Alternative-substrate inhibition and the kinetic mechanism of the β -galactoside/proton symport of *Escherichia coli*

Malcolm G. P. PAGE and Ian C. WEST

Department of Biochemistry, University of Newcastle upon Tyne, Newcastle upon Tyne NE1 7RU, U.K.

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The effects of competing alternative substrates on the rate of uptake by galactoside/proton symport were investigated. These experiments produced a decrease in apparent maximum velocity with increased alternative-substrate concentration that cannot be accounted for by a simple ordered mechanism. This, together with non-linearities in the variation of the apparent kinetic constants with alternative-substrate concentration, can be accounted for by a random mechanism for galactoside and proton binding.

The uptake of exogenous galactosides by *Escherichia coli* occurs with the apparent concomitant uptake of a proton (West, 1970; West & Mitchell, 1972). The apparent reaction can be treated successfully as a two-substrate reaction in which galactoside and symported proton are substrates of a carrier with a single active site (Page & West, 1981). Previous studies have left open the question of whether the substrate binding occurs by an ordered or a random mechanism (Kaczorowski & Kaback, 1979; Page & West, 1981; Wright *et al.*, 1981). The order of substrate binding is important to our understanding of many aspects of the uptake process, and a solution to this problem is highly desirable.

In enzyme systems several approaches have been used to distinguish between ordered and random mechanisms (e.g. Fromm, 1975; Cornish-Bowden, 1976). These are as follows.

(1) Equilibrium exchange, where the flux between the substrate-product pairs is measured. Although this is a very convenient technique for soluble enzymes, its application to galactoside/proton symport is restricted because only galactoside exchange can readily be measured (Kaczorowski & Kaback, 1979).

(2) Product inhibition requires both products to be varied over a range of accurately known concentrations, which would be very difficult because of the inaccessibility and complexity of the internal phase of the cell (Page & West, 1981).

(3) Dead-end inhibition requires an analogue of at least one substrate that cannot react (in this context reaction would be translocation). Such a compound is not yet known for galactoside/proton symport.

(4) Alternative-substrate inhibition is essentially

the same technique as dead-end inhibition, but uses an analogue that can react. There are many such analogues for galactoside uptake.

The interpretation of the results of any of these techniques relies on certain assumptions; in particular, it must be assumed that the steady state is maintained during the measurement of the initial rates and that depletion of substrates or accumulation of products is not affecting the observed rate. Independently of the kinetic mechanism, product inhibition could account for changes in the K^A and V^A of uptake (e.g. Page & West, 1981). For the proton and galactosides that are accumulated, this will appear as a time-dependent slowing down of uptake in each individual run (the proton will be expected to cause inhibition by depolarization of the membrane as well as through accumulation in the internal milieu). The 2-nitrophenyl β -galactoside used to monitor uptake is not accumulated, since it is rapidly hydrolysed (Koch, 1964), but the hydrolysis product (galactose; K^A for permease 10 mM or more; Sandermann, 1977; Wright *et al.*, 1981) might accumulate during the reaction. Thus we would expect to see increased product inhibition as galactosides are accumulated or reaction products built up. Further, when the internal concentration of galactoside becomes large relative to its K^A (internal) we might expect exchange of galactosides instead of simple influx. The kinetics of exchange are different from those of simple influx and so the apparent rate of influx may change. Again, this will be a time-dependent phenomenon. By monitoring influx during only the first few per cent of the reaction, when the rate of uptake is linear, we can avoid most of the effects discussed above (Alberty *et al.*, 1954; Koch, 1974).

We have studied the inhibition of galactoside

uptake caused by alternative substrates in order to gain some information about the kinetic mechanism of uptake. We have observed a non-competitive element in the inhibition caused by several galactosides, which is not consistent with single-site models such as the simple ordered mechanism.

Materials and methods

Escherichia coli ML308 was grown in minimal medium M9 (Anderson, 1946) containing 0.5% glycerol to late-exponential phase in baffled conical flasks. The cells were harvested by centrifugation, washed once with 0.1 M-potassium phosphate buffer (61 mM- K_2HPO_4 /39 mM- KH_2PO_4 , pH 7.0) and resuspended in the same medium. The steady-state rate of 2-nitrophenyl galactoside uptake and hydrolysis was monitored by using a Perkin-Elmer model 156 double-wavelength spectrophotometer. The wavelength pair 420/450 nm was chosen and the analog output collected on chart paper. Experiments were performed by using a 1 ml reaction mixture consisting of 0.1 M-potassium phosphate buffer in which were dissolved the desired concentrations of 2-nitrophenyl β -galactoside and the alternative substrate. The reaction was initiated by the addition of 10 μ l of the stock bacterial suspension (to give a final cell density of 0.5–1 mg dry wt. of bacteria/ml). The reaction was monitored for between 25 and 50 s after the initiation and then 10 μ l of formaldehyde solution (37–40%, w/v) was added and the reaction monitored for another 25–50 s. Formaldehyde was chosen in preference to other commonly used inhibitors of the permease (Hg^{2+} ; Lancaster & Hinkle, 1977; *N*-ethylmaleimide, Fox & Kennedy, 1965; and thiodigalactoside, Kennedy, 1970) because of convenience and because its mode of action was less likely to rely on competition for the active site than the other reagents. The concentration used (about 0.1 M) was chosen for the speed of its effect. Concentrations between 0.03 and 0.25 M did not produce appreciably different rates of residual 2-nitrophenol release in this buffer, although the lower concentrations were less immediate in their effect.

The rate of 2-nitrophenol release was calculated from the initial linear portion of each trace (Fig. 1) after correcting for the continuing rate of 2-nitrophenol formation in the presence of formaldehyde. These initial rates, after correction for cell density, were used to calculate values of the apparent maximum velocity (V^A) and apparent Michaelis constant (K^A) with the equation:

$$\frac{[B]}{v_{\text{obs.}}} = \frac{K^A}{V^A} + \frac{[B]}{V^A} + e \quad (1)$$

where [B] is the concentration of 2-nitrophenyl β -galactoside, $v_{\text{obs.}}$ is the rate of uptake and e is the

Fig. 1. Effect of 4-nitrophenyl α -galactoside on the rate of uptake and hydrolysis of 2-nitrophenyl β -galactoside

Assays were performed as described in the text with (a) 0.2 mM-2-nitrophenyl β -galactoside and (b) 10 mM-2-nitrophenyl β -galactoside. Values against curves are concentrations of 4-nitrophenyl α -galactoside. Tracings of the initial portion of the curve are shown together with the continuing rate of 2-nitrophenol production after the addition of formaldehyde (HCHO).

error in $[B]/v_{\text{obs.}}$. The estimates of K^A and V^A were made by weighted least-squares analysis using five cycles of reiteration with progressively refined weights (Wilkinson, 1961).

Absorbance units were converted into concentrations by using a molar absorption coefficient of $1.6 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at pH 7.0 for 2-nitrophenol.

For experiments in which uncoupler was used, 10 μ M-carbonyl cyanide *m*-chlorophenylhydrazone was included in the reaction mixture and a sample of the stock suspension of bacteria was treated with the same concentration of uncoupler for 2 min before addition to the reaction mixture.

Results

The 'high-affinity' inhibitor 4-nitrophenyl α -galactoside brought about a 50% decrease in the rate of uptake with 1.4 mM 2-nitrophenyl β -galactoside when present over the range 0.01–0.05 mM. The inhibition was not purely competitive, for there were changes in the values of both V^A and K^A (Fig. 2). Replots of $1/V^A$ and K^A/V^A against 4-nitrophenyl α -galactoside concentration were non-linear (Figs. 3 and 8). A large scatter in the collected data originating from (a) a large variability between experiments (errors in determining cell density, cell suspensions in slightly different phases of growth etc.) and (b) the error in determining the small

Fig. 2. Lineweaver-Burk plot of the rate of 2-nitrophenyl β -galactoside uptake and hydrolysis in the presence of 4-nitrophenyl α -galactoside

Initial-rate data obtained from experiments (such as shown in Fig. 1) with three different cell suspensions are plotted against $1/[2\text{-nitrophenyl } \beta\text{-galactoside}]$. Values against curves are concentrations of 4-nitrophenyl α -galactoside in mM.

Fig. 3. Effect of 4-nitrophenyl α -galactoside on the kinetic constants for 2-nitrophenyl β -galactoside uptake

The effect of the alternative substrate on the value of (a) K^A/V^A and of (b) $1/V^A$ is shown. ● represents the values estimated by least-squares regression using the collected data. ○ represents the mean

value of estimates obtained by manual plotting of the data obtained from experiments with eight individual cell suspensions. The range of values from individual experiments is shown by the vertical bars. ▲ represents mean values from a similar set of experiments but in which the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone was present.

velocities at these high inhibitor concentrations, makes it difficult to place a precise interpretation on these non-linearities.

A second 'high-affinity' inhibitor, thiodigalactoside, caused a 50% decrease in the rate of uptake at 1.4 mM-2-nitrophenyl β -galactoside when present over the range 0.01–0.25 mM. Again the inhibition resulted in changes in the values of V^A and K^A (Fig. 4). The non-linearities in the replots were not so pronounced (Figs. 5 and 8) as they were with 4-nitrophenyl α -galactoside.

The 'low-affinity' inhibitor isopropyl β -thiogalactoside was much less efficient as an inhibitor, and a 50% decrease in rate at 1.4 mM-2-nitrophenyl β -galactoside was not achieved until 1–5 mM-isopropyl β -thiogalactoside was present. Again both V^A

Fig. 4. Lineweaver-Burk plot of the rate of 2-nitrophenyl β -galactoside uptake and hydrolysis in the presence of thiodigalactoside

Initial-rate measurements were made in assays described in the text and Fig. 1, with thiodigalactoside as the alternative substrate. Data from experiments with a single cell suspension are shown. Values against curves represent the concentration of thiodigalactoside in mM.

Fig. 5. Effect of thiodigalactoside on the kinetic constants for 2-nitrophenyl β -galactoside uptake

Data from experiments with five cell suspensions were treated as shown in Fig. 4 or as described in the text. The effect of thiodigalactoside on (a) K^A/V^A and (b) $1/V^A$ is shown. ● represents the values obtained by least-squares regression using the collected data. ○ represents the mean value of the estimates obtained by manual plotting of the data (Fig. 4). The range of values obtained in individual experiments is shown by the vertical bars.

and K^A were affected (Fig. 6), but there was little evidence of non-linearity in the replots (Figs. 7 and 8).

Linear competitive inhibition is described by:

$$V_{(i)}^A = V^A \tag{2}$$

$$K_{(i)}^A = K^A(1 + [I]/K_I) \tag{3}$$

Linear mixed inhibition and hyperbolic inhibition are two well-known cases where the inhibitor also affects the V^A . With linear mixed inhibition the replots of $1/V^A$ and K^A/V^A are expected to be linear with positive slopes, whereas with hyperbolic inhibition non-linearities in both plots might be expected (Fromm, 1975; Cornish-Bowden, 1976). The inhibition that we have observed conforms with none of these types. They can be described by the following equations, which are closest to those for hyperbolic inhibition but include a term in $[I]^2$ in eqn. (5):

$$V_{(i)}^A = V^A \frac{(1 + [I]/K_I)}{(1 + [I]/K_{II})} \tag{4}$$

$$K_{(i)}^A = K^A \left(\frac{1 + [I]/K_{III} + [I]^2/K_{IV}}{1 + [I]/K_{II}} \right) \tag{5}$$

The values of V^A , K^A , K_I , K_{II} , K_{III} and K_{IV} are given in Table 1, alongside values taken from the

literature, where the inhibition has been assumed to be competitive.

Discussion

The inhibition caused by alternative substrates for galactoside uptake might be ascribed either to the consequences of the kinetic mechanism or to the effects of product accumulation. The immediacy of the effects prompts the former interpretation rather than the latter one.

The type of inhibition we describe commonly arises in mechanisms where there are two types of binding site (Fromm, 1975; Cornish-Bowden, 1976). Genetic mapping has shown only a single binding site per molecule of permease (Hobson *et al.*, 1977;

Fig. 6. Lineweaver-Burk plot of the rate of 2-nitrophenyl β-galactoside uptake and hydrolysis in the presence of isopropyl β-thiogalactoside. Details are as given in Fig. 4. Values against curves represent the concentration of isopropyl β-thiogalactoside in mM.

Fig. 7. Effect of isopropyl β-thiogalactoside on the kinetic constants for 2-nitrophenyl β-galactoside uptake. Details are as in Fig. 5, except that only three cell suspensions were used.

Fig. 8. Comparison of the effects of the alternative substrates on the kinetic constants for 2-nitrophenyl β -galactoside uptake

The mean value of (a) K^A/V^A and (b) $1/V^A$ obtained in all experiments is plotted against the fraction:

$[\text{Alternative substrate}]/([\text{alternative substrate}] \text{ producing doubling of } K^A/V^A) ([I]/[I]_{50})$

●, ■ and ▲ represent values obtained with 4-nitrophenyl α -galactoside, thiodigalactoside and isopropyl β -thiogalactoside respectively.

Teather *et al.*, 1980). Even so, two types of binding site may arise in the following ways:

(i) More than one galactoside molecule could fit into the active site (cf. Wratten & Cleland, 1965). The active site of the permease is apparently quite large and will accept molecules such as raffinose and dansylaminolactose without serious loss of affinity (Wright *et al.*, 1981).

(ii) The permease could function as a dimer (Lancaster, 1978; Robertson *et al.*, 1980; Lombardi, 1980; Mieschendahl *et al.*, 1981). Co-operativity between monomers can lead to sites of different affinities.

(iii) In the random mechanism, a protonated and an unprotonated site will be present during the steady-state turnover. The steady-state solution of the random mechanism predicts inhibition curves just such as are described by eqns. (4) and (5).

(iv) Robertson *et al.* (1980) have proposed that there are two forms of the permease: one that catalyses active transport with high affinity and one that facilitates diffusion with low affinity for galactoside. Such models give similar results to the random mechanism and, indeed, if the active-transport form is identified with the protonated permease and the facilitated-diffusion form is identified with the unprotonated permease in a random mechanism in which the binary complexes are allowed to translocate, the models are identical. According to the model of Robertson *et al.* (1980), in which the protonmotive force causes the transition between the low-affinity form and the high-affinity form, we would expect the shape of the inhibition curves in the presence and absence of uncoupler to be very different, but they are not (Fig. 3). Ghazi & Shechter (1981) have proposed a modification of the model of Robertson *et al.* (1980) in which the distribution of the carrier between the two forms is not affected by the protonmotive force. This would be in accord with the above experimental result, as is the random mechanism.

Page (1980) reported an apparent inhibition of the lactose-induced proton flux by high lactose concentrations and pointed out that possibilities such as (i), (iii) and (iv) above could account for such results. Robertson *et al.* (1980) and Ghazi & Shechter (1981) have reported dependence of the value of V^A and K^A on the value of the membrane potential and pH gradient, which can be explained by models such as (iii) and (iv), but not by (i). These experimental results cannot be accounted for by the dimer model [possibility (ii) above] unless the kinetic mechanism is one such as is described by possibility (iii) or (iv). The best explanations of the data are thus the random mechanism or a mechanism that is kinetically identical with it.

Kaczorowski & Kaback (1979) interpret their results as indicating an ordered mechanism. Their

Table 1. *Apparent kinetic constants for 2-nitrophenyl β -galactoside uptake in the presence of alternative substrates*
 The values of K^A and V^A for 2-nitrophenyl β -galactoside and the derived constants were obtained by computation using the data presented in Figs. 3, 5 and 7. Values in parentheses were obtained by using the data presented in Fig. 8 (i.e. with only low inhibitor concentrations). (*) indicates that the value of the appropriate constant was too large to be detected by this plot. Units for K and V^A are mM and $\text{nmol} \cdot \text{s}^{-1} \cdot (\text{mg dry wt. of bacteria})^{-1}$ respectively. References: ^aWright *et al.* (1981); ^bOverath *et al.* (1979); ^cSandermann (1977); ^dCarter *et al.* (1968).

Kinetic constant	Substrate ...	4-Nitrophenyl α -galactoside	Thiodigalactoside	Isopropyl β -thiogalactoside
Obtained for 2-nitrophenyl β -galactoside	K^A	1.12	1.41	0.98
	V^A	10	12	10
Derived inhibition constants	K_I	0.60 (*)	2.0 (*)	>500 (*)
	K_{II}	0.07 (0.06)	0.17 (0.03)	28 (50)
	K_{III}	0.07 (0.02)	0.05 (0.05)	2.9 (2.9)
	K_{IV}	0.02 (0.01)	0.08 (0.1)	57 (*)
Kinetic constants reported for whole-cell systems	K^A	0.024 ^a		
		0.05–0.2 ^b	0.44 ^a	
	K_I	<0.1 ^c	0.08 ^d , <0.1 ^c	4 ^c

data, however, can equally well be explained in terms of the random mechanism, as we have previously indicated (Page & West, 1981). Wright *et al.* (1981) have also postulated that the permease functions by a random mechanism, although their particular kinds of experiment do not discriminate between random and ordered mechanisms.

Eddy (1968, 1980, 1981) has distinguished between permease-independent leak pathways and permease-dependent slip reactions in which the accumulated substrate moves out on the permease but without a co-ion. The random mechanism, in which the permease–galactoside complex is allowed to translocate, provides the possibility for galactoside slip. Such a slip reaction can explain the apparent inefficiency of coupling between proton-motive force and galactoside accumulation observed at high galactoside concentrations (Rickenberg *et al.*, 1956; Kepes, 1960, 1971; Booth, 1980; Booth & Hamilton, 1980; Hamilton & Booth, 1982). It must be borne in mind that, for some galactosides, carrier-independent leaks may be significant (Maloney & Wilson, 1973; Booth & Hamilton, 1980), but it is nevertheless noteworthy that those galactosides for which according to our analysis the unprotonated pathway is prominent (i.e. show large effect on V^A ; Fig. 8) are less well accumulated.

The different apparent affinities shown by the galactosides mentioned above for the unprotonated species of the permease parallel their different abilities to protect the essential thiol group of the permease against *N*-ethylmaleimide (Carter *et al.*, 1968), which of course reacts most readily with unprotonated thiol groups. West (1980) has postulated that the essential thiol group is the protonatable group on which symport depends (Page & West, 1981; Bentaboulet & Kepes, 1981). The current findings lend support to this conclusion.

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