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Location of Sulfate-binding Protein in *Salmonella typhimurium*

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A method is described for location of proteins in bacteria. It depends upon two techniques. One technique is the inactivation of the protein by a reagent which is incapable of penetrating the bacterial membrane (permeability barrier). Proteins inside this membrane cannot be inactivated unless the cells are disrupted; proteins on or outside the membrane can be inactivated. The second technique depends upon inactivation of the protein by specific antibody. Antibody should not penetrate the external bacterial wall, and therefore should only inactivate proteins that are on the wall surface. Thus, proteins can be localized inside the membrane, in the wall-membrane area, or outside the wall. One reagent developed for use with the first technique is diazo-7-amino-1,3-naphthalene-disulfonate. It inactivated β-galactoside transport, but not β-galactosidase of intact *Escherichia coli*. Similarly, it inactivated sulfate binding and transport but not uridine phosphorylase activity of *Salmonella typhimurium*. This indicates that the sulfate-binding protein is on or outside the cell membrane, and that uridine phosphorylase is inside the cell. The organic mercurial compounds used also showed that the sensitive parts of the sulfate and α-methyl-glucoside transport systems are less reactive than the sensitive part of the β-galactoside system. Antibody to the sulfate-binding protein inactivated the purified protein but did not inactivate this protein when intact bacteria were employed. Thus, it appears that the sulfate-binding protein does not protrude outside the cell wall. The conclusion that the binding protein is located in the wall-membrane region is supported by its release upon spheroplast formation or osmotic shock, and also by its ability to combine with sulfate in bacteria which cannot transport sulfate into the cell.

Transport proteins should be located on the outer surface of the permeability barrier. There they are accessible to the medium and yet are able to participate in transport through the barrier (membrane). The protein of β-galactoside transport (the M protein) is bound sufficiently firmly so that it is retained by isolated membrane preparations (6). Other transport proteins are released into solution by osmotic shock or by disruption of the bacteria (1, 12, 13); their location cannot be determined by cell fractionation. Release by osmotic shock suggests a surface location (7); but data in support of this hypothesis are meager, being based mainly on detection (by electron microscopy) at the surface of phosphate produced by the enzyme alkaline phosphatase (2, 7).

Cellular location of a soluble protein presents a general problem. An approach to this problem is suggested by the inactivation of transport by protein reagents (6, 15, 17). 4-Acetamido-4′-isothiocyanostilbene-2,2′-disulphonic acid (SITS) should be ideal for this purpose; it was designed to combine with proteins but not to penetrate animal cell membranes (11). Unfortunately, in our studies, this reagent did not inactivate the bacterial transport systems for β-galactosides or sulfate. Hence, another reagent with similar properties was devised, 7-diazonium-1,3-naphthylene disulfonate (diazo-NDS). With this reagent, one can determine whether a protein lies inside the cell membrane or whether it is exposed to the external environment. Diazonium compounds can combine with several amino acid residues, primarily histidine and tyrosine (8). The charged sulfonate groups appear to prevent penetration into the cell, as they do with SITS. The organic mercurials *p*-mercuric benzoate and *p*-mercuric phenylsulfonate were used similarly, as suggested by studies of glucose transport into red blood cells (17).

Antibodies are sufficiently large so that they should not be able to penetrate the cell wall; therefore, only proteins on the outside of the cell wall should react with them.

With combination of the two tests, inactiva-

tion of the protein by a reagent and inactivation by specific antibody, it is possible to determine whether a protein lies on the external surface of the cell, within the wall-membrane area, or inside the membrane. Experiments with these tests are described in this communication.

MATERIALS AND METHODS

Bacterial strains and media. *Escherichia coli* PR7 is a K-12 F^- mutant produced in this laboratory; it is inducible for β-galactosidase and constitutive for alkaline phosphatase. *Salmonella typhimurium cysAabc20* is a sulfate transport-negative mutant of the wild type (LT-2); *S-typhimurium cysCD519* is doubly defective in sulfate activation but is transport-positive (12). *E. coli* was grown on TG medium (5) supplemented with 20 μg of L-methionine per ml, 5 μg of thiamine per ml, 200 μg of casein hydrolysate per ml, and 0.5 mM isopropyl-β-galactoside (IPTG), with 20 mg of glycerol per ml as carbon source. *S. typhimurium* was grown on E salts-glucose with djenkolate as a sulfur source (3, 12) and with 0.5 mg of uridine per ml added when uridine phosphorylase was to be measured. All cultures were grown for 3 hr at 37 C with aeration by swirling, after a fivefold dilution of an overnight culture.

Chemicals. 7-Amino-1,3-naphthylene disulfonic acid (NDS) and sulfanilic acid were obtained from Eastman Organic Chemicals, Kingsport, Tenn.; ^{14}C-α-methyl glucoside, IPTG, and *o*-NO_2-phenyl-β galactoside (oNPG) were obtained from Calbiochem, Los Angeles, Calif.; ^{14}C-thiomethyl-β-galactoside (TMG) was obtained from New England Nuclear Corp., Boston, Mass.; *p*-Cl-mercuri-phenylsulfonic acid (MBS) and *p*-OH-mercuribenzoate (PMB) were obtained from Sigma Chemical Co. St. Louis, Mo.; SITS was obtained from Nutritional Biochemicals, Corp., Cleveland, Ohio.

Diazotization. A 55-mg amount of NDS was dissolved in 3.5 ml of water plus 0.05 ml of concentrated HCl and was cooled on ice-salt; then 0.25 ml of 0.5 M $NaNO_2$ solution was added at −3 C. After 30 min, 55 mg of urea was added to destroy excess nitrite. A 1-ml amount of the diazo-NDS was added to 40 mg of washed wet bacteria in 10 ml of 0.1 M tris(hydroxymethyl)aminomethane (Tris)-chloride buffer, *p*H 7.1. After incubation at room temperature (24 C) for the desired time, the cells were washed by centrifugation. [Bacteria broken by sonic oscillation (Branson Sonifier) or by grinding with two times their weight of levigated alumina were used directly.] Controls contained all components except NDS.

Assays. β-Galactosidase was assayed by oNPG hydrolysis by bacteria treated with toluene plus Sarkosyl (14). β-Galactoside and α-methylglucoside permease assays depended upon measurement of radioactive substrate uptake by intact bacteria. Bacteria were separated from their medium on an HA (Millipore Corp., Bedford, Mass.) filter (9, 14). Radioactive isotope retained was determined with a liquid scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.).

Sulfate transport was measured similarly with dilute bacterial suspensions; samples were taken before 40 sec in order to measure the maximal uptake. Bacteria were washed twice in E salts and were suspended at about 20 mg (wet wt)/ml in E salts plus 2 mg of glucose per ml and 30 μg of chloramphenicol per ml (EGC). Then, at room temperature, 0.1 ml of bacterial suspension was mixed with 0.45 ml of EGC and 0.25 ml of water; 0.3 ml of this preparation was added to 0.1 ml of 0.05 mM $Na_2{}^{35}SO_4$ containing about 10^6 counts/min. Samples (0.3 ml) were applied to HA filters (Millipore Corp.) that had been prewashed with 1 ml of 0.1 mM Na_2SO_4 plus 0.5 mg of bovine fibrinogen per ml in E salts and then with 5 ml of E salts. After the sample had been applied, the filter was washed with 2 ml of 12% sucrose in EGC at 0 C.

Sulfate binding was measured by the resin assay method (12). Uridine phosphorylase was measured by adding 0.05 ml of toluene to about 0.5 mg of bacteria in 0.85 ml of 0.1 M potassium phosphate buffer (*p*H 7.1), adding 0.1 ml of 10.0 mM uridine, and incubating at 37 C for 30 min. Then 0.03 ml of cold 60% $HClO_4$ was added to stop the reaction, and the precipitate was removed by centrifugation; the optical density, at 295 nm, of 0.2 ml of supernatant fluid plus 0.8 ml of 0.1 N NaOH was read. The control lacked uridine.

Penicillin spheroplasts were prepared (9) by incubating growing bacteria for 2.5 hr at 37 C in Penassay Broth (Difco) containing 20% sucrose, 2 mg of $MgCl_2$ per ml, and 1,000 units of penicillin G per ml. Spheroplast formation was confirmed by examination under the microscope and by optical density loss upon 10-fold dilution in water.

Lysozyme spheroplasts were prepared from resting bacteria grown in E salts, glucose, and djenkolate; the bacteria were first washed with 10.3% sucrose in 4 mM Tris-chloride (*p*H 7.4) and then were suspended in 10% of the original volume of this solution. To each milliliter, 0.06 ml of lysozyme (10 mg/ml) was added at 0 min, 0.1 ml of 0.8 M Tris (*p*H 8.1) was added at 25 min, and 0.1 ml of 10 mM EDTA was added at 26 min. At 41 min, the suspension was cooled on ice, and spheroplast formation was checked as above.

To prepare antibody, a 1:1 mixture of a solution containing 0.66 mg of sulfate binder protein in phosphate-buffered saline plus Freund's adjuvant was injected into the footpads of a rabbit once a week for 4 weeks. A subcutaneous booster injection of the same amount was given during the fifth week. A 2-ml amount of a 1:10 dilution of the serum almost completely precipitated 100 μg of binder protein.

For reaction with purified protein, 33 μg of purified binder was mixed with 0.45 ml of ten times diluted antiserum at 24 C. Binding was determined by resin assay after 5 min. For reaction with bacteria, approximately 10 mg (wet weight) of *S. typhimurium cysA-abc20* cells was mixed with 0.05 ml of antibody in a total volume of 0.66 ml. After 1 hr at 24 C, the cells were removed by centrifugation and were assayed for binding by the resin assay. To test the supernatant fluid for antibody, the amount of protein precipitate obtained after 1 day at 0 C, after addition of 33 μg of binder, was determined on the basis of both optical density at 280 nm and with the Folin reagent.

RESULTS

Effects of diazo-NDS on β-galactoside transport and hydrolysis. Intact *E. coli* or *S. typhimurium* cells stained red-brown when they were treated with diazo-NDS. The cells were broken by sonic oscillation or by grinding with alumina, and the pellets of membrane-wall material were separated from the soluble cytoplasm by low-speed centrifugation for 10 min at 10,000 × *g*. The suspended pellets were highly colored but the supernatant fractions were nearly colorless. Thus, the reaction occurred with bacterial surface material.

In *E. coli* cells disrupted by sonic treatment, β-galactosidase was inactivated by diazo-NDS (Table 1). The enzyme was not inactivated when intact bacteria were treated with the reagent. This is consistent with the internal location of the enzyme, which was demonstrated by the low activity of the enzyme in intact transport-negative mutants.

The β-galactoside permease was partly inactivated by treatment of intact cells with the reagent. Activity was demonstrated by two assays. The first was more reliable and was based on the uptake of radioactive TMG. The second assay depended upon the rate of oNPG hydrolysis by intact bacteria and was variable unless the bacteria were stored overnight after treatment with the reagent. Then about 50% loss of activity was observed, which is smaller than the 85% loss determined by radioactive uptake (Table 1). Inactivation of permease suggests a surface location of this enzyme; this is consistent with the membrane location of the galactoside permease M protein (6).

Effects of diazo-NDS on uridine phosphorylase and sulfate binding. Uridine phosphorylase of *S. typhimurium* was chosen as an example of an internal enzyme, since this organism is β-galactosidase-negative. Uridine phosphorylase is not released when *E. coli* cells are subjected to osmotic shock (7). The enzyme was not inactivated when intact bacteria were exposed to the reagent, although it was inactivated in broken-cell preparations (Table 2).

Sulfate transport was strongly inhibited (70%) by 5 mM diazo-NDS. Inactivation of the binder protein may be partly responsible for this finding. Purified binder (6 nmoles) was inactivated 50% by treatment with 180 nmoles of diazo-NDS for 30 min at room temperature. Binding by transport-negative intact cells was also strongly inhibited. Osmotic shock of bacteria treated with 10 to 20 mM diazo-NDS for 60 min released little active binding material. The optical densities of the osmotic-shocked control and treated cells were similar; the treated cells had a definite reddish color. When the bacteria were disrupted before treatment, binding was only moderately inhibited. Thus, the effects of diazo-NDS on sulfate binding and galactoside transport by *E. coli* are similar and suggest surface location of the systems.

TABLE 1. *Inhibition of β-galactoside permease and β-galactosidase*

Reagent	Length of treatment (min)	Activity (% of control)		
		Intact cells		Disrupted cells
		Permease	β-Galactosidase	(β-Galactosidase)
Diazo-NDS, 5 mM	10	11	91	52
	80	8	112	21
Diazo-sulfanilic, 2.5 mM	10		74	23
	60		47	11

TABLE 2. *Inhibition of sulfate binding and uridine phosphorylase*[a]

Reagent	Length of treatment	Activity (% of control)		
		Intact cells		Disrupted cells (uridine phosphorylase)
		Binding	Uridine phosphorylase	
	min			
Diazo-NDS, 5 mM	30	23	99	62
	60	22	89	34
Diazo-sulfanilic, 5 mM	30	27	43	30
	60		30	0

[a] *S. typhimurium cysCD519* was used in these experiments.

Other reagents. Inactivation with diazotized sulfanilic acid was used as a control for the action of diazo-NDS, since it reacts as does diazo-NDS. Diazo sulfanilic acid has no net charge, and thus should penetrate cells more readily than diazo-NDS. It inactivated internal enzymes to some extent (Tables 1 and 2); the difference between the reagents is attributed to the higher permeability of diazo sulfanilic acid.

The position of a protein with regard to the structure of a cell should also be indicated by its reaction with mercurials (17), since the more highly charged of these compounds should not penetrate bacteria readily. Two compounds were tested, MBS and PMB. The binder protein, which has no SH groups, was not much affected by either of these reagents, being at least 80% active in cells or in the free state after exposure to 1

mM reagent. In contrast, transport of sulfate was inhibited, a higher concentration (0.4 mM) of the sulfonate than of the benzoate (0.2 mM) being required (Fig. 1). (The greater transport at a low sulfonate concentration is probably an experimental error.) The benzoate appeared to penetrate more readily than the sulfonate, as was found with red blood cells (17), although a higher reactivity of the benzoate is not excluded. In comparison, TMG transport by *E. coli* was only about 7% active after exposure to only 0.1 mM of either reagent. α-Methyl-glucoside transport by *S. typhimurium* was about half as sensitive as was sulfate transport, being totally inactivated by 0.3 mM PMB, but retaining 70% of its activity after treatment with 0.4 mM MBS. These results suggest that the target of TMG transport, probably the M protein which is attached to the membrane, is the most exposed. A part of the sulfate system other than the binder and a sensitive part of the α-methylglucoside transport system are deeper or are in less reactive environments.

Experiments with antibodies. Antibinder antiserum precipitated the purified binder and also inactivated its binding ability (Table 3). The specificity of the reaction was indicated by gel diffusion of antiserum against purified binder; a single line was obtained.

Addition of antibinder antiserum to intact bacteria which contained about 40 μg of binder

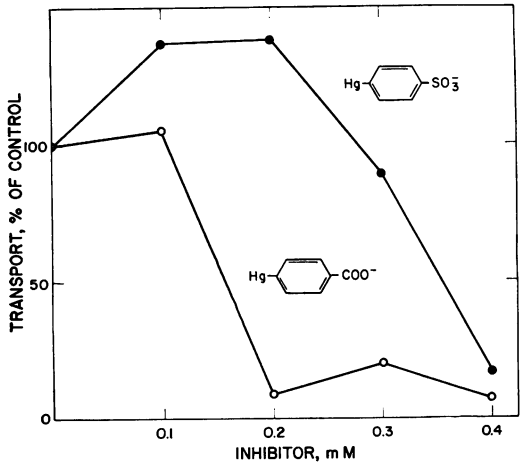

FIG. 1. *Inhibition of sulfate transport by mercurials. Mutant S. typhimurium cysCD519 grown on djenkolate was suspended at 9 mg (wet weight) per ml in E salts at room temperature, and the indicated concentration of either PMB or MBS was added for 30 min. The bacteria were washed by centrifugation at 0 C; then transport was measured. The data are averages of the values obtained at 15 and 30 sec.*

TABLE 3. *Reaction of binder with antibody*

Binder	Antibody	Binding	Visible precipitate
		counts/min	
Purified binder	−	135	−
Purified binder	+	28	+
No binder	+	20	−
Bacteria[a]	−	200	−
Bacteria[a]	+	250	−
No bacteria	+	3	−
Bacteria[b]	−	12	−
Bacteria[b]	+	10	−

[a] Grown on djenkolate.
[b] Grown on cysteine.

(*S. typhimurium cysAabc20* grown on djenkolate) did not clump the bacteria. In addition, the antibody was not removed from solution, as demonstrated by the undiminished ability of the supernatant fluid to precipitate purified binder. These bacteria were not stained by goat fluorescent anti-rabbit antibodies. Furthermore, binding activity of the bacteria was not inhibited. Pretreatment of the derepressed bacteria with EDTA-Tris (10) or with 2% formaldehyde did not permit reaction of binder and antibody, and the residual binding of lysozome spheroplasts was not inhibited. We did not observe any reaction of antiserum in the negative controls, the repressed bacteria grown on cysteine.

Effects of EDTA treatment and spheroplast formation on binding activity of cells. As reported previously (4), spheroplasts lose much of their sulfate transport activity. Spheroplasts made by either the penicillin or the lysozome procedure lost 60% of their binding activity. This activity appeared in the supernatant fluid (Table 4). Apparently, wall material, the structure of which becomes highly defective in spheroplasts, normally holds binder within the cell. Treatment with EDTA-Tris, which removes lipopolysaccharide (10), did not release the binder.

DISCUSSION

Although diazo-NDS was adequate for these experiments, it is not the ideal reagent for locating proteins. It inactivates only a limited fraction of enzymes or transport systems; it failed to inactivate alkaline phosphatase or the α-methyl glucoside transport system of *E. coli*. Other charged reagents that react with different protein groups should be useful for studies of enzyme location. The organic mercurial compounds that we tested and those tested with red blood cells (18) are an example of such reagents.

Reagents too bulky to penetrate the cell wall

TABLE 4. *Binding by spheroplast preparations*

Prepn	Counts/min	Percentage of original
I. Intact bacteria (*S. typhimurium cysAabc20*)	2,280	100
Penicillin spheroplasts, pellet	970	42
Lysed spheroplasts, membrane pellet	208	9
Lysed spheroplasts, supernatant fraction	424	18
II. Intact bacteria (*S. typhimurium cysAabc20*)	500	100
(a) Lysozyme spheroplasts, pellet	190	38
Lysozyme spheroplasts, supernatant fraction	680	136
Lysed spheroplasts, pellet	80	16
Lysed spheroplasts, supernatant fraction	150	30
(b) Lysozyme-Tris alone, pellet	400	80
Lysozyme-Tris alone, supernatant fraction	240	48
Lysozyme, lysed, pellet	220	24
Lysozyme, lysed, supernatant fraction	470	94
(c) EDTA-Tris alone, pellet	570	112
EDTA-Tris, alone, supernatant fraction	0	0
EDTA-Tris, lysed, pellet	770	154
EDTA-Tris, lysed, supernatant fraction	50	10

should also be useful for location. The wall serves as a barrier to medium-sized molecules, such as actinomycin D (10) or oligopeptides (J. W. Payne and C. Gilvarg, Federation Proc. p. 393, 1967), and should exclude antibodies which are much larger. The failure of antibinder antiserum to inactivate the binder in intact cells, as contrasted with its effectiveness against the purified binder, shows either that the bacteria shield their binder from antibody or that the binder is in an unreactive conformation in situ. In contrast, α-amylase produced by the mold *Aspergillus oryzae* reacts with its antibody at the cell surface (16), thereby showing that an exposed enzyme can react as anticipated.

The ability of transport-negative mutants such as *S. typhimurium cysAabc20* to bind sulfate (4) although they do not take it in is indicated by direct tests and by the failure of the organisms to grow at even high sulfate concentrations over long periods of time (12). Therefore, the binder must be on or outside the membrane, for the binding site must be external to the barrier which prevents sulfate from reaching activating and reducing systems for cysteine formation. The external location of the binding site is also indicated by the release of binder upon osmotic shock (12) and the formation of spheroplasts by two different techniques. These observations indicate a loose affinity of binder for the wall-membrane structure. We cannot locate it more precisely by these methods, since the structure of the wall-membrane complex of gram-negative bacteria is not well defined.

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