, to a lower internal concentration when the steady-state is attained. Thus, the mutation results in an apparent increase in the affinity for internal galactoside but it is accompanied by a different response by some of the flux reactions involving internal galactoside to protonation of the permease.

MATERIALS AND METHODS

Growth of bacteria

Escherichia coli ML308 (i^- , z^+ , y^+ , a^+), ML308-22 (i^- , z^+ , y^{UN} , a^+) and ML30 (i^+ , z^+ , y^+ , a^+) were all grown in a minimal medium M9 (Anderson, 1946) containing 0.5% glycerol and harvested in late exponential phase. Strain ML30 was induced by the addition of 50 μ M-isopropylthiogalactoside; protein synthesis was terminated by adding 200 μ g of chloramphenicol/ml. The bacteria were washed once by resuspension in 150 mM-KCl containing 100 μ g of chloramphenicol/ml and once by resuspension in the medium of the experiment, also containing chloramphenicol.

Measurement of transport

Uptake measurements were performed as described previously (Page & West, 1980, 1981, 1982, 1984). Efflux experiments were performed by loading cells by preincubation with different concentrations of radioactive galactosides for 1 h at room temperature and then diluting the suspension with a sufficient volume of medium to ensure that the external concentration of galactoside was $0.05 \times$ the apparent K_m . For example, 1 ml of a cell suspension incubated with 5 mM-thiolactose ($K_m = 0.6$ mM) was diluted with 280 ml of medium. Sodium phosphate buffers were used in the range pH 5.5–8.0 and sodium borate buffers were used in the range pH 8.0–10.0. Equilibrium exchange was performed in the same way except that the dilution medium contained non-radioactive galactoside at the same concentration as the radioactive galactoside in the preincubation medium and that both media contained 30 mM- NaN_3 . Samples were taken at 10–60 s intervals and quenched with 10 mM- HgCl_2 (sampling time < 2 s). Counterflux experiments were performed by incubating cells in medium containing 30 mM- NaN_3 and non-radioactive galactoside at a concentration approx. $10 \times K_m$ (e.g. 6 mM for thiolactose) for 1 h at room temperature. The reaction mixture was diluted 100-fold with medium containing radioactive galactoside to give a final concentration equal to $0.1 \times K_m$. Samples were taken at intervals between 2 and 60 s and quenched with

10 mM- HgCl_2 . In each experiment [^3H]galactosides were used at a specific radioactivity of 1–2 Ci/mol. Quenched samples were collected by filtration through Millipore filters (45 μ m pore size), washed three times with quench solution and radioactivity measured by liquid-scintillation counting. For each experiment a parallel incubation was diluted with medium containing 10 mM- HgCl_2 and samples taken throughout the time-course of the experiment to correct for passive leaks. The components of the protonmotive force were measured using the methods described by Booth *et al.* (1979). Internal volume was estimated to be 1 μ l/mg dry wt. Progress curves of galactoside flux were analysed according to the methods described in the Appendix; substrate dependence of initial rates and pH-dependence of kinetic parameters were analysed by using appropriate weighted linear regression (Cleland, 1979; Page & West, 1981).

Materials

Thiolactose was synthesized following the method of Reed & Goodman (1981). ^3H -labelled galactosides were prepared as described previously (Page & West, 1984). Radioactive materials were purchased from Amersham Buchler and non-radioactive sugars were purchased from Sigma or BDH.

Definitions

K_m , the pH-dependent apparent Michaelis constant for influx.

V_1 , the pH-dependent maximum velocity of influx.

K_p , the pH-dependent inhibition constant describing the competitive effect of internal galactoside on K_m .

K_p' , the pH-dependent inhibition constant describing the non-competitive effect of internal galactoside on V_1 .

V_2 , the apparent unidirectional rate of efflux during net influx.

D , passive diffusion rate constant for galactosides.

K_a , dissociation constant for protons. All ionizations are formally regarded as occurring in the external medium, as this is the variable in all pH-dependence experiments.

K_e , the pH-dependent apparent Michaelis constant for net efflux;

Fig. 1. Time-course of uptake of thiolactose into *E. coli* ML308

(a) Concentration-dependence of the progress curve of thiolactose uptake into the parent. (b) Product-inhibition plot of the data presented in (a). The analysis uses the rate equation [eqn. (3) in the Appendix]. (c) Product-inhibition plot of the data presented in (a). The analysis uses the integrated rate equation [eqn. (6) in the Appendix].

Fig. 2. Time-course of uptake of thiolactose into *E. coli* ML308-22 at pH 6.0

(a) The progress curve for uptake of thiolactose (0.3 mM external concentration) into ML308 (●) and ML308-22 (○). (b) Product-inhibition plot of the data presented in (a) using the integrated rate equation.

V_e , apparent maximum velocity of net efflux.

A_{max} , the maximum internal concentration extrapolated from the dependence of accumulation on external galactoside concentration.

K_{acc} , the concentration of external galactoside giving half-maximal accumulation.

[S], external concentration of galactoside.

[P], internal concentration of galactoside.

Δp_{lac} , the chemical potential gradient for galactosides = $RT \ln ([P]/[S])/F$.

Δp , the protonmotive force expressed in mV.

RESULTS

Analysis of the time-course of uptake: is there product inhibition?

Uptake of thiolactose into the parent strain occurred rapidly at first but slowed down within the first 10 s after mixing; accumulation continued for several minutes until a steady-state was reached where there is no net flux (Fig. 1a). An analysis of the time-course according to the outlines given in the Appendix indicated that there was indeed inhibition from the internal products and that it

Fig. 3. Time-course of uptake of melibiose into *E. coli* ML308, ML308-22 and ML30

(a) Progress curves for melibiose uptake into *E. coli* ML308 (●), ML308-22 (▲) and two different cultures of strain ML30 induced with low levels of isopropylthiogalactoside, as described in the text (■ and ○). (b) Product inhibition plot of the data presented in (a). The analysis uses the integrated rate equation and the kinetic parameters derived from this plot are given in Table 2.

was mixed type with similar values for K_p and K_p' . The analysis using the rate equation (eqn. 5 in Appendix) gave product inhibition constants of about 27 mM for thiolactose ($K_p = 26.5 \pm 2.1$ mM, $K_p' = 27.1 \pm 1.8$ mM; Fig. 1b), whereas the apparent K_m was 0.62 ± 0.11 mM and apparent maximum velocity (V_1) was 210 ± 30 mM $\cdot$ min $^{-1}$. Analysis using the integrated rate equation (eqn. 7 in Appendix) gave a value of 26.8 ± 0.5 mM for the product inhibition constant (K_p), $K_m = 0.37 \pm 0.05$ mM and $V_1 = 180 \pm 10$ mM $\cdot$ min $^{-1}$ (Fig. 1c).

In the mutant the initial rate of uptake of thiolactose collapsed even within the first few seconds of uptake (Fig. 2a) and analysis of the uptake progress curve using the rate equation gave product inhibition constant of about 1.2 mM for thiolactose ($K_p = 1.1$ mM, $K_p' = 1.3$ mM) whereas K_m and V_1 showed less change at 0.34 mM and 150 mM $\cdot$ min $^{-1}$ respectively. Using the integrated rate equation, the corresponding values were $K_p = 1.5 \pm 0.15$ mM and $V_1 = 99$ mM $\cdot$ min $^{-1}$ (Fig. 2b). Similar results were obtained with melibiose (Fig. 3); the time-course of uptake into the mutant showed a much more rapid collapse of the influx velocity than did that of the parent. Analysis using the integrated rate equation showed that, for uptake into the parent $K_p = 2$ mM, $K_m = 0.5$ mM and $V_1 = 193$ mM $\cdot$ min $^{-1}$ whereas, for uptake into the mutant, $K_p = 0.98$ mM, $K_m = 0.2$ mM and $V_1 = 140$ mM $\cdot$ min $^{-1}$.

The efflux velocity, V_2 , was calculated from the derived kinetic parameters and the steady-state level of accumulation, after allowing for the contribution of leaks (eqn. 3 in the Appendix). This gave a series of simultaneous equations which were solved for V_2 and the diffusion constant D at a series of combinations of [S] and [P]. The results always had a considerable scatter but the mean values for D for thiolactose in mutant and the parent were 12.6 μ M $\cdot$ min $^{-1}$ and 15.6 μ M $\cdot$ min $^{-1}$ respectively, whereas the mean values for V_2 for thiolactose were 150 μ M $\cdot$ min $^{-1}$ and 10.2 μ M $\cdot$ min $^{-1}$ for mutant and parent respectively. Although too much weight should not be attached to the values of V_2 obtained from these calculations, it does appear that the rate of unidirectional efflux is greater in the mutant than it is in the parent. This pattern of increased product inhibition (lower K_p and higher V_2) was observed with all galactosides that were tested (Table 1).

The patterns of inhibition observed with the mutant should be contrasted with those observed with partially induced suspensions of ML30 that accumulated approximately the same amount of galactoside as the mutant (Fig. 3). The initial rate of uptake was low and collapsed over the same time-course as in the fully-induced suspension. Analysis of the time-course gave a product inhibition constant that was similar to those obtained with the fully-induced suspension but with values of V_1 and V_2 that were 10-fold lower (Fig. 3 and Table 2).

The pH-dependence of the kinetic parameters of uptake: is the proton-binding site altered?

The kinetic parameters describing the influx reaction (V_1 , K_m , V_1/K_m ; Fig. 4a) appeared to be essentially the same in the parent and the mutant. Although the value of V_1 was somewhat lower for the mutant (by a factor of 0.5–0.9), there were ionizations with apparent pK_a 6.2 and 10.0 which affected its value in both strains. Similarly, K_m was found to be lower for the mutant

Table 1. Kinetic parameters for galactoside uptake at pH 6.0

Uptake assays were performed in 0.1 M-sodium phosphate, pH 6.0. The kinetic parameters are derived using the integrated rate equation (eqn. 3 in Appendix) as shown in Fig. 2.

Substrate	Strain . . .	V_1 (mM $\cdot$ min $^{-1}$)		V_2 (μ M $\cdot$ min $^{-1}$)		K_m (mM)		K_p (mM)	
		308	308-22	308	308-22	308	308-22	308	308-22
Methylthiogalactoside		155 $\pm$ 73	130 $\pm$ 57	6.5	318	1.8 $\pm$ 0.2	0.95 $\pm$ 0.1	32.9 $\pm$ 1.2	2.3 $\pm$ 0.9
Thiolactose		180 $\pm$ 10	95 $\pm$ 23	10	150	0.37 $\pm$ 0.0	0.15 $\pm$ 0.05	26.8 $\pm$ 0.5	1.2 $\pm$ 0.2
Phenylthiogalactoside		127 $\pm$ 36	100 $\pm$ 20	8	320	0.92 $\pm$ 0.1	0.51 $\pm$ 0.1	42.7 $\pm$ 1.5	1.8 $\pm$ 0.3
Galactosylthiogalactoside		94 $\pm$ 21	80 $\pm$ 20	2.4	38	0.06 $\pm$ 0.01	0.04 $\pm$ 0.01	4.0 $\pm$ 0.1	0.08 $\pm$ 0.0
Melibiose		193 $\pm$ 91	140 $\pm$ 23	12	122	0.50 $\pm$ 0.08	0.21 $\pm$ 0.07	21.1 $\pm$ 1.0	0.98 $\pm$ 0.09

Table 2. Kinetic parameters for melibiose uptake into fully or partially induced suspensions of *E. coli* ML30

Induction of ML30 was with 100 μM -isopropylthiogalactoside to obtain full induction and 50 μM -isopropylthiogalactoside to obtain the partial induction. In Fig. 3 uptake by the partially induced culture 1 is shown by squares and uptake by culture 2 is shown by open circles.

	V_1 ($\text{mM} \cdot \text{min}^{-1}$)	V_2 ($\mu\text{M} \cdot \text{min}^{-1}$)	K_m (mM)	K_p (mM)
Fully induced	180	21	0.5	25
Partially induced				
1	50	5.4	0.6	26
2	17	2.2	0.4	25

Fig. 4. pH-dependence of melibiose transport

The pH-dependence of the kinetic parameters describing (a) influx (K_m , V_1), (b) product inhibition (K_p , V_2), (c) efflux (K_e , V_e) and (d) accumulation (see below) are shown for the parent (solid symbols) and the mutant (open symbols). Circles represent the value of K , triangles represent the value of V and squares represent the value of K/V . In (c) the magnitude of the pH gradient is also shown (crosses). In (d) the parameters plotted are the maximum capacity (A_{max} ; circles), the external concentration producing half-maximum accumulation (K_{acc} ; triangles) and $A_{\text{max}}/K_{\text{acc}}$ (squares). All values except the pH gradient in (c) are plotted as the logarithm to base 10.

(again by a factor of 0.5) but there was an ionization with apparent $\text{p}K_a$ 8.3 that resulted in an increase in K_m at alkaline pH in both strains. The pH-dependence of the specificity constant V_1/K_m , which describes the association between substrate and protonated permease to form the ternary complex, was nearly the same in both strains. Thus the mutation does not affect unidirectional influx.

The pH-dependence of the kinetic parameters describing product inhibition of influx (V_2 , K_p , V_2/K_p ; Fig. 4b) did appear to differ between the two strains. In the parent, V_2 showed a marked dependence on pH, with a maximum around pH 7.5, decreasing sharply at lower pH and more gently at higher pH. The ionizations that produced this effect had $\text{p}K_a$ 6.1 and $\text{p}K_a$ 10. In the

mutant, V_2 was similar to that of the parent at high pH (7.5) but rose at lower pH. This appears to be due to a stimulation of V_2 by a protonation with apparent pK_a 8.3. In the parent K_p has a maximum value at pH 7.0 and decreases at higher or lower pH. The decrease at acid pH appeared to be controlled by a protonation with apparent pK_a 6.2 whereas the variation at alkaline pH appeared to be controlled by a combination of an ionization with apparent pK_a 8.3 that increased K_p and the collapse of the pH gradient as the external pH approaches pH 8 (which decreases K_p). In the mutant K_p appeared to be independent of pH so that, whereas at pH 9 the value of K_p was the same for the mutant and parent, at pH 6 the value of K_p was very much lower in

the mutant than it was in the parent. These differences are reflected in the pH-dependence of V_2/K_p ; in the parent V_2/K_p increased between pH 5 and pH 8 then decreased, whereas in the mutant V_2/K_p increased between pH 5 and pH 6 and then decreased at alkaline pH where it had the same value as in the parent.

Efflux from the parent cells occurred with K_e of 46 mM, and V_e of 83 mM·min⁻¹ at pH 10. This did not change significantly until the external pH was less than 8, when K_e started to increase, reaching a maximum of 300 mM around pH 7 (Fig. 4c). At still lower values of external pH, both V_e and K_e decreased. Thus, the pH-dependence of efflux paralleled that of product inhibition. In contrast, efflux from the mutant cells showed little

Fig. 5. Galactoside exchange reactions

(a) Isotope exchange at equilibrium. Isotope exchange for reaction of melibiose (10 mM) with parent (▲) and mutant (●) at pH 6 and with parent (△) and mutant (○) at pH 9.0 are shown. (b) Counterflux reactions with thiolactose are shown for the parent at pH 6 (○) and pH 8.0 (●) and the mutant at pH 6 (△) and at pH 8.0 (▲). The cells were loaded with 10 mM-galactoside and diluted into medium containing 0.1 mM (final concentration) [³H]thiolactose. The curve of counterflux is described empirically by eqn. (1):

$$[P^*]_t = [P^*]_0 e^{-k_{\text{eff}} t} (1 - e^{-k_{\text{in}} t}) \quad (1)$$

$[P^*]_t$ is the instantaneous radioactivity, $[P^*]_0$ is the extrapolated end-point of the influx reaction, k_{eff} is the rate of efflux and k_{in} is the rate of influx. For the parent the relevant parameters are $k_{\text{in}} = 0.046 \text{ s}^{-1}$, $k_{\text{eff}} = 0.015 \text{ s}^{-1}$ at pH 6 and $k_{\text{in}} = 0.0375 \text{ s}^{-1}$, $k_{\text{eff}} = 0.029 \text{ s}^{-1}$ at pH 8.0. For the mutant the parameters are $k_{\text{in}} = 0.046 \text{ s}^{-1}$, $k_{\text{eff}} = 0.004 \text{ s}^{-1}$ at pH 6.0 and $k_{\text{in}} = 0.035 \text{ s}^{-1}$, $k_{\text{eff}} = 0.005 \text{ s}^{-1}$ at pH 8.0. The theoretical curves for influx (---) and efflux from parent (---) and mutant (—) at pH 6.0 are shown. (c) Counterflux reactions with melibiose are shown for the parent (▲) and the mutant (△) at pH 6.0. The cells were loaded with 5 mM-melibiose and diluted into medium containing 0.05 mM (final concentration) [³H]melibiose. The parameters describing the curve are $k_{\text{in}} = 0.03 \text{ s}^{-1}$, $k_{\text{eff}} = 0.008$ for the parent and $k_{\text{in}} = 0.017 \text{ s}^{-1}$, $k_{\text{eff}} = 0.002 \text{ s}^{-1}$ for the mutant. (d) Counterflux reactions with methylthiogalactoside at pH 6. The cells were labelled with 10 mM-galactoside and diluted into medium containing 0.1 mM-[¹⁴C]galactoside. The parameters describing the curve are $k_{\text{in}} = 0.046 \text{ s}^{-1}$, $k_{\text{eff}} = 0.015 \text{ s}^{-1}$ for the parent (▲) and $k_{\text{in}} = 0.041 \text{ s}^{-1}$, $k_{\text{eff}} = 0.004 \text{ s}^{-1}$ for the mutant (△).

pH-dependence and was slower than efflux from the parent (Fig. 4c). Efflux from the mutant resembled product inhibition of influx only in the lack of pH-dependence of the apparent affinity for internal galactoside; the variation of V_2 and V_e for efflux was not the same. West (1980) and Herzlinger *et al.* (1985) have previously noted that efflux from the mutant shows less dependence on pH than does efflux from the parent.

The pH-dependence of accumulation by the parent and mutant has also been examined (Fig. 4d). Accumulation can be described by a half-saturation constant ($K_{acc.}$) and a maximum accumulation ($A_{max.}$) (Rickenberg *et al.*, 1956). The half-saturation constant had the same value as K_m for influx and showed the same pH-dependence. The maximum accumulation by the parent behaved in the same way as V_1 whereas the maximum accumulation by the mutant shows a decrease below pH 8.5 that is consistent with the rise in V_2 .

Galactoside exchange reactions: is the translocation rate of ternary complex altered?

There was little perceptible difference between the rates of isotope exchange at equilibrium determined for mutant and parent at pH 9, although at pH 6 the rate of exchange for the mutant was slightly lower than it was for the parent (Fig. 5a).

Counterflux is the product of two processes: one, the influx of tracer in exchange for efflux of substrate, can be described by kinetic parameters similar to those describing net influx (Hankin *et al.*, 1972) and the other, net efflux of the internal pool, is described by the same parameters as the normal efflux reaction provided that the concentration of external tracer is sufficiently low. In the parent the rate of efflux was found to be low at pH 6 but to increase with external pH (Fig. 5b), which was similar to the pH-dependence of net efflux. The rate of influx showed little variation between pH 6 and pH 7.5, but above this pH decreased. Overall, the apparent efficiency of counterflux at a single, subsaturating concentration of tracer decreases with increasing pH because of the slowing down of influx and speeding up of efflux. In the mutant, the rate of efflux was also low at pH 6 but did not increase significantly with pH, just as was found with the rate of net efflux. The rate of influx was pH-dependent and decreased above pH 7.5, as expected from the pH-dependence of net influx. The lack of pH-dependence of the rate of efflux in the mutant accounts for the modest pH-dependence of the counterflux profile reported here and by Seto-Young *et al.* (1984). It should be noted that the rate of influx into the mutant and the parent were found to have very similar values, whereas the rate of efflux from the mutant was less than that from the parent (Figs. 5b–5d). This resulted in a higher, and slightly later, peak value for counterflux in the mutant and, for some galactosides, an apparently higher initial rate of influx (Figs. 5b and 5d). With other galactosides (thiolactose and melibiose in particular) the initial rate of counterflux was apparently greater in the parent (Fig. 5c). The relative initial rates depended on V_1 for influx and fractional saturation with tracer. Thus, the somewhat higher affinity of the mutant for external galactoside resulted in a higher rate of influx for the same external concentration when subsaturating concentrations were used, but when higher concentrations (relative to K_m) were used the lower V_1 of the mutant resulted in a lower rate of influx. The

discrimination between certain types of galactoside reported by Wright & Seckler (1985) is not supported by these results and it appears that the mutation affects transport of all galactosides in the same way.

The linked assay, which measures transport of 2-nitrophenyl- β -galactoside by following release of 2-nitrophenol after hydrolysis by internal β -galactosidase,

Fig. 6. Time course of 2-nitrophenyl- β -galactoside uptake and hydrolysis in the parent and mutant at pH 7.0

(a) The rate of 2-nitrophenol production in respiring suspensions of *E. coli* ML308 (triangles) and ML308-22 (circles). Open symbols represent the time-course with 0.1 mM-2-nitrophenylgalactoside and closed symbols represent the time-course with 5 mM-galactoside. (b) The rate of 2-nitrophenol production in suspensions of *E. coli* ML308 and ML308-22 poisoned with 30 mM- NaN_3 . Symbols are as in (a). The time points in all curves are calculated from continuous recordings of the absorption (measured using a dual-wavelength spectrophotometer using 420 nm and 450 nm as the wavelength pair) after deducting the absorption change in a suspension that was identical except that uptake had been inhibited with 5 mM-HCHO.

can contain an element of exchange (Page & West, 1982). The initial rate of 2-nitrophenol production by respiring suspensions of *E. coli* ML308 decays during the first 10 s to a lower rate that can continue for some minutes afterwards (Fig. 6a). When cells were poisoned with uncouplers the initial rate of 2-nitrophenol production was very low but it accelerated within the first 5–30 s to form a new steady-state rate which could continue for some minutes (Fig. 6b). The kinetic parameters of uptake during the initial slow phase were found to be different from those of the faster phase: in particular K_m was higher (10 mM instead of 0.9 mM) and V was lower. This behaviour was not observed with the mutant, for the initial rate of nitrophenol production by respiring cells showed little change in the first 1 min of the reaction and nitrophenol production by poisoned suspensions occurred at the same rate, without a lag phase (Fig. 6). The initial rates of uptake into the mutant and during the initial phase of uptake into respiring suspensions of the parent are essentially the same, although at longer times the mutant can appear to be 1.5–2.3 times as fast; in the azide-treated suspensions the difference can be even more marked. Thus it appears that exchange between external 2-nitrophenyl- β -galactoside and internally generated galactose can occur at much lower concentrations of galactose in the mutant than it can in the parent. This result, obtained under conditions where only galactose and galactoside concentrations are changing, strongly suggests that the increased product inhibition in the mutant is due to a higher affinity for internal galactosides rather than a higher affinity for internal protons.

The galactoside:proton stoichiometry: is there any uncoupling of fluxes?

The stoichiometry of galactoside–proton symport was measured in two independent ways. One was by comparing the rate of uptake of galactosides with that of the rate of uptake of protons. It has been shown that the initial rates of uptake of both species into the parent are the same (West & Mitchell, 1973; Booth *et al.*, 1979). This was also true for thiolactose (Fig. 7a) and the results indicated a stoichiometry of 0.95 ± 0.17 . The original observation was that proton uptake into the mutant was much lower than in the parent (West & Wilson, 1973). This was also true when proton-uptake was induced by thiolactose (Fig. 7b). However, the uptake of galactoside was also much lower in the mutant and when the initial rates were compared the stoichiometry was not very different from that of the parent (0.93 ± 0.15). The second method for determining stoichiometry was to compare the steady-state level of accumulation with the magnitude of the protonmotive force. Although the measurements are rather more complex (Booth *et al.*, 1979; Ahmed & Booth, 1981a,b) they do have the advantage that they measure the stoichiometry under the conditions of the kinetic analysis. The protonmotive force at pH 6.0 was apparently composed of 85 mV pH gradient and 95 mV membrane potential for the parent and of 94 mV pH gradient and 81 mV membrane potential for the mutant. The accumulation ratio with 0.01 mM external thiolactose was $329 (\equiv 149 \text{ mV})$ in the parent and $55 (\equiv 102 \text{ mV})$ in the mutant. These values give a stoichiometry ($\Delta p_{lac}/\Delta p$) of 0.83 for the parent and 0.58 for the mutant. With melibiose as substrate the stoichiometries were 0.79 and

Fig. 7. Time course of thiolactose–proton symport in the parent and the mutant

In (a) the uptake of thiolactose (solid symbols) is compared with the pH change (continuous trace) induced by the addition of the thiolactose to an oxygen-starved suspension of the parent (●) or the mutant (▲). In both experiments the bacteria were present at a density of 5 mg dry wt./ml in 3 ml of 2 mM-Mops/100 mM-KCl/50 mM-KSCN at pH 6.5. The suspension was stored in a sealed chamber that had been flushed with a gentle stream of N_2 . A 50 μ l addition of a degassed solution of 300 mM-thiolactose in 150 mM-KCl was added and samples were withdrawn at intervals and quenched by addition to a degassed N_2 -flushed solution of 10 mM-HgCl₂ in 150 mM-KCl. In (b) the semilogarithmic plot of tangents constructed to the pH traces is shown for parent (●) and mutant (▲). This is extrapolated to zero time to give the initial rate of proton uptake (0.62 and 0.43 nmol of H^+ /s per mg dry wt. for parent and mutant respectively). In (c) the progress curve for uptake of thiolactose into parent (●) and mutant (▲) is analysed by using the integrated rate equation. The initial rate of uptake into the parent is 0.65 nmol/s per mg dry wt. and into the mutant it is 0.48 nmol/s per mg dry wt.

0.61 for parent and mutant respectively. At pH 9.0 the parent had 140 mV membrane potential and the mutant had 146 mV membrane potential, with no detectable pH gradient. The accumulation ratio with 0.05 mM-thiolactose was $157 (\equiv 130 \text{ mV})$ in the parent and $123 (\equiv 123 \text{ mV})$ in the mutant. These values correspond to a stoichiometry of 0.93 for the parent and 0.89 for the mutant. With melibiose as substrate the stoichiometries were 0.87 and 0.85 for parent and mutant respectively.

DISCUSSION

The kinetics of uptake of galactosides by a strain having the y^{UN} mutation in the permease have been examined in order to test several hypotheses concerning the phenotype of the mutant. Very strongly curved progress curves were observed, particularly with uptake into the mutant. This rapid collapse of the initial rate of uptake makes it very difficult to determine the true initial rate without recourse to rapid reaction techniques (Page & West, 1984). However, the empirical methods of analysis developed here could be used to obtain estimates for the initial rate from data collected in the conventional time-domain. Using these methods it was observed that the initial rate of uptake into the mutant was not very different from that of uptake into the parent, the rate of uptake into the mutant being no less than half that of the parent. Further, the methods allow values for the affinity for the internal galactoside to be determined under influx conditions, which has not been possible before. The mutant is observed to have an affinity 15–50 times greater than that of the parent for internal galactoside at low pH. This higher affinity has two consequences: (i) the initial rate of influx very rapidly decreases through the effect of product inhibition by the accumulated galactoside giving rise to strongly curved progress curves and (ii) because of the slowing down of influx a lower final level of accumulation is reached (for further discussion of this point see Wright, 1986). Thus, when the stoichiometry of symport is measured during influx, the mutant and parent give approximately the same value but when the stoichiometry is measured at the final steady state the mutant gives a significantly lower value than the parent.

The apparently higher rate of counterflux and 2-nitrophenyl- β -galactoside transport that have been noted for the mutant (Wilson *et al.*, 1970) also appear to stem from the different affinity of the mutant for its internal substrates. The initial rate of influx during counterflux is, in fact, very similar in the parent and the mutant and it is the different rates of efflux that account for most of the difference between the time-course of counterflux in the mutant and in the parent. The difference in efflux rates seems to be modulated by changes in the apparent K_m for efflux, which parallel the changes in apparent affinity for internal galactoside estimated from product inhibition. Examination of the pH-dependence of the various flux reactions showed that there was no change in the pK of the controlling ionizations, although their effects did differ between mutant and parent. Thus, as a result of this analysis, it seems that there is no change in the proton-binding site, no change in the real stoichiometry, a slight change in the rate of turnover and that the only major change is in the apparent affinity for internal galactoside.

The apparent affinity for internal galactoside is controlled by a number of kinetic parameters. These may be summarized as follows: the rates of galactoside association and dissociation from the permease, the protonation state of the permease (Page & West, 1981) and the translocation rate-constants (Geck & Heinz, 1979). The value of $k_{cat.}/K_m$, which gives a lower estimate for the association between galactoside and permease (Fersht, 1985), is essentially the same in parent and mutant (for thiolactose the values are $2.9 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $3.8 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ for parent and mutant respectively) so it appears that the galactoside

association reactions forming the ternary complex in the forward direction are probably not affected by the mutation. In contrast the ratio of $k_{cat.}/K_m$ for the reverse direction is very different between parent and mutant. From the influx data the values of $k_{cat.}$ (for exit)/ K_m are $0.02 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $8.6 \text{ M}^{-1} \cdot \text{s}^{-1}$ for parent and mutant respectively. So, it is clear that it is this reaction (efflux) that the mutation affects. The maximum rate of influx and the rate of isotope exchange at equilibrium, for all galactosides tested so far, are lower by a factor of 1.5–2.0, suggesting that there has not been a significant change in any of the translocation rate constants. The lack of change in the proton dissociation constants suggests that the protonation states of the permease are not altered but the different response to the ionizations does suggest that it is a step associated with protonation that is affected by the mutation. The protonation states of the permease that can be identified by kinetic analysis are summarized in Fig. 8. In the parent strain, at pH 7 the permease appears to exist in the monoprotonated state with predominantly externally accessible sites. The apparent affinity for internal galactoside is thus very low. As ΔpH decreases when pH_e is raised, a greater proportion of the permease will adopt the internally accessible site and the apparent affinity for internal galactoside increases. This is simply due to redistribution of the permease among its intermediates in response to a change in substrate availability in the external medium and can be seen as a relaxation of the competitive inhibition produced by the internal product. At increasingly alkaline pH the permease deprotonates (pK_a 8.3) and the affinity for galactosides drops. Binding of galactoside to the monoprotonated form of the carrier appears to increase the pK_a of the permease by approx. 1 pH unit. At acid pH a second protonation appears (pK_a 6.0–6.3) which decreases the rate of translocation and increases the apparent affinity for internal galactoside but does not affect the affinity for external galactoside. Such an effect of acid pH has been noted before in studies of transport (Robertson *et al.*, 1980; Bentabollet & Kepes, 1981) and of binding (Kennedy *et al.*, 1974).

In the mutant strain ML308-22 the basic scheme of reactions is the same with the exception that the monoprotonated form of the permease (centre of Fig. 8b) appears to be unable to adopt the alternating conformational states that have high affinity on one side and low affinity on the other side; the affinity remains high for galactoside on both sides of the membrane. This increased affinity for internal galactoside could be due to either a change in competitive inhibition by external protons or a defective conformation. The very flat pH-dependence of efflux in the mutant suggests that the external proton is no longer such a good competitor and that the pK_a for the external proton should be lower in the mutant than it is in the parent. But the apparent pK_a for the external proton is the same in the mutant as it is in the parent. Thus it appears that either protonation of the external site fails to trigger the conformational change that causes the redistribution of galactoside binding sites or the conformation of the protein around the binding site is defective in such a way as to allow access of internal galactoside to the externally directed binding site.

Some of this work was performed in the Department of Biochemistry, University of Newcastle upon Tyne, Newcastle

Fig. 8. The protonation states of the lactose permease of *E. coli* strains ML308 and ML308-22

(a) Normal reaction mechanism in ML308. In the absence of galactoside the permease predominantly exists in a monoprotinated state between pH 6.5 and 8.5. This form may bind galactoside at either face of the membrane, the accessibility of the binding site being determined whether the permease has bound a proton at the periplasmic or the cytoplasmic face of the membrane. Thus in the absence of a pH gradient the permease should be accessible at either face whereas, in the presence of a pH gradient (e.g. at pH 7) the binding site will be more often accessible at the periplasmic face of the membrane. Below pH 6.5 the permease is predominantly in a diprotinated state and the effect of competitive inhibition by external pH is less; this appears as an increase in the apparent affinity for internal galactoside (shown as a slight opening towards the cytoplasmic face). Above pH 8.5 the permease is predominantly deprotonated and cannot bind galactoside with high affinity. Binding of galactosides produces an occluded form of the monoprotinated state (square in centre). This deprotonates at a higher pH (pK_a 9.6–10) but enters the deprotonated state at the same pH. Neither the deprotonated nor the diprotinated state appear to be able to translocate galactosides, but the extreme values of pH make it impractical to demonstrate this effectively. The units of the affinity constants are M and for k_{cat} , the units are s^{-1} . The values given are for influx; those in parentheses are for efflux. (b) Altered reaction mechanism in ML308-22. The mutant appears to undergo the same basic set of reactions except that the monoprotinated state does not appear to distribute according to the pH gradient and always has a high affinity for internal galactoside.

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APPENDIX

Analysis of transport progress curves

In the analysis given below it is assumed that the concentration of the external galactoside remains constant. This is justifiable for most experiments for less than 10% of the external galactoside is taken up before the reaction stops. Clearly care should be exercised at low sugar concentrations and at high cell (or vesicle) densities.

Rate equations

- (1) Assuming competitive product inhibition:

$$\frac{d[P]}{dt} = \frac{V_1[S]}{K_m(1 + [P]/K_p) + [S]} \quad (1)$$

- (2) Assuming mixed product inhibition:

$$\frac{d[P]}{dt} = \frac{V_1[S]}{K_m(1 + [P]/K_p) + [S](1 + [P]/K_p')} \quad (2)$$

- (3) The complete reaction:

$$\frac{d[P]}{dt} = \frac{V_1[S] - V_2[P]}{K_m(1 + [P]/K_p) + [S](1 + [P]/K_p')} + D([S] - [P]) \quad (3)$$

This equation is not amenable to simple analysis but it is the negative terms containing V_2 and D that cause the departure from linearity observed in the last 10–25% of the reaction (see Fig. 1). Eqn. (3) can be used to calculate V_2 and D at $d[P]/dt = 0$ when estimates of the other parameters are available.

Analysis using the rate equations

- (1) Competitive inhibition. From eqn. (1):

$$\left(\frac{d[P]}{dt}\right)^{-1} = \frac{K_m(1 + [P]/K_p)}{V_1} \cdot \frac{1}{[S]} + \frac{1}{V_1} \quad (4)$$

Plot $(d[P]/dt)^{-1}$ against $1/[S]$. The linear part of the curve has a slope of $K_m(1 + [P]/K_p)/V_1$ and an intercept of $1/V_1$. A replot of the slope against internal concentration, $[P]$, gives K_m/V_1 and K_p .

- (2) Mixed inhibition. From eqn. (2):

$$\left(\frac{d[P]}{dt}\right)^{-1} = \frac{K_m(1 + [P]/K_p)}{V_1} \cdot \frac{1}{[S]} + \frac{1}{V_1} \cdot (1 + [P]/K_p') \quad (5)$$

Plot $(d[P]/dt)^{-1}$ against $[P]$. The intercept gives the true initial velocity when $[P] = 0$ (intercept = $K_m + [S]/V_1[S]$). The slope has a value:

$$\frac{K_m}{V_1 K_p [S]} + \frac{1}{V_1 K_p'}$$

and therefore a replot of the slope against $1/[S]$ will give the values of K_p and K_p' .

Analysis using the integrated rate equations

(1) Competitive inhibition. From eqn. (1):

$$[P] = \frac{2V_1 K_p [S]}{K_m} \cdot \frac{t}{[P]} - \frac{2K_p (K_m + [S])}{K_m} \quad (6)$$

A plot of $[P]$ against $t/[P]$ has a slope = $2V_1 K_p [S]/K_m$, an intercept on the $[P]$ axis of $-2K_p (1 + [S]/K_m)$ and an intercept on the $t/[P]$ axis of $(K_m + [S])$. A replot of the latter intercept against $1/[S]$ gives K_m and V_1 , and a replot of the slope or the intercept on the $[P]$ axis can be used to estimate the value of K_p .

(2) Mixed inhibition. From eqn. (2):

$$[P] = \frac{2V_1 K_p K_p' [S]}{K_m K_p' + K_p [S]} \cdot \frac{t}{[P]} - \frac{2K_p K_p' (K_m + [S])}{K_m K_p' + K_p [S]} \quad (6)$$

A plot of $[P]$ against $t/[P]$ has a slope = $2V_1 K_p K_p' / (K_m K_p' + K_p [S])$. A replot of $1/\text{slope}$ against $1/[S]$ has a slope of $K_m/2V_1 K_p$ and an intercept of $1/V_1 K_p'$. The intercept of the $[P]$ versus $t/[P]$ plot on the $t/[P]$ axis is $(K_m + [S])/V_1 [S]$. A replot of this against $1/[S]$ gives K_m and V_1 , and hence the values of K_p and K_p' can be calculated from the slopes and intercepts of the replots.

When the product inhibition is symmetrical eqn. (6) simplifies to:

$$[P] = \frac{2V_1 K_p [S]}{K_m + [S]} \cdot \frac{t}{[P]} - 2K_p \quad (7)$$

A plot of $[P]$ against $t/[P]$ thus has a slope of $2V_1 K_p [S]/(K_m + [S])$ and an intercept on the $[P]$ axis of $-2K_p$.

Examples of the use of the integrated rate equation and the rate equation to analyse uptake progress curves are given in Fig. 1 in the text. For the analysis using the rate equation $d[P]/dt$ can be estimated from the progress curve using the method described by S.-L. Yun & L. H. Suelter [(1977) *Biochim. Biophys. Acta* **480**, 1–13], or by constructing tangents to the curve.