Monitoring Conformational Rearrangements in the Substrate-binding Site of a Membrane Transport Protein by Mass Spectrometry

Combined biochemical, biophysical, and crystallographic studies on the lactose permease of *Escherichia coli* suggest that Arg-144 (helix V) forms a salt bridge with Glu-126 (helix IV), which is broken during substrate binding, thereby permitting the guanidino group to form a bidentate H-bond with the C-4 and C-3 O atoms of the galactopyranosyl moiety and an H-bond with Glu-269 (helix VIII). To examine the relative interaction of Arg-144 with these two potential salt bridge partners (Glu-126 and Glu-269) in the absence of substrate, the covalent modification of the guanidino group was monitored with the Arg-specific reagent butane-2,3-dione using electrospray ionization mass spectrometry. In a functional background, the reactivity of Arg-144 with butane-2,3-dione is low (~25%) and is reduced by a factor of approximately 2 by preincubation with ligand. Interestingly, although replacement of Glu-126 with Ala results in a 3-fold increase in the reactivity of Arg-144, replacement of Glu-269 with Ala elicits virtually no effect. Taken together, these results suggest that in the absence of substrate the interaction between Arg-144 and Glu-126 is much stronger than the interaction with Glu-269, supporting the contention that sugar recognition leads to rearrangement of charge-paired residues essential for sugar binding.

The major facilitator superfamily (MFS) is one of the largest families of membrane transport proteins found in archaeal, bacterial, and eukaryotic cell membranes and contains over 1000 members, some of which are clinically relevant (e.g. the glucose transporters). As revealed by phylogenetic analysis, most members of this family have a similar topology consisting of 12 transmembrane helices connected by hydrophilic loops, with both the N and C termini on the cytoplasmic face of the membrane (4–7), and it is functionally and structurally a monomer (7–9).

LaC is selective for disaccharides containing a D-galactopyranosyl ring as well as D-galactose (10–12) but does not interact with D-glucopyranosides or D-glucose (12–14). Therefore, the substrate specificity of LaC is directed toward the galactopyranosyl ring of substrate and, most importantly, toward the C-4 OH of the galactopyranosyl ring. Galactose is the most specific substrate of LaC but has a very low affinity (K<sub>d</sub>app ~ 30 mM) (12), and substitutions at the anomeric position can markedly increase affinity up to 3 orders of magnitude by nonspecific interactions (15).

An important advance with regard to unraveling the mechanism of LaC has been the solution of the x-ray structure of the inward facing conformation of a LaC mutant (C154G) with a bound lactose homologue, β-β-D-galactopyranosyl-1-thio-β-D-galactopyranoside (TDG) (7). The overall structure reveals pseudo 2-fold symmetry between the N- and C-terminal 6-helix domains, as proposed for other members of the MFS (16). Remarkably, similar pseudo symmetry and helical packing were also found simultaneously in the x-ray structure of the P-glycerol-3-phosphate (GlpT) antiporter of *E. coli* (17), another MFS member. This structural similarity is even more interesting because the two proteins have only ~20% homology and catalyze completely different reactions. Interestingly, LaC and GlpT were crystallized in the same inward facing conformation, although the GlpT structure was obtained in the absence of bound ligand.

The sugar-binding site in LaC is located in a large water-filled cavity open only to the cytoplasm at the pseudo 2-fold axis of symmetry situated in the approximate middle of the membrane (Fig. 1A). Arg-144 (helix V) and Glu-126 (helix IV) are the major determinants for sugar binding (Fig. 1B). Arg-144 determines the stereoselectivity of LaC forming a bidentate H-bond with the O-3 and O-4 atoms of the galactopyranosyl ring, whereas Glu-126 probably interacts with O-4, O-5, or O-6 via water molecules. Interestingly, however, indirect biochemical approaches suggest the presence of a salt bridge between Glu-126 and Arg-144 when substrate is not bound (18–22). Furthermore, as predicted (23), the structure exhibits hydrophobic stacking between the galactopyranosyl ring of TDG and...
the indole side chain of Trp-151 (Fig. 1B). The nature of this interaction has been extended recently (24) by luminescence spectroscopy.

Glu-269 also plays an important role in the architecture of the binding site in the inward facing conformation (Fig. 1B), and functional characterization of Glu-269 mutants indicates that this may be a key residue in coupling between sugar binding and H^+ translocation (7, 19, 25–29). Thus, with the exception of aspartic acid, which demonstrates a dramatic alteration in the H^+/TDG stoichiometry (25), all other replacements for Glu-269 do not bind sugar and are completely defective in all modes of translocation (28). In the current structure, Glu-269 forms a salt bridge with Arg-144 and is situated in a position to form an H-bond with the indole nitrogen of Trp-151. Finally, studies combining chemical modification with mass spectrometry suggest an interaction between Glu-269 and the O-3 of p-nitrophenyl-a-d-galactopyranoside (NPGal), probably mediated by a water molecule (30). From the combined data, it seems clear that understanding the role of Glu-269 in the structural organization of the binding site is essential to understanding coupling between substrate and H^+ translocation.

In this paper, we present experiments that describe the interplay among Glu-126, Arg-144, and Glu-269 in sugar recognition. Experimentally, this is accomplished by engineering LacY to retain function and simultaneously produce a unique peptide containing Arg-144 on cleavage with cyanogen bromide (CNBr). Subsequently, the effects of substrate and mutations on the reactivity of Arg-144 can be probed using the Arg-specific reagent butane-2,3-dione (BD) (31) and monitored by ESI-MS. Consistent with the x-ray structure, substrate protects against BD modification of Arg-144. Moreover, when Glu-126, the putative charge partner of Arg-144, is replaced with Ala, the reactivity of Arg-144 with BD increases ~3-fold. Remarkably, replacement of another carboxyl group close to Arg-144 and within 5 Å of the substrate (E269A) elicits no significant effect on the reactivity of Arg-144. Taken together, the observations provide further evidence for a salt bridge between Glu-126 and Arg-144 that is broken during sugar binding, a process involving the rearrangement of interactions between essential residues.

EXPERIMENTAL PROCEDURES

Materials—TDG, NPGal, p-nitrophenyl-a-d-glucopyranoside (NPGal), diisopropylcarbodiimide (DiPC), and BD were purchased from Sigma. α-Dodecyl-β-D-maltopyranoside (DDM) was purchased from Calbiochem. Synthetic deoxyoligonucleotides were purchased from Sigma Genosys, and restriction endonucleases, T4 DNA ligase, and Vent DNA polymerase were from New England Biolabs (Beverly, MA). All other materials were reagent grade and obtained from commercial sources.

Construction of LacY Mutants—Two-step PCR mutagenesis of the genes encoding wild-type and C154G LacY (in plasmid pT7-5 bearing a PstI/SpeI insert), mutant C154G (6), and E269A/R135M/R142S/C154G (10), and E126A/E269A/R135M/R142S/C154G (11). Aliquots (50 μl) of cell suspensions containing 35 μg of protein in 100 mM potassium Pi (pH 7.5), 10 mM MgSO4, and adjusted to an optical density of 10.0 at 600 nm (0.7 mg of protein/ml). Transport was initiated by the addition of [1-14C]lactose (5 μCi/
mmol) to a final concentration of 0.4 mM. Samples were quenched at given times with 100 mM potassium Pi (pH 5.5), 100 mM LiCl and assayed by rapid filtration as described previously (32).

**Bacterial Strains, Growth, and LacY Purification**—*E. coli* T184 (lacZ/H11002Y/H11002) transformed with pT7-5 encoding LacY mutants with a His10 tag was grown in 6 liters of Luria-Bertani broth at 37 °C, respectively, containing ampicillin (100 μg/ml) to an A600 of 0.6 and induced with 0.5 mM isopropyl-1-thio-D-galactopyranoside. Cells were disrupted by

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**Fig. 3. Modification of Arg-144 by BD.** A, mechanism of Arg modification by BD is shown. Reaction of BD with the guanidino group (I) of Arg leads to the formation of a dihydroxyimidazoline intermediate (II), which can be dehydrated to form a new intermediate (III) that forms the stable product (IV) with no further change in mass. B, collision-activated dissociation of the engineered decapeptide 135SNFEFGSARM145 (I) and BD-modified 135SNFEFGSARM145 (II) is shown. Annotated peaks in the spectra include the doubly charged parents (m/z 549.2 and 592.2) and the respective b and y ion series. In II, BD is very unstable during collision-activated dissociation and is often lost to produce b-BD and y-BD series. C, time course is shown of 135SNFEFGSARM145 modification by BD in mutants R135M/R142S/C154G (1) and E126A/R135M/R142S/C154G (2). Purified LacY (~40 μM) in DDM (pH 8.0) was incubated with 20 mM BD at 30 °C, leading to a mass shift of 86 Da. The total ion current from peptide 135SNFEFGSARM145 modified by BD divided by the total ion current from unmodified and modified 135SNFEFGSARM145 was used to calculate the percentage of modification of Arg-144. No measurable difference in the ionization efficiency of the unmodified and modified peptides was detected. Each point represents the average of two independent experiments.
passage through a French pressure cell, and the membrane fraction was harvested by ultracentrifugation. Membranes were solubilized by DDM to a final concentration of 2%, and LacY was purified by cobalt affinity chromatography (Talon Superflow™, Palo Alto, CA) as described previously (33). One aliquot (100 μl) of the aqueous protein solution at a concentration of 1–2 mg/ml was diluted 1:3 (v/v) with MeOH and mixed briefly. CHCl₃ (100 μl) was added and mixed, yielding a single phase. Phase separation was accomplished by adding water (200 μl) and mixing vigorously. The phases were separated by centrifugation (10,000 g for 2 min), yielding a precipitate at the interface. The bulk of the upper aqueous methanol phase was then carefully aspirated, and methanol (300 μl) was added. After gently mixing, insoluble protein in the single phase mixture was recovered by centrifugation at 10,000 × g for 1 min. For cleavage of the protein, after drying, the pellet was resuspended in a saturated solution of CNBr in 90% formic acid (34) and left for 2 h in the dark. Formic acid was removed in a vacuum centrifuge, and the pellet was left overnight in 0.5 ml of 0.1% trifluoroacetic acid in water. The sample was dried again and resuspended in 60% formic acid immediately prior to RP-HPLC.

**HPLC**—For the final purification of LacY after covalent modification with DiPC or BD, HPLC was used prior to ESI-MS in an in-line setup. To separate the CNBr peptides generated from LacY, a polyethylene/divinylbenzene co-polymer column (5 μm, 300 Å, 150 × 2.1 mm) (PLRP/S, Polymer Labs) at 40 °C was used for reverse phase chromatography (35). Following equilibration in a mixture of 95% solvent A (0.1% trifluoroacetic acid in water) to 5% solvent B (0.1% trifluoroacetic acid in CH₃CN/isopropanol (1:1)) for 5 min, the percentage of solvent B was increased to 40% over the next 25 min and further to 100% over the subsequent 120 min at a flow rate of 0.1 ml/min. All chromatographic
separations were performed at 40 °C using a modified ABI 120A dual syringe pump HPLC equipped with a post-detector (A280) splitter for backpressure regulation.

Electrospray Ionization Mass Spectrometry—ESI-MS was performed using a PerkinElmer Life Sciences Sciex API III triple quadrupole instrument operating in the positive ion mode as described previously (36). The orifice potential was ramped from 60 to 120 V across the mass range (600–2300 a.m.u.) for CNBr fragments. Tandem mass spectrometry fragment ion spectra were obtained by splitting the flow into the API III during RP-HPLC and collecting peptides of interest for infusion (3 nl/min) into a ThermoFinnigan LCQ Deca ion trap instrument with a 33-gauge stainless steel needle source at 3.3 kV. The nomenclature used for fragment ions is N-terminal fragments (b type) and C-terminal fragments (y type) (37, 38).

RESULTS

Engineering LacY to Probe the Reactivity of Arg-144—The group-specific modification reagent BD reacts specifically with the guanidino group of Arg enabling the environment of Arg-144 to be probed in a functional background (see Fig. 3A). Following modification of LacY by BD and cleavage with CNBr, the peptides produced are resolved by RP-HPLC in-line with ESI-MS. There are 12 Arg residues in LacY, three of which are in the CNBr fragment containing Arg-144 (30), thus making quantification of Arg-144 modification challenging. Based on previous mutagenesis studies (39), LacY was engineered so that following cleavage with CNBr a small peptide containing only Arg-144 was produced by replacing two non-essential residues (R135M/R142S). To examine the environment of Arg-144 under conditions in which the predicted charge pair between Glu-126 and Arg-144 is broken by sugar binding, Glu-126 was replaced with Ala to generate mutant E126A/R135M/R142S. Likewise, to probe the effect of another substrate-binding residue (Glu-269) on the reactivity of Arg-144, Glu-269 was replaced with Ala to generate mutants E269A/R135M/R142S and E269A/E126A/R135M/R142S. Mutants R135M/R142S, E126A/R135M/R142S, E269A/R135M/R142S, and E269A/E126A/R135M/R142S were generated in the wild-type background (to assess transport activity of the mutants) and in the

![Graphical Abstract](image_url)
mutant C154G background (for comparison with the x-ray structure).

*E. coli* T184 (lacZ− Y−) expressing wild-type LacY catalyzes lactose accumulation at a high rate to a steady state of 150 nmol/mg of protein in 10 min, which corresponds to a 100-fold concentration gradient (Fig. 2A). Similarly, mutant R135M/R142S demonstrates a similar rate and steady-state level of lactose accumulation, although mutants E126A/R135M/R142S, E269A/R135M/R142S, and E269A/E126A/R135M/R142S do not significantly accumulate lactose to levels higher than T184 transformed with a vector lacking the LacY gene (Fig. 2A). Like mutant C154G (40), mutant R135M/R142S/C154G demonstrates a very low level of lactose accumulation and, as expected, mutants E126A/R135M/R142S/C154G, E269A/R135M/R142S/C154G, and E269A/E126A/R135M/R142S/C154G do not accumulate lactose (Fig. 2B). However, purified mutant R135M/R142S/C154G binds TDG, whereas purified mutants E126A/R135M/R142S/C154G, E269A/R135M/R142S/C154G, and E269A/E126A/R135M/R142S/C154G do not (data not shown).

CNBr cleavage of all R135M/R142S LacY constructs followed by RP-HPLC in-line with ESI-MS reveals a new peptide with a measured monoisotopic mass ([M+H]+) of 1097.2 compared with the expected monoisotopic mass 1097.5. Notably, most of the observed ions existed in a doubly charged state with an m/z of 549.2 compared with the expected 549.25. Collision-activated dissociation of the doubly charged parent ion at m/z 549.2 revealed fragment ions consistent with the sequence 135SNFEFGSARM145 (Fig. 3B, I). BD reacts with mutant R135M/R142S/C154G, and cleavage with CNBr reveals that approximately 20% of the peptide 135SNFEFGSARM145 is modified rapidly; however, the reaction is completed at ~25% mod-

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**Fig. 6.** BD modification of mutants E269A/R135M/R142S/C154G and E269A/E126A/R135M/R142S/C154G. Purified E269A/R135M/R142S/C154G LacY (~40 μg) in DDM (pH 8.0) was incubated for 30 min at 30 °C with 2% Me_SO (I) and 20 mM BD in the absence (II) or presence (III) of 20 mM NPGal. Similarly, purified E269A/E126A/R135M/R142S/C154G LacY (~40 μg) in DDM (pH 8.0) was incubated for 30 min at 30 °C with 2% Me_SO (IV) and 20 mM BD in the absence (V) or presence (VI) of NPGal. For clarity of presentation, the data are displayed as an integrated mass spectrum of scans 230–280 (23.6–28.7 min) containing the unmodified and BD-modified peptide 135SNFEFGSARM145 (m/z 549.22 and 592.22, respectively) in the m/z range between 500 and 800 Da. The mass spectra are representative of two independent experiments.

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**Fig. 7.** BD modification of mutants E269A/R135M/R142S/C154G and E126A/E269A/R135M/R142S/C154G. Time courses are shown of 135SNFEFGSARM145 modification by BD in mutants E269A/R135M/R142S/C154G (1) and E126A/E269A/R135M/R142S/C154G (2). Comparison is made with mutants R135M/R142S/C154G (3) and E126A/R135M/R142S/C154G (4) (see Fig. 3C) shown in dashed lines. Purified LacY (~40 μg) in DDM (pH 8.0) was incubated with 20 mM BD at 30 °C, leading to a mass shift of 86 Da. The total ion current from peptide 135SNFEFGSARM145 modified by BD divided by the total ion current from unmodified and modified 135SNFEFGSARM145 was used to calculate the percentage of modification of Arg-144. No measurable difference in the ionization efficiency of the unmodified and modified peptides was detected. Each point represents the average of two independent experiments.
loss of a charge pair between Arg-144 and Glu-269 in steps V
Modifications to the proposed mechanism (7) are: (i) introduction of a charge pair between Glu-126 and Arg-144 in step III
Hydrogen bonds are shown as filled lines
ligand NPGal reduces the rate of modification of the decapeptide 268GELLNASIM276 in 30 min (Fig. 5, compare C with D). Preincubation with NPGal has little effect on DiPC modification (Fig. 5, compare V and VI).

Influence of Position 269 on the Reactivity of Arg-144—The x-ray structure of LacY indicates that Arg-144 forms a salt bridge with Glu-269 in the presence of substrate. Therefore, the influence of Glu-269 on the reactivity of Arg-144 with BD was examined in the absence of substrate in mutants E269A/R135M/R142S/C154G and E269A/E126A/R135M/R142S/C154G.

Following reaction of mutant E269A/R135M/R142S/C154G with BD for 30 min, 33% of peptide 135SNFEFGSARM145 is modified (Fig. 6, compare I with II). Preincubation with the high affinity ligand NPGal has no significant effect on BD modification of the decapetide 135SNFEFGSARM145 is modified by BD to over twice the extent (data not shown). In mutant E126A/R135M/R142S/C154G, 268GELLNASIM276 is modified by DiPC at approximately the same rate (~30% in 30 min) (Fig. 5, compare IV with V); however, in this mutant, preincubation with NPGal has little effect on DiPC modification (Fig. 5, compare V and VI).

DISCUSSION
The x-ray structure of an inward facing conformation of LacY with bound substrate (7) provides a framework for understanding the molecular basis of substrate recognition and translocation by LacY, a paradigm for the MFS family of secondary membrane transport proteins. By integrating the structure with biochemical and biophysical analyses, a model for the mechanism for lactose/H+ symport has been proposed (7). Influx consists of six steps starting from the outward facing conformation as shown in Fig. 8: (I) protonation of LacY; (II) binding of lactose; (III) a conformational change that results in the inward facing conformation; (IV) release of substrate; (V) release of proton; and (VI) return to the outward facing conformation. Using the x-ray structure of the inward facing conformation with substrate bound as a structural foundation, mass spectral techniques have been exploited to gain detailed in-
sights into local alterations in structure induced by substrate binding.

One surprise from the x-ray structure is the absence of a salt bridge between Glu-126 and Arg-144. Previous functional studies demonstrate that the carboxyl and guanidino side chains at positions 126 and 144, respectively, are critical for substrate binding (19, 20) and translocation (41). Furthermore, close physical proximity of Glu-126 and Arg-144 has been inferred from site-directed spectroscopic studies with E126C/R144C LacY (42) and spontaneous disulfide formation (22). Finally, the reactivity of Cys-148 (helix V) is reduced dramatically by removal of the carboxyl group from position 126 (19). However, interpretation of all of these experiments must be placed within the context of either an inactive background (E126C/R144C) or changes in reactivity of an indirect reporter (Cys-148).

In this study, by exploiting chemical modification and the ability of HPLC in conjunction with mass spectrometry to solve complex mixtures, the environment of Arg-144 is analyzed directly on a functional background that permits analysis of substrate effects on native irreplaceable side chains. In a functional background, in which Arg-144 and Glu-126 are likely charge-paired, the Arg-specific reagent BD reacts with Arg-144. Furthermore, preincubation with a substrate analog reduces the rate of modification by a factor of two. However, when the charge pair is disrupted (E126A), the reactivity of Arg-144 with BD increases markedly. Two findings indicate that the interaction between Glu-126 and Arg-144 is being monitored primarily; (i) E126A causes a relatively large change that the interaction between Glu-126 and Arg-144 is being disrupted (E126A), the reactivity of Arg-144 decreases the rate of modification by a factor of two. However, removal of the carboxyl group from position 126 (19).

From a mechanistic perspective, one interesting feature of the x-ray structure (Fig. 1B) is the relative proximity of Arg-B and Glu-269. and (ii) E126A causes essentially no change in the reactivity of Glu-269.

To further explore the role of the Glu-126–Arg-144 salt bridge in substrate binding, we examined the effects of mutations at these positions. In a biological context, we chose to study mutants that disrupt the Glu-126–Arg-144 salt bridge because these mutations have been shown to affect substrate binding and/or translocation. Previous functional studies with E126C/R144C LacY have demonstrated that the Glu-126–Arg-144 salt bridge is critical for substrate binding and translocation.

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