Direct Measurement of the Binding of Labeled Sugars
to the Lactose Permease M Protein*

(Received for publication, July 20, 1973)

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SUMMARY

Previous studies of the interaction of sugars with the membrane protein (M protein) component of the lactose permease system have employed an indirect procedure based on the protection of a reactive cysteine in the M protein against attack by labeled N-ethylmaleimide when certain sugars are bound. A method has now been devised for measuring the binding of labeled sugars to M protein directly, avoiding the use of N-ethylmaleimide. The particulate, membrane-containing fraction is equilibrated with 50 μM β-[3H]galactosyl-1-thio-D-β-galactoside (thiodigalactoside), in phosphate buffer containing 32P equal to the total count of 3H. The membrane fragments are then sedimented by high speed centrifugation, and, after careful removal of the supernatant (without washing), the pellet is resuspended in a detergent solution and counted. The absorption of thiodigalactoside to the membrane fragments is revealed by an increment in the ratio of 3H to 32P. Control experiments reveal that 32P equilibrates with at least 95% of the total aqueous compartment of the pellet. The increment in the ratio of 3H:32P is, therefore, not the result of the preferential filling of cell-free vesicles by the radioactive sugar. This binding reaction is not affected by energy poisons such as azide. Membranes from cells that have not been induced for the lac system and not affected by energy poisons such as azide. Membranes from cells that have not been induced for the lac system and thus contain no M protein have no specific binding sites for thiodigalactoside as defined by this assay.

The specific binding of thiodigalactoside involves a limited number of saturable binding sites. The value for half-saturation with thiodigalactoside is about 67 μM, in excellent agreement with previous studies of the affinity of the M protein for thiodigalactoside obtained with the indirect N-ethylmaleimide procedure. The site for binding thiodigalactoside (Site II) has also been found to have a surprising affinity for certain α-galactosides, such as p-nitrophenyl-α-galactoside, for which the $K_d$ is about 7 μM. Other properties of the system are described.

A procedure is described for the preparation and purification of tritiated thiodigalactoside.

Previous studies (1) of the interaction of sugars with the lactose permease M protein revealed that substrates for the permease could be classified into two distinct groups. Substrates of Class I, such as thiomethyl-β-galactoside, thiosopropyl-β-galactoside, and lactose itself, have little or no affinity for the site leading to the protection of a reactive cysteine of the M protein. Binding of substrates of Class II, notably thiodigalactoside (β-D-galactosyl-1-thio-D-β-galactoside) and melibiose, protects the reactive cysteine from attack by labeled N-ethylmaleimide. The kinetics of the interaction of sugars of Class II with the M protein can be measured by studying the kinetics of protection against attack by N-ethylmaleimide, but the procedure is necessarily indirect and laborious. A direct procedure for the measurement of the interaction of sugars with the lactose permease M protein would obviously be desirable.

There is now a copious literature on the binding of substrate by water-soluble "binding proteins" thought to be essential elements of various bacterial transport systems (2). In the assay of such binding proteins, the concentration of the protein must approach that of the substrate, if the bound substrate is to be a significant fraction of the total. Concentration of the binding Protein to the degree needed for the assay is usually easily achieved by a few steps of purification. However, proteins imbedded in the membrane structure cannot be subjected to such preliminary purification and concentration. We have now employed F'-lac strains of Escherichia coli, with copies of the lac genes on the episome as well as on the chromosome, in which the concentration of M protein in the membrane fragments is more than double that of wild type, and have measured the binding of sugar at the highest possible concentration of membrane fragments, that is, in the pellet obtained by high speed centrifugation after equilibration with 3H-labeled sugar. Since the binding of sugar is readily reversible, the pellet cannot be washed. The buffer in the system for equilibration with sugar is therefore labeled with 32P. The specific binding of sugar is revealed by an increment in the ratio of 3H:32P. Control experiments reveal that the 32P equilibrates with at least 95% of the total volume of the pellet; alteration of isotope ratio, therefore, cannot be the result of equilibration of the internal volume of cell-free vesicles with the sugar. Some aspects of this work have been briefly described in a review of the lactose permease system of E. coli (1).

MATERIALS AND METHODS

Preparation of Tritiated Thiodigalactoside—Since thiodigalactoside is a specific and useful substrate for the study of the lac system, the preparation of a suitable tritiated form has been described. The method involved the tritiation of a natural thiodigalactoside and isolation of the tritiated product by chromatography. The purified tritiated thiodigalactoside was then used in the binding assay described above.

* This work has been supported in part by Grant GM-13952 from the United States Public Health Service, National Institute of General Medical Sciences.

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permease, its preparation in labeled form, based on the enzymatic procedure of Avigad (3), will be briefly described.

Thiodigalactoside was enzymatically oxidized to the 6-aldehyde in an incubation mixture that contained sodium phosphate buffer of pH 7.5 (0.05 M), thiodigalactoside (final concentration 0.05 M), catalase (Worthington; 10 units), and galactose oxidase (Miles Laboratory, 250 units) in a final volume of 5.0 ml. The incubation mixture was stirred in a 40 ml polyethylene centrifuge tube with a magnetic stirrer in a bath at 28° for 24 hours. The reaction mixture was chilled in an ice bath in a well ventilated hood. Caution: tritium gas may be evolved in subsequent steps. Trifluoracetic acid (50 μmoles, containing 10 mCi of 3H) was added, dissolved in 0.5 ml of an ice-cold solution of 1 mM sodium hydroxide, and the reaction mixture was stirred for 90 min in the ice bath. Unlabeled sodium borohydride (19.7 mg dissolved in 1 ml of 1 mM NaOH) was then added, and stirring was continued at 0° for 60 min further. The reaction mixture was acidified to pH 2.8 by the slow addition of 2 ml of 1 M H3PO4. The acidified mixture was stirred at 0° for 15 min further and then neutralized with 4 ml of 1 M NaOH. Absolute ethanol was added to bring the ethanol concentration to 75%, and the mixture was allowed to warm to room temperature overnight in the hood. The magnetic stirrer was removed and the suspension centrifuged at 20,000 × g for 20 min at 0°. The precipitate was discarded. A sample of the supernatant was assayed for radioactivity and found to contain a total of about 1.9 × 10⁶ dpm.

The labeled thiogalactoside was purified by partition chromatography on Dowex 1 sulfate, in a procedure based on that of Samuelson and Swenson (5). Dowex 1 sulfate (8% cross-linked) was suspended in 75% ethanol (v/v) and made up into a column (1.2 × 46 cm). The column was washed with several bed volumes of 75% ethanol. A portion of the thiodigalactoside (25 ml) in 75% ethanol was then passed over the column. The column was then eluted with 75% ethanol at the rate of 15 ml per hour. Minor peaks amounting to 1 or 2% of the radioactivity emerged at 70 ml and 200 ml respectively. Nearly all of the radioactivity applied to the column emerged as a major fraction with a peak at 420 ml.

The fractions containing the radioactive thiodigalactoside were taken to dryness in a rotatory evaporator. A portion of the dried material was weighed, dissolved, and counted. It had a specific radioactivity of 6.7 × 10⁶ dpm per μmole, indicating that essentially all of the radioactivity of the crude product was present as labeled thiodigalactoside. The purity of the labeled product was tested by ascending paper chromatography in the system 2-propanol-0.1 M aqueous ammonium acetate (70:30, v/v). All of the radioactivity was recovered in a single spot with an Rf of 0.34, identical with that of authentic samples of thiodigalactoside. The purity of the labeled material was judged by this test to be greater than 99%.

Essentially similar procedures were used to prepare labeled p-nitrophenyl-a-galactoside.

**Bacterial Strains**—The following strains, all derivatives of K-12, were used in this study:

- A-324 pro lac i- z+ y+ P-
- A-3244 pro lac i- z+ y+P' pro lac i- z+ y+P' lac +
- A-3245 pro lac i- z+ y+P' pro lac i- z+ y+P' lac +

The following ML strains were also used:

- ML 306 lac i- z+ y+
- ML 308-225 lac i- z+ y+

The parent A-324 strain was originally a gift from Dr. S. Luria. All strains were grown on minimal medium supplemented with thiamine (and also with proline where needed). Glycerol (1%) was the carbon source. Inducible strains were induced with 0.5 mM isopropylthiogalactoside as indicated.

**Preparation of Cell-free Fractions**—Cells were harvested during logarithmic growth at a cell density of about 5 × 10⁸ per ml. The cells were chilled, harvested by centrifugation, and washed with Buffer I, containing 50 mM Tris chloride, pH 7.4, 1 mM magnesium sulfate, 0.1 mM EDTA, and 10 mM β-mercaptoethanol. The cells in a concentrated suspension (about 10¹⁰ per ml) were disrupted by intermittent sonic irradiation in a MSE sonicator in 5-ml portions in an ice bath.

The suspension was largely freed of unbroken cells by centrifugation at 7000 × g for 15 min at 0°. Surviving intact cells (less than one in 10⁸) were counted on nutrient agar plates.

In some experiments the cell-free, low speed supernatant fraction was used without further fractionation. In other experiments, the membrane-containing particulate fraction was obtained by further centrifugation at 40,000 × g for 60 min, washed in the centrifuge with Buffer I, and resuspended in Buffer II in a volume equal to that of the original cell-free supernatant.

The results with both types of preparation were essentially the same.

**Measurement of Binding of Radioactive Sugars to Membrane Particles**—Portions (0.8 ml) of the cell-free preparations were mixed with 0.2 ml of 0.5 mM potassium phosphate buffer, pH 7.0, containing 250 μM thiogalactoside (100 units per mg). The final concentration of phosphate was thus 0.1 M, and that of the labeled thiodigalactoside was 50 μM. The system also contained 32P such that the total count of 32P was about equal to that of 3H. Control vessels also contained 5 or 10 mM added unlabeled thiodigalactoside to displace the radioactive sugar from specific binding sites. After 15 to 30 min at 28°, the suspension in a total volume of 1 ml was centrifuged in 2-ml centrifuge tubes, 8 mm in diameter, at 28,000 × g for 1 hour at 0°. The supernatant was removed, and the inner surface of the tubes was carefully wiped without disturbing the pellet. The pellet, containing about 4.5 mg of protein, was taken up without washing in 0.8 ml of 5% solution of Triton X-100. Triplicate samples (0.20 ml) were mixed with 0.8 ml of water and were counted after the addition of 10 ml of Triton-toluene counting solution (6) in a Packard liquid scintillation spectrometer set for efficient discrimination between 3H and 32P.

**Other Materials**—Methyl-a-D-galactopyranoside was synthesized by the method of Frasn and Mills (7). Phenyl-a-L-galactoside was synthesized by the method of Clancy (8). Thigalactoside was purchased from Schwarz-Mann (Orangeburg, N.Y.) and p-nitrophenyl-a-galactoside from the Pierce Chemical Co. (Rockford, Ill.).

Other materials and methods have previously been described (9).

**EXPERIMENTAL RESULTS**

**Specificity of Binding Test**—The binding of radioactive thiodigalactoside to membrane fragments from induced and uninduced cells of strain A-3244 was tested in the experiment shown in Table I. The specific binding of thiodigalactoside to the membrane particles derived from the induced cells is shown by the strikingly higher ratio of 3H to 32P in the membrane preparation from such cells. In uninduced cells containing no M protein, the addition of an excess of unlabeled thiodigalactoside has no significant effect on the 3H:32P ratio.
The specific binding of thiodigalactoside to the membrane particles is not affected by 10 mM sodium azide (Table I) and thus is not the result of the energy-dependent concentration of the sugar in cell-free vesicles, as described by Kaback and his colleagues (10).

**Equilibrium of 32P with Membrane Fragments**—The possibility was considered that the binding of radioactive sugar in an experiment such as that of Table I might be the result of the equilibration of the sugar with the internal volume of cell-free vesicles (10). Such a process might occur only in induced cells and would not be expected to be blocked by azide. If, however, 32P also equilibrates with the membrane vesicles, no alteration in the 3H:32P ratio should occur as a result of the equilibration of radioactive sugar. This point was tested in experiments in which the amount of 32P per ml of water in the pellet was determined in a procedure similar to that of Table I. The water content of the pellet containing the membrane fragments was collected by high speed centrifugation as described under "Materials and Methods." Portions (0.8 ml) of these fractions, containing about 4.5 mg of membrane protein, were equilibrated with 50 mM labeled thiodigalactoside in 0.1 M phosphate buffer, pH 7.0, containing 32P about equal in total count to the total 3H. The pellet containing the membrane fragments was collected by high speed centrifugation as described under "Materials and Methods," and triplicate samples were assayed for 3H and 32P. The amounts of radioactive sugar bound to saturable sites were calculated from the difference in total tritium between the pellet in the experimental tube and, in the control to which 10 mM unlabeled thiodigalactoside had been added, corrected for differences in the volume of the pellets as measured by their content of 32P. The induced cells were grown in the presence of 0.5 mM thioisopropyl-β-galactoside, using the N-ethylmaleimide labeling method (9). The experiments were carried out with extracts of the constitutive strain A-3245, in a procedure similar to that of Table I, except that higher concentrations of labeled thiodigalactoside were used, and the time of incubation before centrifugation was varied as shown.

**Optimum pH for Binding**—Fig. 1 illustrates the effect of variation of the pH of the test system on the specific binding of radioactive thiodigalactoside. The optimum pH is about 6.0.

**Time Course of Binding**—The procedure employed to measure the binding of radioactive sugars to the cell-free particulate membrane fractions does not permit an analysis of the binding at short time intervals. However, it was essential to determine whether the system had attained equilibrium under the conditions chosen for the standard test. Results shown in Table II reveal that this is the case.

**Saturation of Binding Sites for Radioactive Thiodigalactoside**—When the concentration of radioactive thiodigalactoside in the test system was varied from 15 to 90 μM, the double-reciprocal plot of amount of radioactive sugar bound versus concentration in the test system revealed that there are a limited number of saturable sites for specific binding (Fig. 2). The system was half-saturated at 67 μM, in good agreement with the value (68 to 75 μM) obtained for the half-saturation of M protein with thiodigalactoside, using the N-ethylmaleimide labeling method (9).

The specific binding of thiodigalactoside to the membrane particles is not affected by 10 mM sodium azide (Table I) and thus is not the result of the energy-dependent concentration of the sugar in cell-free vesicles, as described by Kaback and his colleagues (10).

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### Table I

**Specific binding of labeled thiodigalactoside to particulate, membrane-containing fraction of strain A-3244**

| Experiment | Ratio of 3H:32P in pellet | Thiodigalactoside bound |
|------------|--------------------------|-------------------------|
| 1. Thiodi[3H]galactoside (50 μM) | 1.416 | 690 |
| 2. Thiodi[3H]galactoside (50 μM) + 0.01 M sodium azide | 1.408 | 695 |
| 3. Thiodi[3H]galactoside (50 μM) + 10 mM unlabeled thiodigalactoside | 0.924 | 0 |
| Experiment 2 (uninduced cells) | | |
| 1. Thiodi[3H]galactoside (50 μM) | 0.965 | 0 |
| 2. Thiodi[3H]galactoside (50 μM) + 10 mM unlabeled thiodigalactoside | 0.942 | 0 |

### Table II

**Time course of binding**

| Time (min) | Thiodigalactoside bound (pmoles) |
|-----------|----------------------------------|
| 1. 100 μM thiodi[3H]galactoside | 15 | 789 |
| 2. 200 μM thiodi[3H]galactoside | 15 | 922 |

**Optimum pH for Binding**—Fig. 1 illustrates the effect of variation of the pH of the test system on the specific binding of radioactive thiodigalactoside. The optimum pH is about 6.0.
FIG. 2. Saturability of binding sites for thiodigalactoside (TDG). The experiment was carried out with cell-free, low speed supernatant fraction from A-3245, essentially as described in the experiment of Table I, except that the concentration of labeled thiodigalactoside in the test system was varied from 15 pM to 90 pM as indicated. The radioactivity in the pellet at each concentration was compared with its control containing labeled thiodigalactoside at the same concentration plus 10 mM unlabeled thiodigalactoside. The reciprocal of the thiodigalactoside bound is plotted against the reciprocal of thiodigalactoside concentration in the test system.

TABLE III
Affinity of other sugars for thiodigalactoside-binding site

The experiment was carried out as described in Table I, using an extract of A-3245, except that 5 mM unlabeled thiodigalactoside was used in the control tube, and other sugars, also at 5 mM, were tested for their ability to displace thiodigalactoside from its specific binding site.

| Additions            | Thiodigalactoside bound (pmoles) |
|----------------------|----------------------------------|
| None                 | 400                              |
| 5 mM melibiose       | 19                               |
| 5 mM thiomethylgalactoside | 396                           |
| 5 mM thioisopropylgalactoside | 385                           |
| 5 mM a-methylglucoside | 410                            |
| 5 mM galactose       | 405                              |
| 5 mM glucose         | 410                              |

370 pmoles per mg of membrane protein from induced cells of strain A-3244. These values for the F'-lac strains, containing lac genes on the episome as well as the chromosome, are about three times higher than those obtained in an earlier study (9) with the N-ethylmaleimide technique, of the abundance of M protein in ML strains, containing lac genes on the chromosome only. When ML 308-225 was tested in an experiment similar to that of Table I, it was found to bind 114 pmoles of thiodigalactoside per mg of membrane protein, as compared with a value of 119 pmoles of M protein by the N-ethylmaleimide procedure (9).

Displacement of Thiodigalactoside by Other Sugars—If there are two distinct sites for the binding of sugars to the M protein, sugars of Class I should not displace radioactive thiodigalactoside from its binding site, while sugars of Class II should compete with the labeled thiodigalactoside for Site II and thus reduce the binding of the radioactive sugar. This prediction was tested in the experiment of Table III. Melibiose effectively displaced thiodigalactoside from its specific binding site. Thiomethyl-β-galactoside and thioisopropyl-β-galactoside had only slight effects, directly demonstrating that melibiose and thiodigalactoside are bound to a site for which these Class I sugars have little affinity.

Affinity of α-Galactosides for Site II—Fox discovered that p-nitrophenyl-α-galactoside is surprisingly effective in protecting the M protein against attack by N-ethylmaleimide. An equimolar concentration of p-nitrophenyl-α-galactoside greatly reduced the binding of thiodigalactoside (Table IV), indicating that the affinity of this α-galactoside for Site II is considerably greater than that of thiodigalactoside.

Labeled p-nitrophenyl-α-galactoside was prepared by the enzymatic procedure described for thiodigalactoside. Its specific binding was tested as a function of concentration in an experiment essentially similar to that of Fig. 2, in which 5 mM unlabeled thiodigalactoside blocked specific binding in the control. The binding sites were found to be half-saturated at 7 μM p-nitrophenyl-α-galactoside, about one-tenth the concentration found for thiodigalactoside.

The affinity of Site II for several α-galactosides is summarized in Table V. Since the sugars other than p-nitrophenyl-α-galactoside had little affinity, the values were used to estimate the degree to which each of the α-galactosides bound to Site II.

1 C. F. Fox, personal communication.
Inhibition of specific binding of thiodigalactoside (TDG) by detergent. The experiment was carried out essentially as in Table I, except that varying amounts of Triton X-100 were added as indicated.

toside were not available in labeled form, their affinity for Site II was estimated from their ability to compete with labeled thiodigalactoside in experiments similar to those of Table IV. An aromatic aglycone greatly increases the affinity of the sugar for Site II; the p-nitrophenyl derivative has an affinity almost 10 times higher than that of phenyl-α-galactoside. The p-nitrophenyl residue itself cannot be a determinant for specific binding since p-nitrophenyl-α-glucoside had no detectable effect in displacing radioactive thiodigalactoside from Site II.

Inhibition of Binding by Triton X-100—The effect of varying concentrations of the non-ionic detergent Triton X-100 on the specific binding of thiodigalactoside was measured in the experiment shown in Fig. 3. Even small amounts of the detergent markedly inhibit binding. Fifty per cent inhibition was observed at 0.2 mg per ml, less than is needed for extraction of significant amounts of protein.

Stability of Cell-free Preparations—Frozen cell-free extracts stored at -15°C slowly lost their specific binding capacity for thiodigalactoside over a period of weeks. Extracts heated at 55°C for 10 min retained 90% of the binding capacity. Heating at 65°C for the same time, however, almost completely abolished specific binding.

DISCUSSION

The present results offer direct confirmation of the finding that substrates for the lac permease system may be divided into two distinct classes on the basis of their affinity for a site (Site II) on the M protein that leads to the protection of a reactive cysteine. Surprisingly, it now appears that α-galactosides have a higher affinity for Site II than β-galactosides. Indeed, of a large number of β-galactosides tested, only thiodigalactoside (β-β-galactosyl-1-thio-β-D-galactoside) has an affinity for Site II comparable to its affinity for the permease system in vivo. This prompts the hypothesis that the transport of β-galactosides, such as lactose, may be regulated by the internal level of some α-galactoside. Accordingly, UDP-galactoside and galactose 1-phosphate were tested for their ability to displace labeled thiodigalactoside, but were found to have little or no affinity for Site II (data not shown).

The method of measuring binding of substrate to membrane fragments described here has already been applied by Hsu and Fox (11) and by Wong et al. (12) in studies of the lac system. The substitution of tritiated p-nitrophenyl-α-galactoside for thiodigalactoside increases the sensitivity and range of the assay considerably. In principle, the method should be generally useful for the study of binding of other substrates to membrane-localized receptors.

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J. Biol. Chem. 1974, 249:33-37.

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