Dependence on pH of substrate binding to lactose carrier in *Escherichia coli* cytoplasmic membranes

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Lactose permease in *Escherichia* mediates proton-substrate cotransport. The molecular mechanism of this process is not understood. We examined the effect of proton concentration on the binding of a substance analogue to the carrier. The dissociation constant of *p*-nitrophenyl-α-galactoside from the carrier was dependent on pH, with an apparent pK_a of 9.7.

Cotransport *Galactoside* *Ligand binding* *Permease* *Protonmotive force* *Uncoupling*

1. INTRODUCTION

Uptake of lactose into *Escherichia coli* cells is one of the most extensively studied transport processes since the discovery, in 1956, that its uptake against a concentration gradient is mediated by the product of the *lacY*-gene [1]. A decade later, the protein was identified and partially purified in denatured form [2]. Successful reconstitution of partial transport activity from solubilised membrane protein and lipids has been reported in [3]. This, as well as other studies, became possible only with the availability of over-producing strains [4]. The source of energy of active transport has been identified 10 years ago to consist of proton–lactose cotransport [5,6]. Yet, the expected pH-dependence of substrate binding, as it has been demonstrated in proline uptake in *E. coli* [7,8], could not be detected with substrates of the lactose permease system in vesicles obtained from enriched plasma membranes [9]. Binding of ligand and of protons were therefore judged to be independent events. In kinetic studies of substrate uptake into whole cells, a pH-dependence of the K_m has been described in [10,11]. We have now extended the binding study of 4-nitrophenyl-α-galactoside (αNPG) to the carrier into an alkaline pH range. Applying the formalism described earlier [7], we observed that:

(i) Ligand binding is pH-dependent, with a pK_a of 9.7;

(ii) The apparent inactivation, observed at moderately high pH, is reversible.

Although identification of the proton with that proton apparently cotransported is not possible as yet, the result obtained may be significant to attain a better understanding of the molecular mechanism of active lactose transport.

2. MATERIALS AND METHODS

E. coli strain T185, constructed as in [4] for the amplification of the *lacY* gene, was grown at 37°C in a 60-l fermentor in M9 minimal salt medium [12], supplemented with 0.4% casamino acids, 0.4% succinate and 0.4% lactate. When the A_{550} reached 0.15 units, 1 g of isopropylthiogalactoside was added to induce expression of lactose permease. Cells were grown for about 3 generations after addition of inducer, and then collected by centrifugation. Spheroplasts were prepared by EDTA and lysozyme treatment as in [13]. Cytoplasmic membranes were obtained by density gradient centrifugation of membrane vesicles into

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a 43% (w/w) sucrose cushion after disrupting spheroplasts by sonication on ice (10 min total, using a Branson sonic oscillator (100 W) with intermittent cooling). Cytoplasmic membranes were collected by ultracentrifugation and suspended in distilled water at ~50 mg protein/ml.

Cytoplasmic membrane vesicles were dialysed overnight at 4°C against 40 mM buffers with one change. Usually, triethylamine glycine was used and adjusted to the pH values indicated. Experiments with Tris–HCl (pH 8.0) and Na-carbonate (pH 10.3) did not reveal significantly different results. All buffers contained 0.5 mM dithiothreitol. Binding of αNPG to the permease was measured in a microequilibrium dialysis apparatus, holding 100-μl solutions in both chambers [8]. Aliquots of 50–100 μl of the membrane suspension (2–4 mg protein) were used in one of the chambers, with [^{3}H]αNPG, labelled as in [14], in the other (final spec. act. 15 Ci/mol). Six different concentrations between 8–250 μM were used. After 2–3 h of incubation at room temperature, aliquots from both chambers were assayed for radioactivity. Binding activity was measured in the presence or absence of 50 mM NaN_3. Similar binding activity was observed, as it was in the presence of 50 μM carbonylcyanide *m*-chlorophenylhydrazone (CCCP). Protein was measured as in [15], with bovine serum albumin as standard.

3. RESULTS AND DISCUSSION

Fig.1 demonstrates the pH dependence of the binding of 4-nitrophenyl-α-galactoside to isolated plasma membrane vesicles. The representation is based on the expression

$$K_d = K_S (1 + K_H/[H^+]),$$

where: K_d = the apparent dissociation constant of the examined ligand from the complex, determined from double reciprocal plots of 6 substrate concentrations at any pH;

K_S and K_H = the dissociation constants of each of the two components, substrate (K_S) from the ternary complex, and protons (K_H)

From the results shown, K_S is calculated to be 16 μM, and K_H corresponds to 0.15 nM, or an apparent pK_a of 9.7.

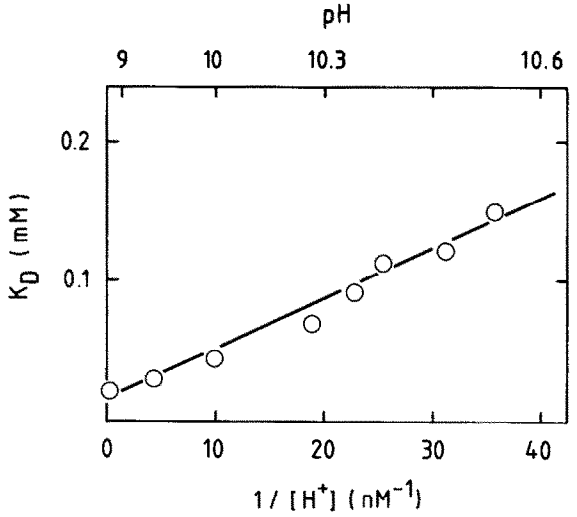

Fig.1. Dependence of the dissociation constants (K_D) of αNPG to lactose carrier on proton concentration (indicated as reciprocal values; for the rationale, see text). The binding of [^{3}H]αNPG to cytoplasmic membranes from *E. coli* strain T185 was measured using 6 different concentrations of αNPG at each pH value, as in section 2. Initial concentrations of αNPG were between 8–250 μM. Apparent dissociation constants (K_d) for αNPG were obtained at each pH value by double reciprocal plotting of carrier-bound αNPG *vs* concentrations of free αNPG.

The following ancillary tests have been performed:

(i) Decomposition of ligand is unlikely to affect the results at high pH. Exposure of αNPG to pH 10.5 for 5 h resulted in 3%, and to pH 11.0 for 2 h in 5% decomposition. These values were determined spectrophotometrically to detect 4-nitrophenol, and by paper chromatography to quantify released galactose [14].

(ii) Calculations of the number of binding sites indicate little variation over the pH range tested (0.9–1.1 nmol/mg protein over 8.0–10.5), and agree with previous studies [9].

(iii) Partial irreversible inactivation of the binding at alkaline pH occurred only above pH 10.7. After exposure to pH < 10.7, original binding activity was recovered upon readjustment to pH 8 by dialysis. Exposure to pH 11.1 (Na_2CO_3 buffer), or pH 11.5 (NaP_i buffer) caused apparently irreversible inactivation of 10 and 20% upon readjustment to pH 8.

(iv) Differences in binding activities in the presence of uncoupling agents (NaN_3, CCCP; see section 2) were not detected.

We therefore infer that binding rather than uptake was determined. This is consistent also with the independence of the number of binding sites measured on pH.

We conclude that binding of αNPG to the lactose carrier protein indeed depends on the proton concentration of the aqueous bulk phase. This result is not surprising since in the pH-range tested in [9], protonation of the permease was nearly complete. The result further demonstrates that the increased K_m [10,11] of transport in the alkaline pH range may reflect decreased affinity of ligand, rather than merely a kinetic phenomenon. Although the observed protonation may be that of the carrier, alternative interpretations are possible. Protonation of other membrane components, such as phosphatidyl-ethanolamine ($pK_a \simeq 10$), or contaminants, e.g., components of the periplasm or from outer membranes, can not be excluded. To distinguish among the various alternatives, and to identify the residue whose protonation is coupled to active transport, two approaches may be taken.

(1) The protein of the uncoupled phenotype [16] may be investigated by studying its sequence.

(2) Reconstitution of transport from homogeneous components [3] could provide the key.

We are attempting to obtain highly active protein in a homogeneous state.

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