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ENERGY-DEPENDENT MASKING OF SUBSTRATE BINDING SITES OF THE LACTOSE PERMEASE OF *ESCHERICHIA COLI*

MARTINE BENARD-BENTABOULET and ADAM KEPES

Laboratoire des Biomembranes, Institut de Biologie Moléculaire, Tour 43 – 2 place Jussieu, 75005 Paris (France)

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SUMMARY

A method was devised to measure the number of specific substrate binding sites of lactose permease in membrane preparations derived from mechanically disrupted *Escherichia coli.*

The method consists of incubation with radioactive thiodigalactoside (galactosyl β-D-thiogalactoside, TDG) followed by precipitation with 80% saturated $(NH_4)_2SO_4$ and washing with the same solution.

The measurement gave reproducible results, easy to correct for a moderate nonspecific binding, but active transport, when it occurred, resulted in excess counts.

The radioactivity bound to the pellet was shown to depend on the presence of intact *lac y* gene product.

Addition of ascorbate and phenazine methosulfate (PMS) stimulated active transport into the membrane vesicles. This could be inhibited by cyanide and by uncoupling agents and under these conditions the number of available binding sites was strongly diminished, while the inhibitors alone did not bring about a similar decrease.

The decrease of available substrate binding sites was reversed by removal of oxygen or by washing out the respiratory substrates.

The decrease in available binding sites is interpreted as reflecting one of the energy coupling steps which during *in vivo* active transport prevents the mobile carrier from being available for outflux, but the detailed interpretation of the reported results raises a number of problems connected with the energy cycle of active transport

INTRODUCTION

The lactose permease of *Escherichia coli* behaves as a transport system with a mobile carrier capable to transport its substrate inward and outward and capable of coupling opposite fluxes[1]. The affinity site of the carrier is located on the protein product of the *lac y* gene, which is firmly bound to the membrane.

However, the mobile carrier characteristics only prevail under the energy-inhibited conditions, while during normal energy metabolism the transport is essen-

Abbreviations: TMG, methyl β-D-thiogalactopyranoside; TDG, β-D-galacto-1-*S*-thio-β-D-galactoside; PMS, phenazine methosulfate.

tially unidirectional from outside in and the possibility for the carrier to support outflux and flux coupling are diminished if not abolished[2]. All models built by several workers agree upon a concept of energy coupling which includes at some stage the unavailability of the binding site of the carrier for outflux, as a result of a reaction which occurs on the carrier protein. The nature of this reaction is, however, highly controversial: oxidation reduction[3], protonation or H^+ dissociation[4] or some group exchange with a high-energy metabolite[5,6] have been advocated.

In order to approach this problem it was important to be able to detect and to measure the available binding sites of the carrier and to explore which type of possible energy yielding reactions are capable of producing a decrease of this number. In the present article an $(NH_4)_2SO_4$ precipitation technique is proposed which apparently "freezes" the carrier substrate complex in a membrane preparation and permits the washing out of free substrate. The number of substrate binding sites, measured by this technique, can be decreased reversibly by addition of ascorbate and phenazine methosulfate (PMS) in the presence of oxygen. The oxidation of these substrates, as shown recently, energizes lactose transport in protoplast ghost membrane vesicles[7]. With a membrane preparation, derived from mechanical disruption of exponentially growing cells, the same phenomenon is accompanied by a decrease in the number of available binding sites. Although the binding technique does not rest on grounds of absolute safety and the observations at this stage raise more questions than they can solve, it was thought of interest to report them in some detail.

MATERIALS AND METHODS

Bacteria *E. coli* K12 strain 3300 *Lac* i^- z^+ y^+ was grown on medium 63 supplemented with glycerol and casamino acids in 15- or 30-l fermentors under forced aeration. It was harvested during the exponential growth phase, washed with a buffer containing 10 mM potassium phosphate (pH 6.8), 1 mM $MgCl_2$, 0.1 mM dithiothreitol.

Membrane preparation

The pellet resuspended in phosphate–$MgCl_2$–dithiothreitol buffer was introduced into a Ribi cell fractionator and disrupted by extrusion under 23000 lb/inch^2 pressure (approx. 1600 atmospheres) at temperatures between 5 and 15 °C. The crude extract was centrifuged in a Sharples continuous centrifuge at 50000 rev./min and the pellet was discarded. The supernatant was supplemented with 10 mM $MgCl_2$ and 10 mM $CaCl_2$ and centrifuged again at the same speed. The second pellet, which represents the major part of the fraction often called light membranes, was resuspended in the standard buffer, at a concentration of approx. 50 mg protein/ml, frozen is small portions in liquid nitrogen, and kept frozen at −20 °C. It was utilized after thawing and appropriate dilution in phosphate–$MgCl_2$–dithiothreitol buffer.

Washing of the membranes, when necessary, was done on small columns of agarose 150 M built in Allynh tubes of 50-ml bed volume, on which 2–4-ml portions of membrane suspensions could be harvested in about 15 min.

The binding assay

Incubation samples containing 1–4 mg membrane protein, 10–200 μM ^{35}S-

labelled β-D-galacto-1-*S*-thio-β-D-galactoside (TDG) (C.E.A., France) and various additions in a final volume of 500 μl were quickly pipetted into 4.5 ml 90% saturated neutralized ice-cold $(NH_4)_2SO_4$. The precipitate was centrifuged 5–25 min later (when a number of samples had been collected) for 5 min at 6000 rev./min in a Sorvall bench centrifuge. The supernatant was discarded, the tubes carefully dripped and the pellet was washed twice with 3 ml 80% $(NH_4)_2SO_4$ by centrifugation at the same speed. The final pellet was resuspended in 0.5 ml 1% Triton X-100 solution, transferred to scintillation vials and counted with 5 ml Bray's scintillation mixture in a Nuclear Chicago scintillation counter.

Protein was measured by the method of Lowry *et al.*[8] using bovine serum albumin as a standard.

Respiration was measured polarographically with a Clark electrode.

In vivo transport was measured in aerated suspensions after addition of 1 mM [^{14}C]methyl β-D-thiogalactoside (TMG) by the millipore filtration technique.

RESULTS

The binding assay

Previous reports used a centrifugal technique to measure TDG binding sites sedimentable with the membranes. [^{32}P]orthophosphate was used to measure the water content of the pellet and the binding of ^{3}H-labelled TDG was calculated from the increase of ^{3}H/^{32}P ratio[9]. Using diploid strains which carry two copies of the *lac* gene, Kennedy[9] reported up to 40% increases of the isotope ratio in the pellet compared to the supernatant. We tried the use of tritiated water with ^{35}S-labelled TDG and found with a haploid strain 12–18% isotope enrichments in the pellet, compared to a control with excess nonradioactive TDG. Besides the low degree of accuracy due to the small enrichment, the results varied when the reference used for the isotope ratio was the supernatant, or the pellet of membranes inactivated with *N*-ethylmaleimide.

This method was sufficient to demonstrate the specificity of binding, its dependence on the *lac y* gene and its approximate stoichiometry, but could not be improved for a more quantitative use and for kinetic experiments.

The $(NH_4)_2SO_4$ precipitation was suggested by the success of this technique in the measurements of repressor–inducer binding[10].

It was found that $(NH_4)_2SO_4$ at concentrations of 60% saturation or higher precipitates substantially all membrane from a suspension and that radioactive TDG added to the suspension remained attached to the precipitate. Membrane samples containing 1–4 mg protein were necessary to record a sufficient number of counts which made the millipore filtration too slow to be practical and therefore the centrifugation of the precipitate was adopted.

Table I shows the dependence of the recorded counts on the number of washes, and Fig. 1 shows, under standard conditions, their independence of time and temperature of incubation and their proportionality to the amount of membrane in the sample. The figure also shows that addition of excess nonradioactive TDG in the incubation mixture displaces the bound ^{35}S-labelled TDG. The background levels vary with membrane preparations from 15 to 35% of the total counts, but remain reproducible within one preparation.

TABLE I

DEPENDENCE OF THE RECORDED COUNTS ON THE NUMBER OF WASHES

The experiment was carried out with 10 mg/ml membrane protein, 10 mM phosphate buffer, pH 6.8, at 14 °C. Samples of 0.5 ml were precipitated with 80% saturated $(NH_4)_2SO_4$. Washing by resuspension in 3 ml 80% saturated $(NH_4)_2SO_4$ followed by recentrifugation at 4 °C took approx. 10 min.

Addition	*Treatment after precipitation*	^{35}*S-labelled TDG (pmoles/mg protein)*
None	Centrifugation	254
	Centrifugation + 1 wash	54
	Centrifugation + 2 washes	30
	Centrifugation + 3 washes	23
	Centrifugation + 4 washes	20
TDG, 1 mM	Centrifugation + 2 washes	4

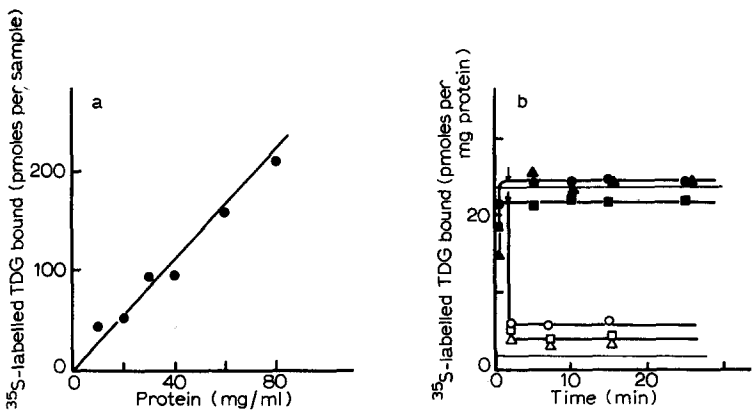

Fig. 1. (a) Proportionality of recorded counts in the $(NH_4)_2SO_4$ precipitate to the amount of membrane in the sample. Incubation mixture contained 10 mM potassium phosphate buffer, pH 6.8, 1 mM $MgCl_2$, and the indicated amounts of membrane. 40 mM azide and 0.03 mM *N,N'*-dicyclohexylcarbodiimide were added 10 min before starting reaction with 0.025 mM TDG. Each point represents the average of two samples (0.3 ml each) which were taken 6 min and 12 min after the addition of ^{35}S-labelled TDG. The background measured in presence of 1 mM nonradioactive TDG was subtracted. (b) ^{35}S-labelled TDG binding *vs* incubation time at various temperatures. Experiments were carried out at pH 6.8 with 0.05 mM ^{35}S-labelled TDG and 10 mg/ml membrane protein. ■—■, 0 °C; ▲—▲, 14 °C; ●—●, 24 °C; arrows indicate time of addition of 1 mM nonradioactive TDG. □—□, 0 °C; △—△, 14 °C; ○—○, 24 °C. The upper and lower thin lines represent the ^{35}S-labelled TDG binding at 14 °C under standard conditions with membranes of fully induced *E. coli* 3000 (upper line) and not induced *E. coli* 3000 (lower line), respectively.

The difference between the radioactivity bound to the pellet before and after addition of excess nonradioactive TDG was taken to measure the binding of TDG to permease sites, provided that the binding was immediate (in less than 30 s), the chase was immediate and the amount was reasonable. This is distinct from mea-

TABLE II

REQUIREMENTS FOR ACTIVE TRANSPORT

The complete reaction mixture contained 10 mM phosphate buffer, pH 6.8, 10 mg/ml membrane protein, 20 mM ascorbate, 0.1 mM PMS and 0.025 mM ^{35}S-labelled TDG and was vigorously agitated at 15 °C. An anaerobic condition was obtained by transfer of an aliquot into a non-agitated test tube. An independent polarographic experiment showed that anaerobiosis was achieved after 2 min. The background radioactivity has been subtracted.

Conditions	*^{35}S-labelled TDG retained in the precipitate 8 min after addition (pmoles/mg protein)*
Complete system	86
PMS omitted	19
Ascorbate omitted	17.5
Oxygen omitted	17.5
Ascorbate + PMS omitted	18

TABLE III

TEST OF VARIOUS SUGARS AS COMPETITORS OF TDG BINDING TO PERMEASE SITES

0.02 mM ^{35}S-labelled TDG was incubated with 8 mg/ml membrane protein in phosphate–$MgCl_2$–dithiothreitol buffer containing 40 mM NaN_3.

Additions	*^{35}S-labelled TDG bound to pellet relative to control*	
	1 mM	*10 mM*
None	100	100
D-Galactosyl β-D-thiogalactoside	14	10
Methyl β-D-thiogalactoside	49	19
Melibiose	31	11
Isopropyl β-D-thiogalactoside	52	24
Phenyl β-D-thiogalactoside	37	16
Lactose	38	14
D-Galactose	63	38
D-Glucose	92	104
α-Methyl-D-glucose	76	81
Trehalose	95	100
Maltose	95	73
Sucrose	97	94
D-Glucuronic acid	86	80
N-Acetyl glucosamine	84	83
D-Fructose	112	91
L-Arabinose	98	83
D-Xylose	111	90
meso-Inositol α-D-galactoside	90	88

surement of accumulated TDG which increased with time, was chased progressively and resulted in much higher amounts of TDG in the pellet than compatible with the expected number of binding sites. It is realized that these criteria are not very stringent but the significance of our method of measuring binding sites is reinforced by the consistency of results reported below.

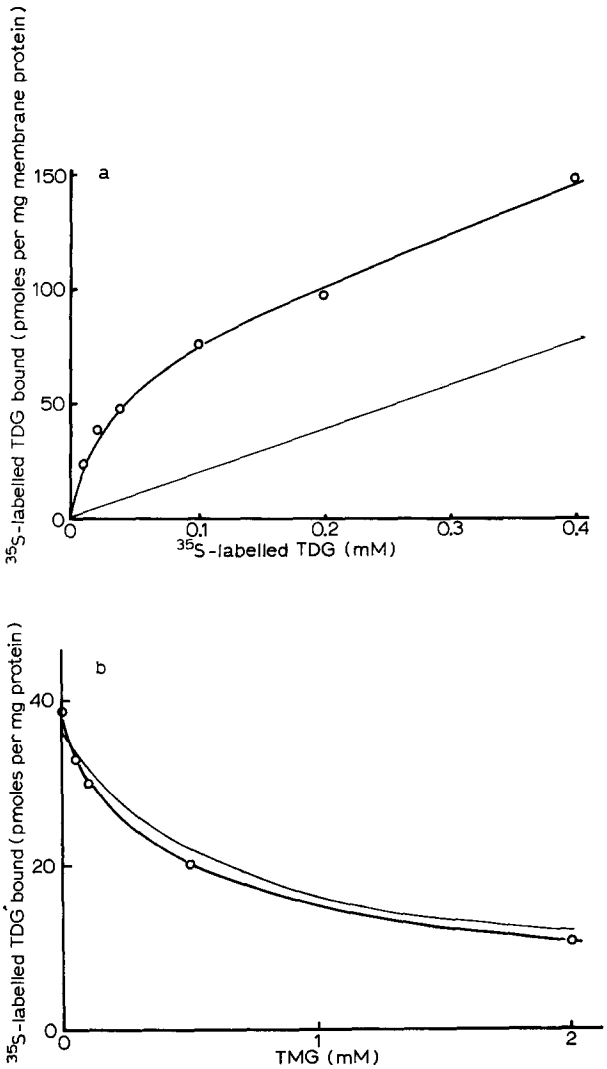

Fig. 2. (a) ^{35}S-labelled TDG bound to pellet *vs* ^{35}S-labelled TDG concentration. Membrane samples were incubated in 10 mM phosphate buffer (pH 6.8) containing 1 mM Mg^{2+} and the indicated concentration of ^{35}S-labelled TDG. The thin straight line is the estimated contamination. (b) Competition of increasing concentrations of TMG with the ^{35}S-labelled TDG binding. Experiments were carried out at pH 6.8 with 0.02 mM ^{35}S-labelled TDG and the indicated amounts of unlabelled TMG. The thin line is calculated on the basis of K_m values measured during *in vivo* active transport[11].

Measurement of transport

Accumulation of radioactive TDG, when it occurs, is measured in the same conditions as above. Fresh membranes often exhibit accumulation without any addition, and in this case the measurement of binding requires either low temperature (0–4 °C) or the presence of energy poisons, or both. Membranes, either fresh or conserved frozen, exhibit active transport of TDG when incubated at temperatures between 15 and 37 °C with ascorbate and PMS in the presence of oxygen. This is a check on the functional integrity of permease in our membrane preparations. Table II shows that the omission of any one of the substrates, ascorbate, PMS or oxygen, abolishes the active transport, but not the binding of TDG to permease sites.

It must be realized that during transport the measurements give the sum of TDG bound to the sites *plus* the TDG accumulated in the membrane vesicles and we have at present no means to measure these two amounts independently.

The binding phenomenon depends on the presence of permease

This is testified by (1) the absence of binding sites on membranes of the inducible strain *E. coli* 3000 when noninduced but their presence when induced (experiments not shown), (2) the substrate specificity as explored by competition with

TABLE IV

SENSITIVITY TO *N*-ETHYLMALEIMIDE AND PROTECTION BY AN EXCESS OF SUBSTRATE

The experiments were carried out either in the presence of 1 mM cyanide or in the presence of 1 mM cyanide, 20 mM ascorbate, 0.5 mM PMS, 10 min before various additions for pretreatment. *N*-Ethylmaleimide and nonradioactive TDG were used at 1 mM final concentration. After 15 min at room temperature, the pretreated membranes were filtered on 150 M agarose as described in Material and Methods. The ^{35}S-labelled TDG binding and transport were tested on the eluted membrane samples in the presence of 0.05 mM ^{35}S-labelled TDG (column 3). The decrease of the binding sites was measured after addition of 20 mM ascorbate, 0.5 mM PMS, 1 mM cyanide (column 4); and the transport measured 8 min after the addition of ascorbate PMS provided a vigorous agitation was maintained (column 5).

Pretreatment		*^{35}S-labelled TDG bound to the pellet (pmoles/mg protein)*		
Additions	*Ascorbate + PMS*	*Average of two samples*		*8 min after addition of ascorbate + PMS*
		Cyanide	*Ascorbate + PMS + cyanide*	
None	—	21.5	10.5	80
	+	21.5	13	110
N-Ethylmaleimide	—	4	4	9.5
	+	6	6	12
TDG	—	20	8	49
	+	16	8	85
TDG + *N*-ethylmaleimide	—	17.5	8.5	62.5
	+	6.5	6.5	48

a number of sugars (Table III and Fig. 2b), (3) the binding affinity of ^{35}S-labelled TDG which resembles closely the K_m of *in vivo* transport (Fig. 2a), (4) its sensitivity to thiol reagents and the protection provided by excess substrate against thiol inactivation (Table IV), (5) the number of sites detected which is of the right order of magnitude, although somewhat lower than expected from independent determinations based upon the comparison with gene copies of *z* (β-galactosidase) and *a* (transacetylase). The deficit might be due to losses during preparation, but also possibly to the masking of a part of the sites by some energy-coupling reaction similar to the one described below.

The binding phenomenon does not depend on transport

This is demonstrated by the finding of a constant amount of binding at high and low temperature and the insensitivity of the number of binding sites to respiratory inhibitors, uncouplers, or a combination of the two (Table V).

TABLE V

DECREASE IN BINDING SITES IN THE PRESENCE OF ASCORBATE+PMS AND VARIOUS INHIBITORS

The experiment was carried out with 10 mg/ml membrane protein, 10 mM phosphate buffer (pH 6 8) and 0.025 mM ^{35}S-labelled TDG.

Additions	*^{35}S-labelled TDG bound to the pellet (pmoles/mg protein)*	*^{35}S-labelled TDG bound to the pellet 10 min after addition of 20 mM ascorbate + 0.05 mM PMS*	*Known properties of the inhibitor**
None	18.0	93.5	
Cyanide, 1 mM	17.0	9.5	a** b
Azide, 40 mM	17.5	8.5	a d e f
2-Heptyl-4-hydroxy-quinoline-*N*-oxide, 0.1 mM	17.5	60.5	c
N, *N'*-Dicyclohexyl-carbodiimide, 0.03 mM	18.5	90.0	a f
2,4-Dinitrophenol, 3 mM	18.0	8.0	a d e
Carbonyl cyanide *m*-chloro-phenylhydrazone, 0.2 mM	15.5	7.0	a d e
Cyanide, 1 mM + azide, 40 mM	16.5	8.0	a b d e f

* a: inhibits active transport *in vivo* (aerobic glycerol medium). b: inhibits cytochrome oxidase. c: inhibits NADH oxidase and succinic oxidase. d: uncoupler. e: proton conductor. f: inhibits ATPase[14].

** According to Wong *et al.*[12] and unpublished experiments from this laboratory. Schairer and Haddock[13] report partial inhibition, but transport substrate was used at 0.02 K_m so that energy requirements were reduced.

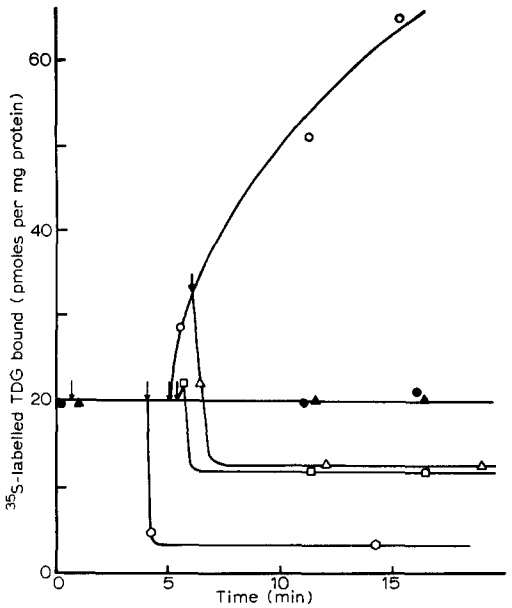

Fig. 3. Decrease in the number of ^{35}S-labelled TDG binding sites observed by addition of ascorbate and phenazine methosulfate in the presence of cyanide. The experiment was carried out at 15 °C with vigorous agitation in the presence of 0.05 mM ^{35}S-labelled TDG. Arrows indicate times of suitable additions. (a) ●—●, no addition; (b) ▲—▲, 1 mM cyanide added to (a); (c) (open hexagon), 1 mM nonradioactive TDG added to (a); (d) ○—○, 20 mM ascorbate and 0.5 mM PMS added to (a); (e) □—□, 20 mM ascorbate and 0.5 mM PMS added to (b); (f) △—△, 1 mM cyanide added to (d).

Reversible decrease in the number of binding sites

As shown on Fig. 3, addition of ascorbate and PMS to cyanide-inhibited membranes caused a clear decrease in the number of detected binding sites. The same final result was observed when cyanide was added to membranes which have accumulated TDG due to the presence of ascorbate and PMS. Fig. 4 shows that the presence of cyanide, ascorbate, PMS and oxygen are all three required to bring about the decrease of the detectable binding sites. The decrease in the presence of nitrogen was small and the decrease observed with oxygen was reversed when nitrogen was bubbled for 20 min (Fig. 4b). The decrease in detectable binding sites is therefore reversible. A second way to reverse the effect is by washing the membrane preparation by Sepharose gel filtration. Table IV, Line 2 shows this reversion, together with the decrease of TDG protection against *N*-ethylmaleimide inactivation, when the number of binding sites was decreased by ascorbate PMS respiration (Table IV, Line 8). This confirms previous observations *in vivo* of decreased substrate protection in energy-rich conditions[14].

Table V, second column, shows that uncoupling agents, azide, carbonyl cyanide *m*-chlorophenylhydrazone, 2,4-dinitrophenol, can replace cyanide in order to demonstrate a decrease in binding sites in the presence of ascorbate PMS.

Fig. 5 shows the pH dependence of the ascorbate PMS effect, and Fig. 6 shows the effect with various concentrations of TDG and of PMS. The dependence on

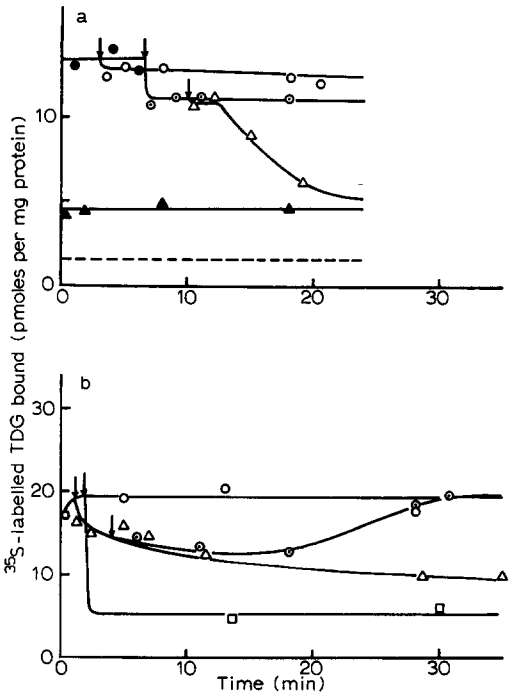

Fig. 4. Reversion of the decrease in the number of ^{35}S-labelled TDG binding sites obtained when using nitrogen. (a) Nitrogen was bubbled in the reaction mixture containing 1 mM cyanide and 10 mg/ml membrane protein in phosphate buffer at pH 6.8 for 10 min. At zero time, 0.05 mM ^{35}S-labelled TDG was added (●—●). A part of the mixture was transferred to oxygen (○—○); another part was mixed with 20 mM ascorbate and 0.5 mM PMS (⊙—⊙); a part of this last reaction mixture was submitted to vigorous aeration (△—△). In a parallel experiment, membranes were incubated in presence of 1 mM cyanide, 20 mM ascorbate, 0.5 mM PMS and vigorous agitation for 10 min before 0.05 mM ^{35}S-labelled TDG was added (▲—▲). The dotted line represents the background after chase with 1 mM TDG. (b) The control reaction mixture contained 10 mg/ml membrane protein in phosphate buffer, 40 mM azide, 0.05 mM ^{35}S-labelled TDG and oxygen (○—○); 20 mM ascorbate and 0.5 mM PMS added to control (△—△); the later mixture submitted to nitrogen bubbling (⊙—⊙); 1 mM nonradioactive TDG added to control (□—□).

ascorbate concentration was not obvious in the range 1–20 mM. The effect of PMS is roughly noncompetitive, although minor variations in apparent K_m values were observed. Moreover, as shown on Fig. 6b, PMS shows both a threshold and a saturation. At saturation, the binding sites were decreased only by 60–75%.

When ascorbate and PMS were present without respiratory inhibitor, active transport was limited in time, and when it stopped a final state corresponding to decreased binding sites was reached. Addition of ascorbate alone was sufficient to reinitiate active transport (Fig. 7).

DISCUSSION

There is at present no rational explanation why $(NH_4)_2SO_4$ precipitation can immobilize specific noncovalent protein–ligand complexes. There are a few empirical

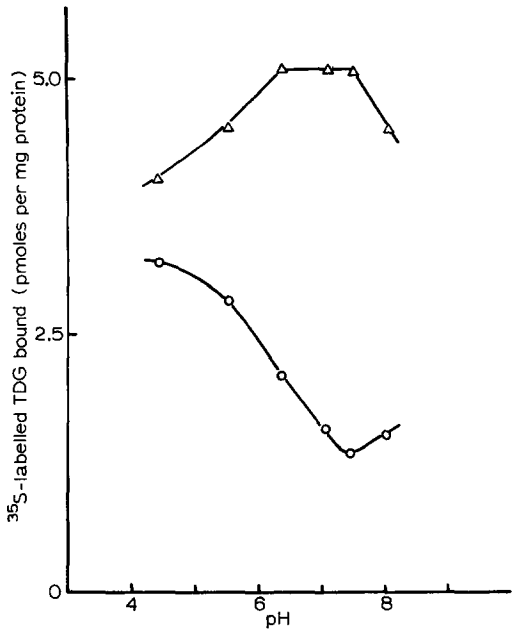

Fig. 5. pH dependence of the ascorbate PMS effect. The reaction mixture contained 10 mg/ml membrane protein in 100 mM potassium phosphate buffer at the suitable pH between pH 4.5 and pH 8, 1 mM Mg^{2+} and either 1 mM cyanide (△—△) or 1 mM cyanide, 20 mM ascorbate, 0.5 mM PMS (○—○) were added 10 min before addition of 0.01 mM ^{35}S-labelled TDG. Each point is the average of three samples taken at 2 min, 6 min and 10 min after addition of ^{35}S-labelled TDG. The background measured with 1 mM nonradioactive TDG was subtracted.

examples where such an effect was observed and fruitfully exploited; *lac*-repressor–inducer complex[10], CAP protein–3′,5′-cyclic AMP complex[11,16], galactose binding protein–galactose complex[17].

In the above instances the proteins were purified and independent methods, such as equilibrium dialysis, provided a check for the validity of $(NH_4)_2SO_4$ precipitation as a means to assess protein–ligand interactions.

Unfortunately, such is not the case with permease-containing membrane particles. These have to be complex in order to include the parts of all possible energy-generating mechanisms, respiration and oxidative phosphorylation. The production of closed vesicles, desirable if the effect of proton gradients has to be scrutinized, undesirable in view of the measurement of binding sites uncontaminated by a substrate pool, is actually beyond control. Should the experiments aim to detect substrate binding sites without regard to the energy-coupling reactions, it would be preferable to attempt solubilization and purification of the permease protein, but until now all attemps to solubilize lactose permease lead to a loss of detectable substrate binding.

Therefore only the self-consistency of the results obtained with a complex preparation can lend support to the validity of the $(NH_4)_2SO_4$ precipitation as a method to measure the substrate binding sites of lactose permease. In view of the reported results on specificity, concentration dependence, requirement for an essential

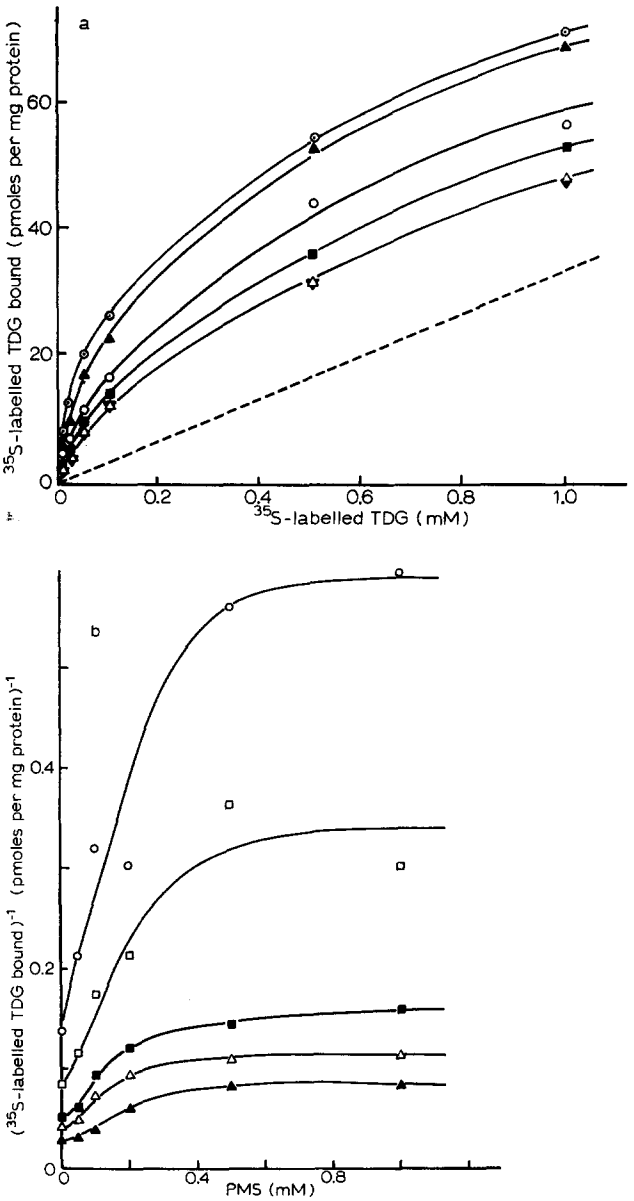

Fig. 6. Effect on TDG binding of ascorbate PMS with various concentrations of ^{35}S-labelled TDG and of PMS. The reaction mixture contained 10 mg/ml membrane protein, 100 mM phosphate buffer, pH 7.0, 20 mM ascorbate and 0–1 mM PMS 10 min before addition of 0.05–1 mM ^{35}S-labelled TDG. Each point is the average of two samples taken 2 min and 4 min after the addition of ^{35}S-labelled TDG. (a) [^{35}S]TDG retained in the $(NH_4)_2SO_4$ precipitate *versus* ^{35}S-labelled TDG concentration. PMS: ⊙—⊙, 0 mM; ▲—▲, 0.05 mM; ○—○, 0.1 mM; ■—■, 0.2 mM; △—△, 0.5 mM; ▼—▼, 1 mM. The presumed background is shown by the dotted line. (b) Dixon plot of the same data. The presumed background has been subtracted from each experimental value. ^{35}S-labelled TDG: ○—○, 0.01 mM; □—□, 0.02 mM; ■—■, 0.05 mM; △—△, 0.1 mM; ▲—▲, 0.5 mM.

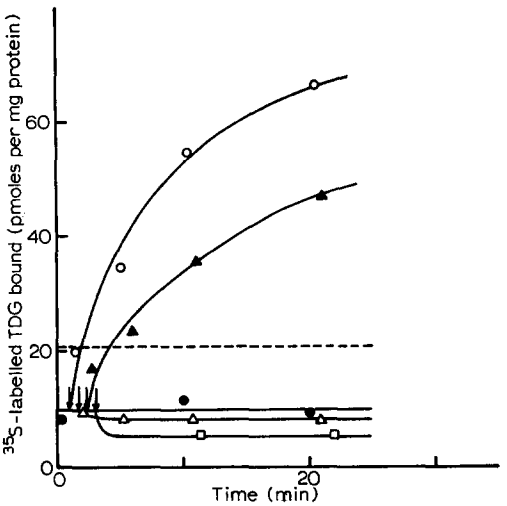

Fig. 7. Reinitiation of active transport by addition of ascorbate. The reaction mixture containing 10 mg/ml membrane protein in 10 mM phosphate buffer at pH 6.8; 20 mM ascorbate and 0.02 M PMS was maintained with vigorous agitation for 1 h. Then, 0.05 mM ^{35}S-labelled TDG was added (●—●). Further additions are indicated by arrows: ○—○, 20 mM ascorbate; △—△, 0.02 mM PMS; ▲—▲, 20 mM ascorbate and 0.02 mM PMS; □—□, 1 mM nonradioactive TDG. The amount of TDG bound in a control experiment without pretreatment is indicated by the dotted line.

thiol group protected by excess substrate, dependence on the expression of an intact *lac y* gene, a trivial physicochemical artefact can be ruled out. When pool formation can also be ruled out, *e.g.* in the presence of energy inhibitors used singly or in combination, at low temperature, the precipitated counts are, in all likelihood, substrate molecules bound to specific sites. If all bound substrate molecules are not detected, a part being lost during precipitation and washing, the proportionality factor in well-controlled conditions is sufficiently reproducible to provide a fair estimate of the binding sites and a decrease in precipitable radioactivity under the influence of energy-yielding reactions is more significant in terms of decrease in high affinity sites than an increase which would be suspect of pool formation.

The analysis of the experimental results in terms of energy coupling to the transport mechanism is still very difficult. The active transport implies a cyclic mechanism with at least two reactions, one making the binding sites unavailable for exit or masking reaction, and the other masking the sites available again for uptake, the unmasking reaction. Presumably, the observed proportion of available sites results from a steady state brought about by these two reactions. It seems unlikely that the permease with the masked site be simply the oxidized or the reduced form, since both oxidizing and reducing equivalents are necessary to bring about the effect.

Table VI shows the apparent lack of correlation between the energy-dependent masking of permease–substrate sites and the redox state of the bulk of the respiratory chain.

Therefore, it seems that an electron flux is the necessary condition of the masking reaction. But what is the nature of the electron flux in the presence of 1 mM

TABLE VI

THE ENERGY-DEPENDENT MASKING OF PERMEASE–SUBSTRATE SITES AND THE REDOX STATE OF THE BULK OF THE RESPIRATORY CHAIN

Addition	*Respiratory chain*	*Available sites*
Cyanide	Reduced	+
Oxygen	Oxidized	+
Nitrogen	Reduced	+
Cyanide + oxygen	Reduced	+
Ascorbate + PMS + oxygen (ascorbate exhausted)	Oxidized	Decreased
Ascorbate + PMS + nitrogen	Reduced	+
Ascorbate + PMS + oxygen + cyanide	Reduced	Decreased

cyanide? Either it is going through a pathway distinct from the major respiratory chain, or, if not, a very small fraction of the normal flux is sufficient to decrease the steady state of available sites. To elucidate this point, ascorbate PMS is certainly not the most favorable respiratory substrate since its autooxidation prevents measurement of very small electron fluxes. The hypothesis of requirement of a proton gradient for the masking reaction is weakened by the fact that uncoupling agents which are proton conductors still allow this step to occur in the presence of ascorbate PMS.

It is also difficult to understand why, when the masking reaction stops by exhaustion of ascorbate, the sites remain masked (respiratory chain presumably oxidized) whereas after removal of oxygen (respiratory chain reduced) they return to the high affinity state.

If ascorbate PMS promotes the site-masking reaction as an energy-yielding substrate, the most puzzling paradox is the impossibility to find an energy poison which can prevent this reaction.

For a better analysis of the system, methods should first be refined so as to be able to measure the rate of reaction rather than the final steady state. Also, means should be found to measure the rate of the unmasking reaction, which is at present impossible because the removal of oxygen and agarose wash, the only methods to observe this reaction, are slow and ill controlled. Finally, it would be desirable to freeze the system in either the energized or in the de-energized state, and find out if any covalent change occurred in the permease protein.

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