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Characterisation in vivo of the reactive thiol groups of the lactose permease from *Escherichia coli* and a mutant; exposure, reactivity and the effects of substrate binding

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The reactivity and accessibility of the reactive thiol groups of the native lactose permease and a mutant have been studied in a number of circumstances and with a number of reagents, in particular using the specific thiol-disulphide exchange reaction. Seven different reactive states of the thiol in the native protein have been characterised by their different second-order rate constants. Interconversion between these states is dependent on the magnitude of the protonmotive force, pH and substrate binding. In the absence of galactoside, reactivity is controlled by an ionisation with apparent pK_a 9.3. This pK_a is not affected by the protonmotive force, but it is lowered in the presence of external galactoside. The conformation adopted by the permease when in equilibrium with saturating galactoside appears to be different from that of the intermediate that accumulates during net turnover. In the former state, the reactivity of the thiol group is depressed, whereas in the latter state it is enhanced. The thiol group of the native protein is buried in a hydrophobic environment that has a dielectric constant considerably lower than that of water. The environment is not greatly perturbed by changes in the magnitude of the protonmotive force, but it is affected by the binding of galactoside. In a strain which carries the Y^{UN} mutation (Wilson, T.H. and Kusch, M. (1972) Biochim. Biophys. Acta 255, 786–797), two reactive thiols were characterised. The more reactive of the two is more exposed than the thiol group of the native molecule and is in an environment that has a dielectric constant close to that of water. The less reactive thiol appears to be more deeply buried than that of the native protein. Thus the mutation appears to produce a conformation change in the central portion of the polypeptide chain that results in greater exposure of the reactive thiol to the aqueous environment.

Introduction

The uptake of exogenous galactosides into *Escherichia coli* is catalysed by the lactose permease [1]. The driving force for accumulation is the protonmotive force [2,3] which is exploited by proton-galactoside symport [4]. It is clear that the magnitude of the protonmotive force influences the kinetics of proton-galactoside symport [2,5–7], but it is not yet clear how it does so. Three types of effect have been discussed [8]: an affinity effect, a velocity effect and a proton well. With the affinity effect, a change in the affinity for galactoside is postulated to be brought about by a

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change in the rate constants for substrate association or dissociation as a result, for example, of a change in the conformation of the protein. Examples of models of this type are the protonmotive-force-dependent oligomerisation of the lactose permease suggested by Kaback [9], the conformation change suggested for the mitochondrial phosphate carrier by Fonyo [10] and the dithiol-disulphide interchange suggested by Konings and Robillard for a number of transport systems, including the lactose permease [11,12]. The velocity-effect type of model ascribes the change in apparent affinity to protonmotive force dependent changes in the translocation rates of the fully 'loaded' or fully 'unloaded' permease. Theoretical analysis of transport kinetics shows that the apparent substrate affinity is a function of the rate constants for substrate association and dissociation and of those for translocation [13]. The effect of the protonmotive force in the velocity-effect model is to alter the distribution of the permease between the intermediates of the transport cycle. Clearly, if the different intermediates constitute different conformations of the proteins, an effect of the protonmotive force on the chemical reactivity of particular amino-acid side-chains may be apparent. The proton-well model describes a change in the local activity of the symported proton at its binding sites on either side of the membrane brought about by an electrochemical potential difference between the binding sites and the bulk aqueous phase (a 'proton well' [14,15]). Any ionisable group at the proton binding site will be affected by changes in the local proton activity and should show a shift in apparent pK_a with changes in the protonmotive force. Such an effect has been reported for the sugar transport system of *Chlorella* [16].

One of the most sensitive ways of investigating the environment of an ionisable group is through the study of its chemical reactivity, and considerable use has been made of this technique for probing the molecular mechanism of transport systems [17]. Protonmotive-force-dependent changes in the reactivity of two types of functional group have been reported for the lactose permease [18,19], but the permease has not yet been studied in its native state, in whole cells. We have examined the reactivity of the thiol group of the permease because of its obvious importance in active transport [20]. The results demonstrate that there are a number of different reactive states reflecting at least three different conformations of the permease. It appears unlikely that a disulphide involving the reactive thiol is formed in any of these conformations and there is no protonmotiveforce-dependent shift in pK_a that would suggest that the thiol lies in a proton well. Indeed, the protonmotive force does not seem to affect the environment of the thiol group at all, although it slightly alters the intrinsic reactivity of this group.

Materials and Methods

Growth of bacteria

Escherichia coli ML308 (i^-, z^+, y^+, a^+), ML308-22 (i^-, z^+, Y^{UN}, a^+) and ML308-225 (i^-, z^- y^+, a^+) were all grown in minimal medium M9 [21] containing 0.5% glycerol, and harvested in late exponential phase. The bacteria were washed once by resuspension in 150 mM KCl and once by resuspension in the medium of the experiment.

Measurement of uptake

Uptake of galactoside was measured by two types of procedure: (a) spectrophotometric determination of the 2-nitrophenol produced by intracellular galactoside hydrolysis and (b) a radioactive stop-point assay.

(a) Spectrophotometric assay. Only those strains that have β-galactosidase activity (z^+) could be assayed by this method. A 3 ml reaction mix was prepared from buffer (2.5 ml) and a stock bacterial suspension (0.1 ml). 2-Nitrophenyl β-galactoside (0.4 ml) was added to give the desired concentration. For concentrations greater than 5 mM, the 2-nitrophenyl β-galactoside was dissolved in the appropriate buffer and added before the bacteria. Absorbance changes were followed using a Perkin-Elmer Model 156 Dual-Wavelength Spectrophotometer, with the signal wavelength set at 420 nm and the reference wavelength set at 480 nm.

(b) Radioactive stop-point assay. The reaction mixture for each time point was created by rapidly mixing 0.25 ml of a bacterial suspension (1 mg dry wt.$\cdot$ ml^{-1}) with 0.25 ml of the substrate using the mixing block from a quench-flow apparatus (Hi-

Tech Ltd., Salisbury, U.K.). The reaction mixture was injected into a glass tube in which there was a rapidly rotating magnetic follower. Samples were quenched by the rapid injection of 0.5 ml of 10 mM $HgCl_2$ in sodium phosphate buffer (pH 7). The quench solution was delivered from the quench-flow apparatus and the interval was timed by the control unit of the apparatus. Samples were collected at 1 s intervals up to 10 s after initiation and thereafter at 5 s intervals (delivery of the quench solution was triggered manually for these longer times). The quenched samples were centrifuged immediately for 60 s and the pellets resuspended in 0.5 ml water. The radioactivity of the sample was measured by liquid scintillation counting in a 1 : 2 mixture of Triton X-100 and toluene containing 4 $g \cdot l^{-1}$ diphenyloxazole and 0.3 $g \cdot l^{-1}$ 1,4-bis(5-phenoxazol-2-yl)benzene. For each time-course, a parallel run was made in which 10 mM 4-nitrophenyl α-galactoside (a tight-binding competitive inhibitor) was added to the substrate solution in order to allow correction for passive uptake of radioactivity (not resulting from the operation of the permease).

Measurement of rates of reactions

The rate of reaction was determined as the rate of inactivations of the uptake process. To measure the rate of inactivation in the absence of galactosides, a 3 ml reaction mixture was prepared from buffer (2.5 ml), stock bacterial suspension (0.45 ml) and stock solution of inactivating reagent (0.05 ml). The inactivating reagent was usually added last in order to initiate the reaction. The concentration of the reagent was 5 mM. Samples were taken at 1 min intervals for the first 5 min and then at 5 min intervals up to 60 min. Each sample was diluted 16-fold with 45 ml of 0.1 M sodium phosphate bufrer (pH 7.0) and centrifuged for 3 min in a bench centrifuge. The dilution slowed the inactivation at least 12-fold and an insignificant amount of reaction occurred during the centrifugation. Pellets were immediately resuspended in 3 ml of 0.1 M sodium phosphate buffer (pH 7.0) and assayed for uptake by one of the methods described above.

To measure the rate of reaction during net turnover in the presence of galactoside, the two uptake assays were modified as follows. In the spectrophotometric assay, uptake was followed for 10 s after initiation and then 0.01 ml of the stock solution of the reagent was rapidly injected and monitoring continued until no further change in the rate of production of 2-nitrophenol was apparent (usually after 60–100 s). At this point, either 4-nitrophenyl α-galactoside (to 10 mM) or formaldehyde solution (to 60 mM) was added. Both reagents block any further turnover of the permease with 2-nitrophenyl β-galactoside and therefore give an estimate of the amount of hydrolysis occurring through passive diffusion. Both reagents gave similar background rates below pH 8, but formaldehyde increased the leakiness of the cells at higher values of pH.

In the stop-point assay using radioactive galactosides, uptake was followed by sampling at 1 s intervals for up to 10 s using reaction mixtures created by rapid mixing in order to measure the initial rate of uptake. Then a number of reaction mixtures were prepared, incubated for 10 s and then mixed with inactivating reagent. Incubation was continued for up to 120 s with time-points at 5 s intervals between 10 and 60 s and at 20 s intervals thereafter. After 120 s, further uptake via the permease was blocked by the addition of formaldehyde solution (to 60 mM) and three further samples were taken at 20 s intervals.

Calculation of the rates of reactions

In the absence of galactoside, the uptake activity remaining at any point in the reaction with inactivating reagent could be measured directly. Pseudo-first-order rate constants for inactivation were obtained by plotting the logarithm of the fractional residual activity against reaction time.

In the presence of galactoside, the residual activty had to be assessed indirectly. In the spectrophotometric assay tangents were drawn to the absorbance traces at 2 s intervals to calculate the instantaneous rate of uptake. In the stop-point assay, a continuous curve was first produced by interpolation between the experimental points and tangents constructed to that curve at the experimental time-points. The uptake rates thus obtained were plotted as before in order to determine the pseudo-first-order rate constants for inactivation.

Second-order rate constants for inactivation were obtained by plotting the pseudo-first-order rate constants gainst the concentration of inactivating reagent for several different concentrations of inactivating reagent.

Preparation of reagents and manipulation of the protonmotive force

Stock solutions of the inactivating reagents were usually prepared in ethanol at a concentration which would allow an addition of between 0.01 and 0.05 ml to the reaction mixture. Acetone was used to dissolve *N*-ethylmaleimide and 2,2′-dithiobis(5-nitropyridine). Solutions of 6,6′-dithiodinicotinic acid and 5,5′-dithiobis(2-nitrobenzoic acid) were prepared in the medium in which the experiments were to be performed. Where an addition of organic solvent was made, the same addition of pure solvent was made to the controls. Bacterial suspensions were prepared containing approx. 10 mg dry wt. bacteria per ml in the buffer in which the experiment was to be performed. Storage at alkaline pH appeared to have no adverse effect on uptake activity in the time-course of these experiments (up to 90 min total exposure time). Storage at low pH in the presence of uncouplers produced an increase in leakiness and eventual lysis of the cells. Uncouplers were therefore added at the earliest possible time that avoided bursting. Thus, azide was added 20 min prior to the start of the experiment, gramicidin A (in EDTA-treated cells [22]) was added 10 min prior to the start of the experiment and carbonyl cyanide *m*-chlorophenylhydrazone was added 3 min prior to the start of the experiment. The effectiveness of the uncouplers in decreasing the protonmotive force, and also the ability of the cells to maintain a protonmotive force during the modification was routinely checked qualitatively using acridine orange and 8-anilinonaphthalene-sulphonate to measure the pH gradient and the membrane potential, respectively. The response of these dyes was quantitated by comparison with measurement made with 5,5-dimethyl[2-^{14}C] oxazolidine-2,4-dione and [^{3}H]methyltriphenylphosphonium using the methods described in Ref. 23.

Results and Discussion

The time-course of inactivation in ML308 and ML308-225

All of the reagents listed in Table I inhibited the uptake of 2-nitrophenyl β-galactoside into ML308 monophasically and completely. The

TABLE I

SECOND-ORDER RATE CONSTANTS FOR THE INACTIVATION OF 2-NITROPHENYL β-GALACTOSIDE UPTAKE INTO *E. COLI* ML308

The second-order rate constants were determined in the absence of galactoside using method (a) as described in Fig. 1. Slopes of the replots (Fig. 1b) were obtained by weighted least-squares analysis. The medium was 0.1 M sodium phosphate (pH 7) in each case.

Reagent	Rate of inactivation ($M^{-1} \cdot s^{-1}$)	
	in respiring cell suspensions	in the presence of uncoupler
(1) 2-Hydroxy-5-nibrobenzylbromide	0.17 ± 0.03	3.3 ± 0.2 [b]
(2) Diamide	0.07 ± 0.02	0.9 ± 0.07 [b]
(3) 6,6′-Dithiodinicotinic acid	1.4 ± 0.2	2.5 ± 0.2 [b,c] (0.04) [a]
(4) 2,2′-Dithiobis(5-nitropyridine)	10.1 ± 1.1	23.4 ± 0.9 [b]
(5) 2,2′-Dithiodipyridine	44.9 ± 3.5	102.7 ± 0.4 [b] (0.05)
(6) 4,4′-Dithiodipyridine	300 ± 15	851 ± 24 [b]
(7) 5,5′-Dithiobis(2-nitrobenzoic acid)	1.1 ± 0.2	0.6 ± 0.1 [b,c] (0.05)
(8) *N*-Ethylmaleimide	17.3 ± 2.6	9.7 ± 1.8 [b] (0.1)
(9) 4-Diazonium benzenesulphonate	250 ± 31	0.9 ± 0.07 [c]

[a] Figures in parentheses refer to the rate of inactivation observed in the presence of 2 mM thiodigalactoside.

[b] 50 mM NaN_3.

[c] 40 μM carbonyl cyanide *m*-chlorophenylhydrazone.

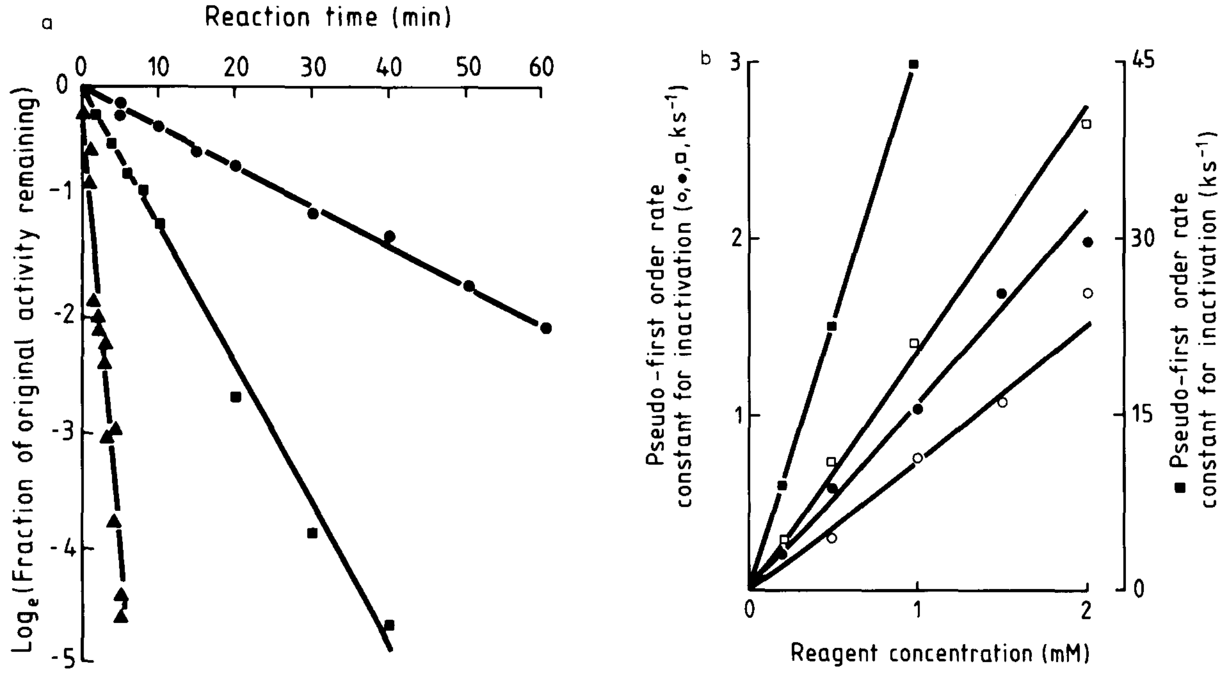


Fig. 1. The time-course of inactivation of uptake into *E. coli* ML308. (a) Semilogarithmic plots of fractional activity against reaction time. Activity was determined by method (a) and the medium was 0.1 M sodium phosphate (pH 7). The inactivating agents were 0.5 mM 5,5′-dithiobis(2-nitrobenzoic acid) (●), 2 mM 5,5′-dithiobis(2-nitrobenzoic acid) (■) and 0.5 mM 2,2′-dithiodipyridine (▲). (b) Typical plots of inactivation rate constants against reagent concentration. The pseudo-first-order inactivation rate constants, determined from semi-logarithmic plots like those shown in (a) are plotted against the concentration of the inactivating reagent. Second-order rate constants were estimated from the slope of the lines in this plot. The inactivating reagents were 2-methylnaphtho-1,4-quinone (○), 5,5′-dithiobis(2-nitrobenzoic acid) (●), 6,6′-dithiodinicotinic acid (□) and 2,2′-dithiodipyridine (□). Note the different scale for the rate of inactivation of 2,2′-dithiodipyridine.

semi-logarithmic plots of log (fractional activity) against reaction time were linear for at least 90% of the reaction (Fig. 1a) and the pseudo-first-order rate constant was proportional to reagent concentration up to 2 mM (Fig. 1b). Thus there was no evidence of saturation of the rate of reaction in this concentration range which would be the result of formation of adsorptive complexes between protein and reagent [24]. The intermediacy of such complexes having dissociation constants greater than 2 mM cannot be excluded, and might indeed the expected in chemical modification reactions [25]. The monophasic reaction is consistent with the reaction of a single residue with complete loss of activity after modification, but the possibilities that (i) there is more than one essential group that reacts with the same time-course or that (ii) there are other essential groups that react more slowly, cannot be excluded.

When the kinetic parameters of 2-nitrophenyl β-galactoside were determined in samples taken from a reaction mixture at zero time and at 1-, 2- and 4-times the half-time of inactivation, only a change in the apparent maximum velocity of uptake (V^{A}) was observed; there was not significant change in the apparent affinity (K^{A}) in the concentration range 0.1–30 mM ($K^{A} = 0.9$ mM). Such measurements were made using 5,5′-dithiobis(2-nitrobenzoic acid), 2,2′-dithiodipyridine, *N*-ethylmaleimide, and 2-methylnaphtho-1,4-quinone. In membrane vesicles derived from ML308-225, 2-methylnaphthoquinone has been reported to cause an increase K^{A} for uptake of lactose without affecting V^{A}. However, when uptake of lactose into suspensions of ML308-225 that had been treated with the quinone was assayed using substrate concentrations up to 50 mM (100-times K^{A}), V^{A} was found to decrease to zero with no

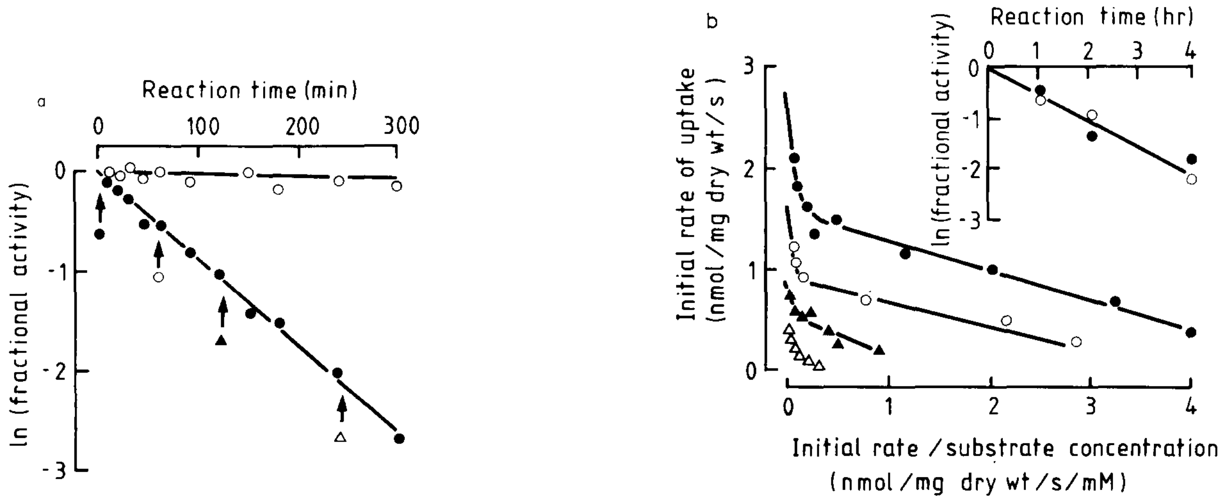
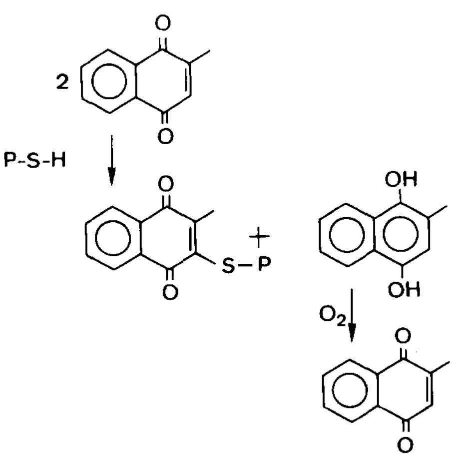


Fig. 2. The kinetics of uptake into *E. coli* after modification with quinones. (a) Semilogarithmic plot of fractional activity against reaction time. Uptake activity was measured using the stop-point method with 1 mM lactose as substrate. Closed circles represent inactivation with 0.5 mM 2-methylnaphtho-1,4-quinone and open circles represent incubation with 0.5 mM tetramethylbenzoquinone in 0.1 M sodium phosphate (pH 7). At the time shown by the arrows, samples were taken for the determination of the apparent kinetic parameters of uptake. (b) The kinetics of lactose uptake after modification. Eadie-Hofstee plot of the rate of uptake against rate (substrate concentration). The intercept on the abscissa gives the apparent maximum velocity of uptake, and the slope is equal to $-K_m$. [^{14}C]Lactose concentrations in the range 0.05–30 mM were employed for the assay of uptake, which was by the stop-point method described in the text. The symbols correspond to those juxtaposed to the arrows in (a) and indicate the origin of the sample in the inactivation time-course. In the inset, V^A for uptake via the apparent high-affinity component (○) and that for uptake via the apparent low-affinity component (●) are plotted as a fraction of the initial value against reaction time.

detectable effect on K^A (Fig. 2).

Quinones may react with thiol residues in two ways: by adduct formation (Scheme I) or by direct oxidation (Scheme II) [26]. Konings and Robillard [11] have reported that several quinones readily modify uptake of lactose into membrane vesicles, causing an affinity change without loss of maximal rate; this was attributed to direct oxidation (Scheme II). However, we found that 2,3,5,6-tetramethylbenzo-1,4-quinone, which is unable to form adducts and can react only by direct oxidation, did not inhibit uptake (Table I), whereas the structurally similar 2-methylnaphtho-1,4-quinone, which can react by adduct formation, was able to inhibit uptake (Table I). Since the redox potentials of these two compounds are similar [27], the most obvious conclusion is that the prinicple pathway for the reaction of 2-methylnaphtho-1,4-quinone with the reactive thiol of the permease is through adduct formation and not by direct oxidation.



Scheme I.

$$\mathrm{P\text{-}S^- + Q \rightarrow P\text{-}S^{\cdot} + Q^{\cdot -}}$$
$$\mathrm{P\text{-}S^- + Q^{\cdot -} \rightarrow P\text{-}S^{\cdot} + Q^{2-}}$$
$$\mathrm{2\ P\text{-}S^{\cdot} \rightarrow P\text{-}S\text{-}S\text{-}P}$$
$$\mathrm{Q^{2-} + 2\ H^+ \rightarrow QH_2}$$

Scheme II.

Inactivation of uptake by oxidation in air [1,28,29] or by diamide (Ref. 30 and references therein) proceeds more rapidly when the cells are in an energy-deficient state (deprived of carbon-source [27] or in the presence of uncouplers (Table I)). This inactivation results in a decrease in V^A to zero.

The rate of reaction with the various disulphides listed in Table I showed a marked trend; the lowest reactivity was observed with the charged reagents (reagents 1 and 2 in Table I), a 10-fold higher rate of reaction was observed when the carboxyl group is replaced by a nitro group and at least a 30-fold increase in rate is observed when there was no substituent (reagent 4). Steric factors may contribute somewhat to the different reactivities but, since the carboxyl and nitro groups are very similar in size, much of the difference may be due to electrostatic effects. It seems, therefore, that there is a hydrophobic diffusion barrier around the reactive thiol. Support for this notion comes from consideration of the relative rates of reaction of 2,2′-dithiodipyridine (reagent 4) and 4,4′-dithiodipyridine (reagent 5). The ratio of the rates of reaction with these two reagents has been shown to be indicative of the dielectric constant of the reaction site [31]. As can be seen from Table I, the ratio $k_{4,4'\text{-DTP}}/k_{2,2'\text{-DTP}}$ is approx. 7 in the absence of uncoupler and 8 in the presence of an uncoupler. These values lie between the values obtained for the buried thiol groups in ficin [31] and papain [32,33] and indicate that the thiol group is located in an environment that has a much lower dielectric constant than that of water.

The effects of a protonmotive force on the kinetics of inactivation

Two groups of reagents were distinguished by the effect of the protonmotive force on their reaction rate: those reagents for which the rate of reaction was greater in the presence of an uncoupler than it was in its absence (reagents 1–6 in Table I) and those reagents for which the rate of reaction was greater in the absence of an uncoupler than it was in its presence (reagents 7–9 in Table I). The effect of uncouplers on the reactivity of the thiol group remained constant between pH 6 and pH 10 (Fig. 3). Thus for 5,5′-dithiobis(2-nitrobenzoic) acid, the reactivity was lower by a factor of 2.0 ± 0.3 and for 2,2′-dithiodipyridine the reactivity was greater by a factor of 2.7 ± 0.3 in the presence of uncouplers, irrespective of the pH. This suggests that the membrane potential is the more significant component of the protonmotive force in causing this effect. Although the values of the pH-independent rate constants (see below) were affected by the protonmotive force, there seemed to be no effect on the apparent dissociation constant (Table II). It is surprising that the dipyridyl disulphides and 5,5′-dithiobis(2-nitrobenzoic acid) should have opposite responses to the change in the protonmotive force. It may be

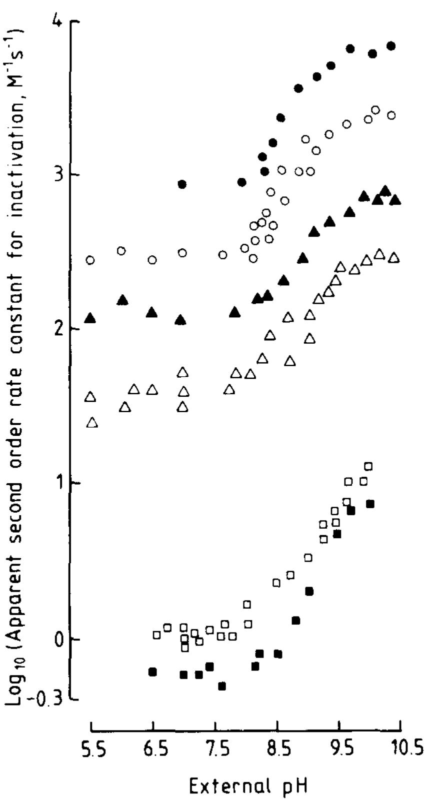


Fig. 3. The pH dependence of the rate of inactivation of uptake into *E. coli* ML308. The second-order rate constants for inactivation of 2-nitrophenyl β-galactoside uptake, determined as shown in Fig. 1, are plotted logarithmically against the pH of the medium in which the experiments were performed. Between pH 5.5 and pH 8.1, 0.1 M sodium phosphate was used as buffer, and between pH 7.9 and pH 10.5, 0.1 M sodium borate was used as buffer. Uptake and its inactivation occurred slightly more slowly in borate buffers than they did in phosphate buffers at the same pH. Either 50 mM NaCl (open symbols) or 50 mM NaN_3 (solid symbols) was present in the reaction mixture. The inactivating reagents were (○, ●) 4,4′-dithiodipyridine, (△, ▲) 2,2′-dithiodipyridine, (□, ■) 5,5′-dithiobis(2-nitrobenzoic acid).

TABLE II

THE PARAMETERS OF THE pH DEPENDENCE OF INACTIVATION OF UPTAKE INTO *E. COLI* ML308

The pH-dependence of the second-order rate constant for the inactivation of uptake of 2-nitrophenyl β-galactoside was investigated as described in the legend to Fig. 3. The parameters in the table were obtained by fitting the data to Eqn. 1. + indicates that 50 mM NaN_3 was present and − indicates that the NaN_3 was replaced by 50 mM NaCl.

Reagent	k_X ($M^{-1} \cdot s^{-1}$)		k_{XH} ($M^{-1} \cdot s^{-1}$)		pK_a	
	+	−	+	−	+	−
(3) 6,6′-Dithiodinicotinic acid	21.1 ± 0.6	10.9 ± 1.1	2.5 ± 0.3	1.4 ± 0.2	9.37 ± 0.12	9.35 ± 0.05
(4) 2,2′-Dithiobis(5-nitropyridine)	167.2 ± 3.5	72.4 ± 5.7	23.5 ± 2.1	10.0 ± 1.6	9.27 ± 0.21	9.31 ± 0.10
(5) 2,2′-Dithiodipyridine	780.2 ± 10.7	302.0 ± 17.3	103.4 ± 5.7	45.6 ± 2.3	9.25 ± 0.07	9.30 ± 0.09
(6) 4,4′-Dithiodipyridine	6610.1 ± 26.3	2530.0 ± 10.5	850.1 ± 17.2	302.4 ± 15.7	9.09 ± 0.05	9.05 ± 0.10
(7) 5,5′-Dithiobis(2-nitrobenzoic acid)	8.3 ± 0.5	15.2 ± 0.9	0.58 ± 0.02	1.1 ± 0.09	9.57 ± 0.09	9.59 ± 0.10

due in part to the greater lipophilicity and ease of penetration of the membrane of the dipyridyl disulphides [34]. In metabolizing cells the disulphides will be continuously reduced by cytoplasmic thiol-regenerating systems [35,36] and this may deplete the effective concentration at the reaction site on the permease. This will occur to a lesser extent with the less penetrating disulphide, 5,5′-dithiobis(2-nitrobenzoic acid), and in this respect it is significant that the slowly penetrating 6,6′-dithiodinicotinic acid [37] shows a less marked acceleration in the presence of an uncoupler than do the lipophilic disulphides. There is very little to suggest the cause of the apparent increase in reactivity that occurs with, for example, *N*-ethylmaleimide, in the presence of a protonmotive force; there is indication of a change in dielectric constant consequent upon a conformation change. The increase in reactivity may be due to alterations in the structure of the lipid bilayer [38–41] or to a general increase in the exposure of proteins to the aqueous medium [42,43] that have been reported to occur in the presence of a membrane potential.

The pH dependence of the kinetics of inactivation

The apparent second order rate-constants increase at alkaline pH (Fig. 3). The pH-dependence of the apparent rate of inactivation (k_{obs}) is given by Eqn. 1:

$$k_{obs} = \frac{k_{XH}}{(1+K_a/[H^+])} + \frac{k_X}{(1+[H^+]/K_a)} \qquad (1)$$

The values of the pH-independent rate constants (k_{XH} and k_X) and the apparent dissociation constant (K_a) are given in Table II. The base form (k_X) reacts some 8-fold faster with the dipyridyl disulphides than the protonated form (k_{XH}) does and it reacts approx. 14-fold faster with 5,5′-dithiobis(2-nitrobenzoic acid) than does the protonated form. The apparent pK_a measured by reaction with the disulphides is in the range 9.0–9.6. This is in the same range as the apparent pK_a of an ionisation affecting V^A of uptake [8] and the apparent pK of an ionisation affecting the apparent equilibrium constant for galactoside binding to membrane vesicles [44]. The pK_a determined for uptake could represent deprotonation of the ternary proton-galactoside-permease complex [8] to form a non-translocating galactoside-permease binary complex and the equilibrium-binding constant could reflect the same process. The observation that the binding affinity, rather than the number of binding sites, changes [43] suggests that the permease is not being simply inactivated by this ionisation.

Thiol reactivity in the presence of galactosides

Whilst it is well known that many galactosides can decrease the reactivity of the thiol group towards many of the reagents listed in Table I [45], it is now apparent that this is true only in certain circumstances. The reactivity of the thiol has been investigated under four conditions: (a) at equilibrium, in the presence of an uncoupler, with galactoside on both sides of the membrane; (b) during the steady state of accumulation, in the absence of an uncoupler, with galactoside on both sides of the membrane; (c) during the initial phase of influx in the presence of an uncoupler, with galactoside only in the external medium ('downhill' galactoside transport) and (d) during the initial phase of active uptake, in the absence of an uncoupler, with galactoside only in the external medium. In the first two experiments, in conditions under which the permease is apparently at equilibrium with galactoside on both sides of the membrane, the reactivity of the thiol group with all of the reagents listed in Table I is very low, the rate of reaction being at least 10-times less than the rate measured in the absence of galactoside. Under both conditions (a) and (b), the dependence of the rate of reaction on galactoside concentration has been shown to obey Eqn. 2 [20]:

$$k_{obs} = \frac{k_{XH}}{(1+[S]/K_d)} \tag{2}$$

In the presence of an uncoupler (condition (a)), the values of the apparent galactoside affinity constant (K_d) determined using Eqn. 2 are similar to those obtained by measuring binding to the permease in membrane vesicles [45]. In condition (c), when the permease is saturated with galactoside only at the external face of the membrane, the reactivity of the thiol group is not depressed, but is very slightly enhanced (compare k_X, Table II with k_{XS}, Table III). In the presence of the high galactoside concentrations needed to saturate the permease under these conditions, the rate of passive diffusion across the membrane becomes significant and the cells rapidly move into condition (a). Nevertheless, by making measurements early in the time-course of uptake (as described in the legend to Fig. 4), we have been able to show that the rate of inactivation, in the presence of saturating concentrations of galactoside, is scarcely altered (an increase of 1.02–1.50-fold in the rate of reaction is observed, Table III). The reactivity of the thiol group is definitely enhanced when the cells are in condition (d), i.e., during turnover in the presence of a protonmotive force. The increase in rate due to galactoside is saturable, with an apparent galactoside affinity constant that is similar to, although somewhat higher than, K^A for uptake. The substrate dependence of the apparent second-order rate constant therefore takes the form shown in Eqn. 3.

$$k_{obs} = \frac{1}{1+[S]/K'_S}\left[\frac{k_X}{1+[H^+]/K_a} + \frac{k_{XH}}{1+K_a/[H^+]}\right] + \frac{1}{1+K'_S/[S]}\left[\frac{k_{XS}}{1+[H^+]/K'_a} + \frac{k_{XHS}}{1+K'_a/[H^+]}\right] \tag{3}$$

$$K'_S = K_S(1 + K_{ia}/[H^+])$$

The pH-independent rate constants, k_X and k_{XH}, are those defined by Eqn. 1, while the pH-independent rate constants, k_{XS} and k_{XHS}, char-

TABLE III

THE PARAMETERS FOR THE SUBSTRATE DEPENDENCE OF THE RATE OF INACTIVATION OF 2-NITROPHENYL β-GALACTOSIDE UPTAKE IN *E. COLI* ML308

The rates of inactivation were determined by the experiments described in the legends to Figs.1 and 4 and pH dependence according to the legend to Fig. 3. The second order rates of inactivation were fitted to Eqn. 3 at each value of pH and the pH-independent constants obtained by fitting the rate constants obtained to Eqn. 1. + indicates the presence of 50 mM NaN_3; − indicates that NaN_3 was replaced by NaCl; n.d. indicates that the rates of inactivation were not determined. The value of K^A was obtained from Eadie-Hofstee plots of the initial rate of uptake against substrate concentration.

Reagent	k_{XS} ($M^{-1} \cdot s^{-1}$)		k_{XHS} ($M^{-1} \cdot s^{-1}$)		pK'_a		K_s (mM)	K^A (mM)
	+	−	+	−	+	−	−	−
(5) 2,2′-Dithiodipyridine	800 ± 24	354 ± 17	111 ± 10	296 ± 18	8.94 ± 0.05	8.65 ± 0.12	1.7 ± 0.3	1.0 ± 0.3
(6) 4,4′-Dithiodipyridine	7050 ± 100	4967 ± 50	1045 ± 53	4764 ± 67	9.04 ± 0.09	8.97 ± 0.20	2.0 ± 1.1	1.2 ± 0.5
(7) 5,5′-Dithiobis(2-nitrobenzoic acid)	10 ± 0.9	20.5 ± 1.2	0.9 ± 0.05	15 ± 0.1	9.09 ± 0.02	9.00 ± 0.01	1.5 ± 1.0	0.9 ± 0.2
(8) *N*-Ethylmaleimide	n.d.	n.d.	18 ± 1.3	30 ± 2.7	n.d.	n.d.	1.2	0.8

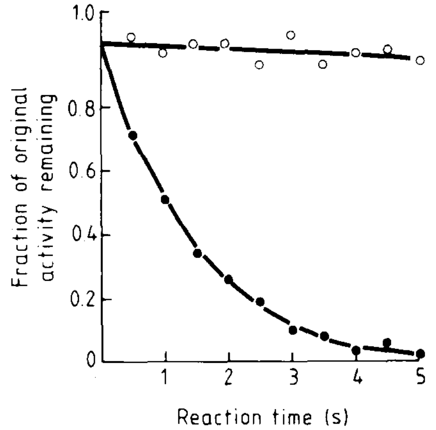


Fig. 4. The time-course of inactivation of uptake of 2 nitrophenyl β-galactoside into ML308 in the presence and absence of a substrate. Conditions were arranged so that a direct comparison of the inactivation rates is possible. (a) ○, Inactivation in the presence of a galactoside. A cell suspension (4 mg dry wt.$\cdot$ml^{-1}) was mixed with 2-nitrophenyl β-galactoside (1.5 mM). After a delay of 0.15 s (during which the initial transient of uptake is completed) the solution was mixed with 2,2′-dithiodipyridine plus 2-nitrophenyl β-galactoside (1.5 mM final concentration) and flow was stopped. The increase in $A_{420-480}$ was followed using a Perkin-Elmer Model 156 Dual-Wavelength Spectrophotometer interfaced to the Hi-Tech Multi-Mixing Stopped-Flow Module in which the rapid mixing was performed. (b) ●, Inactivation in the absence of the galactoside. The same cell suspension (4 mg dry wt.$\cdot$ml^{-1}) was mixed with 2,2′-dithiodipyridine (1.25 mM) and at the times shown it was rapidly ejected into 90 vol. of 0.1 M sodium phosphate buffer (pH 7) containing 1 mg/ml bovine serum albumin. The samples were centrifugated for 5 min as for method (a) (less than 5% inactivation occurs during centrifugation and subsequent work-up using these conditions). The assay was extended to 60 s by manual triggering in order to estimate the rate of inactivation. For (a) this is calculated to be 0.34 s^{-1} ($= 276$ M$^{-1}\cdot$s^{-1}) and for (b) 0.0046 s^{-1} ($= 37$ M$^{-1}\cdot$s^{-1}). In both (a) and (b) the concentrations and cell densities refer to the final reaction mixture.

acterise the reaction in the presence of saturating external galactoside, where half-saturation occurs at a substrate concentration equal to K_S'. The value of K_S' is itself pH-dependent and is affected by an ionisation with apparent p$K_{ia} = 8.3$. This ionisation constant has approximately the same value as that determined from the pH-dependence of the K^A for influx [8]. The ionisation constant, K_a, in Eqn. 3 is that defined by Eqn. 1, whilst the constant K_a' characterises the pH-dependence of the reaction in the presence of saturating external galactoside. The value of pK_a' lies in the range 8.7–9.0 and is therefore up to 0.5 unit lower than the corresponding value of pK_a. Such an effect on the ionisation constant of reactive groups has been noted for the modification of the erythrocyte glucose carrier and was then identified with a substrate induced conformation change [46].

The pattern of depressed reactivity when at equilibrium with substrate but enhanced reactivity during net turnover that has been described here is also observed with the glucose carrier. With the lactose permease the reactivity in the protonated state is enhanced by the substrate by up to 16-fold (compare k_{XH} in Table II with k_{XHS} in Table III) whereas in the base state the enhancement is only a factor of 2 (k_X in Table II; k_{XS} in Table III). Ionisation of the thiol therefore appears to influence its reactivity much less during turnover than it does in the resting state. However, there are a number of problems encountered with the determination of the reactivity at high pH in the presence of galactoside. Firstly it is more difficult to saturate the permease (the value of K_S' increases according to Eqn. 3) and at the higher concentrations of galactoside there is more non-specific entry. Secondly, at pH 9, V^A of turnover starts to decrease, as previously described under the effects of pH. The ionisation that causes the decrease in turnover rate could also decrease the contribution of the reactive form of the permease to the intermediates that accumulate during turnover, thus limiting the enhancement at alkaline pH. Finally, since it is possible that the reactive thiol is a binding site for the translocated proton, it could be that the base-state of the thiol is the reactive intermediate that accumulates during turnover even at values of pH where the protonated state might be expected to predominate.

The ratio of the reactivities with 4,4′-dithiodipyridine and 2,2′-dithiodipyridine when the cells are in condition (c), i.e., in the presence of external galactoside and an uncoupler, is similar to that observed in the absence of the galactoside. Thus the ratio $k_{XH(4,4'\text{-DTP})}/k_{XH(2,2'\text{-DTP})}$ is 9.4 and the ratio $k_{X(4,4'\text{-DTP})}/k_{X(2,2'\text{-DTP})}$ is 8.8; this modest increase of 10% matches the 10% increase in rate that is observed. In contrast, the ratio of reactivities when the cells are in condition (d), i.e., in the presence of external galactoside and in the absence of uncoupler, is considerably increased. The ratio $k_{\mathrm{XH(4,4'\text{-}DTP)}}/k_{\mathrm{XH(2,2'DTP)}}$ is 14 and the ratio $k_{\mathrm{X(4,4'\text{-}DTP)}}/k_{\mathrm{X(2,2'\text{-}DTP)}}$ is 16, which are nearly double the corresponding values in the absence of galactoside. The effect of galactoside enhances the reactivity with 4,4-dithiodipyridine about twice as much as it enhances the reactivity with 2,2′-dithiodipyridine, suggesting that, although the thiol becomes more reactive to both reagents, its environment becomes more hydrophobic and thus depresses the reaction with 2,2′-dithiodipyridine.

Substrate-induced increases in reactivity of essential groups have been noted for a number of transport systems [47–50]. In every case the effect has been attributed to the accumulation of an intermediate of the transport cycle that has a conformation different from the resting state of the protein. This interpretation is directly applicable to the lactose permease in the following manner. Binding of substrate to form the ternary proton-galactoside-permease complex promotes a conformational change occluding both galactoside and the reactive thiol. Release of the galactoside occurs at the other side of the membrane, leaving the permease in a conformation which has a very reactive thiol. This intermediate relaxes through a rate-limiting step to the ground state having the normally reactive thiol. The obvious candidates for the very reactive intermediate are the internally located pools of proton-permease complex and the unloaded permease. It is unlikely that the ternary proton-galactoside-permease complex accumulates to a significant extent during turnover, since this would lead to protection of the thiol and thus it is unlikely that the rate-limiting step is translocation of the ternary complex as has been suggested [7].

The time-course of inactivation in ML308-22

E. coli ML308-22 has a mutation which affects the activity of the permease. The phenotype is characterized by an apparent uncoupling of the flux of protons from that of galactoside [51]; galactosides are transported rapidly but are very poorly accumulated [52]. This strain reacts with some of the inhibitors discussed above in a manner that is very different from that of its parent. In particular, the reaction is characterised by a greater rate and a marked biphasicity [53]. In the absence of substrate, the biphasicity was very marked with 5,5′-dithiobis(2-nitrobenzoic acid) but not with 2,2′-dithiodipyridine. However, in the presence of external substrate, the reaction became noticeably biphasic with all reagents (Table IV and Fig. 5).

The dependence of the fractional residual activity ($v_t/v_t = 0$) on reaction time (t) takes the form shown in Eqn. 4:

$$v_t/v_{t=0} = C_1\cdot e^{-k_1t} + C_2\cdot e^{-k_2t} \tag{4}$$

The pseudo-first order rate constants, k_1 and k_2, were found to be linearly proportional to concentrations of inactivating reagent of up to 1.5 mM (the highest concentration investigated). The apparent second-order rate constants were found to be sensitive to the presence or absence of an uncoupler and to depend on the external galactoside concentration (Table IV). The coefficients C_1 and C_2 were independent of the nature and concentration of the reagent, the presence or absence of uncoupler and the presence or absence of galactoside. This strongly suggests that there are two independent groups of thiols reacting [58]. The thiol group reacting in the fast phase of inactivation showed less difference between the rates of reaction with polar and non-polar reagents; for example the thiol group in the parent strain reacts with 4,4′-dithiodipyridine 270-times more rapidly than it does with 5,5′-dithiobis(2-nitrobenzoic acid), but in the mutant the rate of reaction with 4,4′-dithiodipyridine is only 40-times more rapid than the rate of reaction with 5,5′-dithiobis(2-nitrobenzoic acid). The difference is due mostly to an increase in the reactivity towards the polar reagents and there is little difference between the reactivities towards 4,4′-dithiodipyridine. In the parent the ratio $k_{\mathrm{4,4'\text{-}DTP}}/k_{\mathrm{2,2'\text{-}DTP}}$ has a value of 7 at pH 7 and in the absence of uncoupler, whereas in the mutant the ratio is only 2. Therefore it appears that the thiol group reacting in the fast phase is in a more exposed position and is in a more aqueous environment than the reactive thiol of the parent.

The thiol reacting in the slow phase of inactivation appears to be at least as deeply buried as the reactive thiol of the parent permease. It shows a lower reactivity overall, it is markedly less reactive with charged reagents than it is with uncharged reagents and, finally, the ratio $k_{\mathrm{4,4'\text{-}DTP}}/k_{\mathrm{2,2'\text{-}DTP}}$

TABLE IV

KINETIC PARAMETERS FOR THE INACTIVATION OF UPTAKE INTO *E. COLI* ML308-22 IN THE PRESENCE AND ABSENCE OF GALACTOSIDE

The rate of inactivation of 2-nitrophenyl β-galactoside uptake at pH 7.0 was determined in experiments such as those shown in Fig. 5. The values of the pre-exponential coefficients, C_1 and C_2, and of the apparent second-order rate constants, k_1 and k_2, were obtained by fitting the data to Eqn. 5. The values of k_1 and k_2 are expressed as $M^{-1} \cdot s^{-1}$. The figures in parentheses were obtained by fitting the data to Eqn. 5 taking $C_1 = 0.71$ and $C_2 = 0.29$.

Reagent	Absence of galactoside				Presence of saturating external 2-nitrophenyl β-galactoside			
	C_1	C_2	k_1	k_2	C_1	C_2	k_1	k_2
(3) 6,6′-Dithiodinicotinic acid	0.67 ± 0.02	0.33 ± 0.05	5.6 ± 0.4	0.8 ± 0.01	0.73 ± 0.02	0.27 ± 0.01	16.2 ± 0.5	0.07 ± 0.09
(5) 2,2′-Dithiodipyridine	1.0	–	135	–	0.71 ± 0.02	0.29 ± 0.01	450 ± 10	28 ± 2.7
			(134.3)	(16.2)				
(6) 4,4′-Dithiodipyridine	1.0	–	308	–	0.69 ± 0.02	0.31 ± 0.02	1025 ± 72	175 ± 5
			(301)	(78)				
(7) 5,5′-Dithiobis-(2-nitrobenzoic acid)	0.75 ± 0.03	0.25 ± 0.01	7.2 ± 0.7	0.7 ± 0.02	0.69 ± 0.02	0.31 ± 0.03	14.0 ± 0.2	0.01 ± 0.02

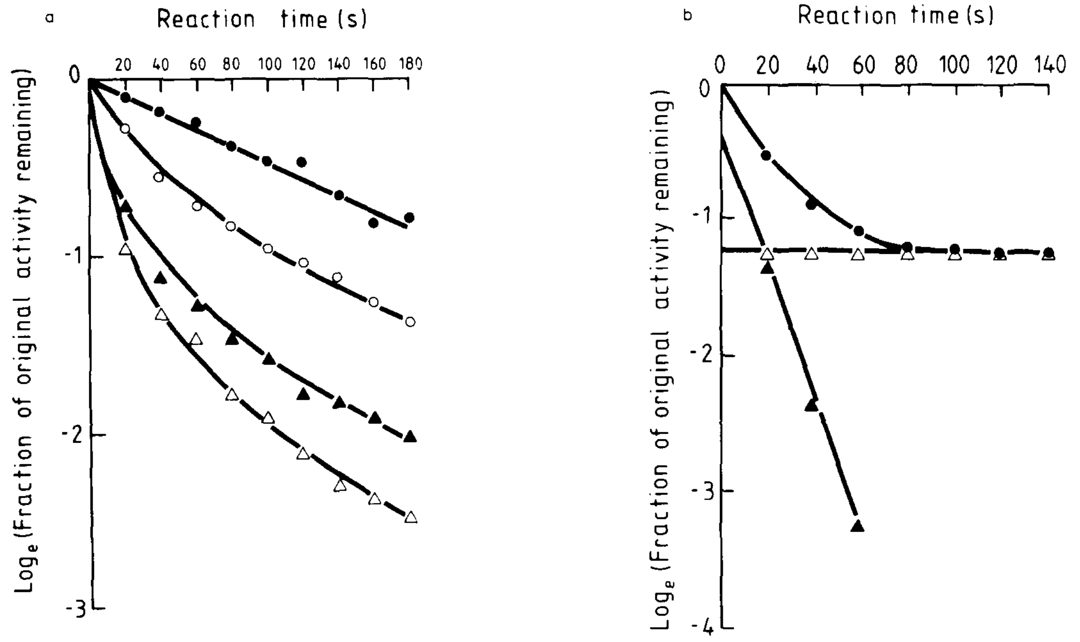


Fig. 5. The time-course of inactivation of active transport into *E. coli* ML308-22. The inactivation was followed in presence of the 2-nitrophenyl β-galactoside. The medium was 0.1 M sodium phosphate (pH 7). (a) The effect of 2-nitrophenyl β-galactoside concentration on the rate of inactivation of utpake into ML308-22 by 20 μM 2,2′-dithiodipyridine. (b) The time-course of inactivation by 4.6 mM 6,6′-dithiodinicotinic acid of 2-nitrophenyl β-galactoside uptake into ML308-22 at saturating galactoside concentration. ●, Plot of the experimental data; △, plot of the reaction of the slower-reacting thiol groups; ▲, plot of the reaction of the faster-reacting groups (calculated according to Ray and Koshland [53]).

is approximately 9 under the same conditions where a ratio of 7 was measured for the parent.

In general, the reactive states characterised for the parent are also observed with mutant (see Fig. 6). Thus, the values of k_1 and k_2 are affected by the magnitude of the protonmotive force, by equilibration with galactosides and by turnover of the permease in the presence of galactoside. However, the effect of saturation with galactoside on the reactivity of the thiol groups is less than it is in the parent strain. Equilibration with galactoside on both sides of the membrane decreases the rate of reaction much less than it does in the parent (Fig. 6), whilst, during turnover, the reactivity is enhanced only 3-fold and there is little change in the relative reactivity with 4,4′-dithiodipyridine and 2,2′-dithiodipyridine. The slowly reacting residue appears to become even less accessible, particularly to charged reagents and, from the relative rates of reaction with 4,4′-dithiodipyridine and 2,2′-dithiodipyridine, to move into a more hydrophobic environment.

The difference in thiol-reactivity between parent and mutant suggest that the conformation of the protein around the reactive thiol group Cys-148 [54] has been altered such that (i) this residue is more exposed and (ii) a neighbouring cysteine residue, which is also important for full activity, becomes more reactive than it is in the parent (where it is normally obscured by reaction of Cys-148). All the cysteine residues in the parent lie between residues 117 and 355, whereas the mutation has been suggested to be within 30 residues from the C-terminus (residue 417) by genetic mapping [55]. Therefore, it seems that the altered reactivity is the consequence of an indirect alteration in protein conformation.

The low value of k_2 for charged disulphides in this mutant makes it possible to study the kinetics of uptake via the partially modified permease. The inhibition of uptake is due to a decrease in V^A and there is no detectable increase in the value of K^A (Table V). Thus, it appears that modification of the fast-reacting thiol decreases the turnover

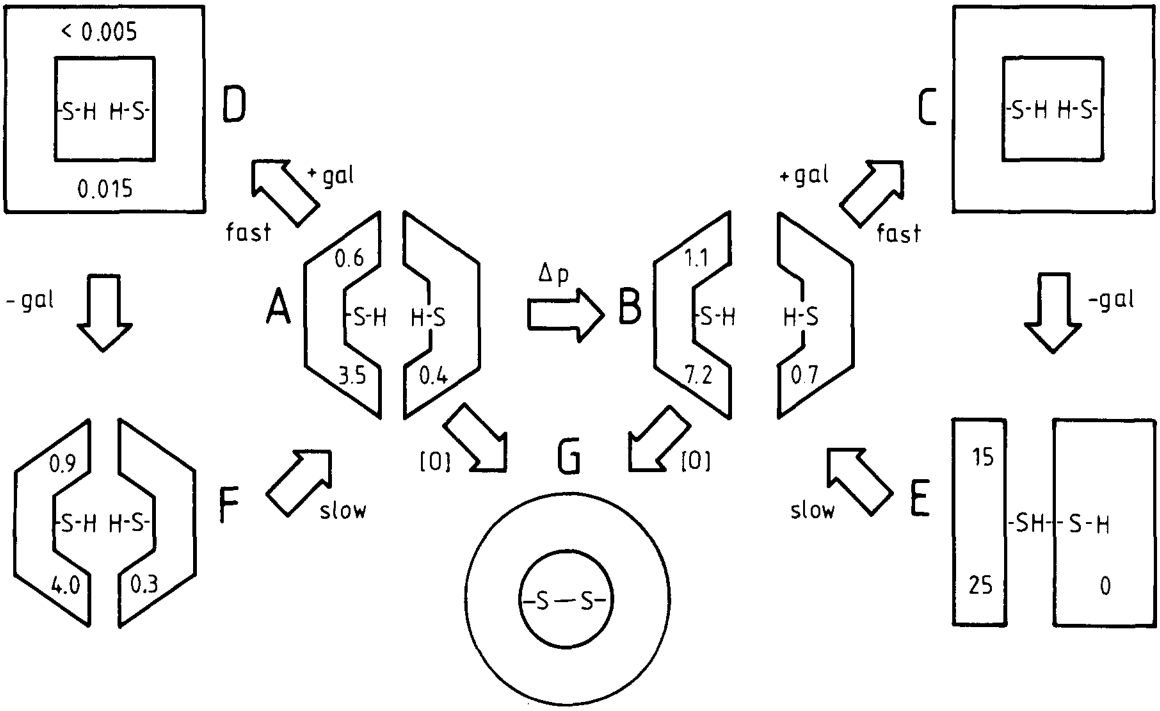


Fig. 6. The reactions of the thiol groups in the lactose permease with 5,5′-dithiobis(2-nitrobenzoic acid) at pH 7.0. In the resting state (A) the permease is suggested to have two reactive thiol groups that are buried in a hydrophobic pocket; one (right-hand block) is relatively unreactive. Imposition of a protonmotive force (Δp) produces a slightly more reactive conformation shown by the more open hexagon (B). Binding of galactoside promotes a conformation where the thiol groups are occluded (C, D), although in the mutant there is still a measurable rate of inactivation. During turnover this relaxes to the resting state via intermediates (E, F) with more reactive thiol groups. Oxidation forms a disulphide between the two thiols and inactivates the permease (G). In each block the upper number is the second-order rate constant for the reaction of the parent and the lower number is the second-order rate constant for reaction of the mutant.

TABLE V

THE APPARENT KINETIC PARAMETER FOR UPTAKE OF 2-NITROPHENYL β-GALACTOSIDE INTO *E. COLI* ML308-22 AFTER PARTIAL MODIFICATION

A suspension of *E. coli* ML308-22 was incubated with 1 mM 6,6′-dithiodinicotinic acid until the fast phase of inactivation (Fig. 5) was complete. The suspension was then diluted 100-fold with buffer and centrifuged. The pellet was washed twice by resuspension in buffer and then assayed for uptake by the spectrophotometric method in 0.1 M sodium phosphate (pH 7).

	Before modification	After modification
Apparent velocity (nmol/mg dry wt. per s)	8.6	2.5
Apparent K_m (mM)	1.4	0.5

number of the permease without affecting the binding-site for galactoside. The creation of a defective permease by the modification of a functional thiol group clearly parallels results obtained with mutants in which Cys-148 was replaced by a non-reactive amino acid (Ser or Gly) by site-directed mutagenesis of the parent molecule [56,57]. In these mutants the activity was decreased to 10–25% of that of the parent and could be inhibited further by modification of a slow-reacting thiol [56,57]. These observations clearly suggest that there are at least two thiol residues in both the parent and the mutant which can affect activity, but that in the parent the residues are much less reactive. This suggestion is supported by the slow air-oxidation and inactivation observed by

Koch [27], by the results obtained with diamide (Refs. 29, and references therein) and the inhibition with phenylarsine oxide reported by Robillard and Konings [12]. The factors affecting reactivity (such as the magnitude of the protonmotive force, presence of galactoside in equilibrium or during turnover) on the parent also affect reactivity of the thiol in the mutant (see Fig. 6). The differences between the relative values of the rate constants characterising the different reactive states suggest that the mutant may undergo slightly different conformational changes during its catalatic cycle, which might be associated with the defective accumulation of galactoside. The results to date do not indicate a particular role in substrate binding or energy transduction for the reactive thiols, although it is clear that Cys-148 cannot be a unique gating residue. Both residues appear to be intimately involved in the conformational changes that take place during proton-galactoside symport and could be involved in the binding of either galactoside or proton. It remains to be seen whether the thiol residues have an active role in transport or whether, as in the arabinose binding protein [59,60], it is that they are located in functionally important parts of the molecule.

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