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Publication Date
2012-01-01

Peer reviewed|Thesis/dissertation
Dynamics of the L-fucose/H$^+$ symporter revealed by fluorescence spectroscopy

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science in Integrative Biology and Physiology

by

Junichi Sugihara

2012
ABSTRACT OF THE THESIS

Dynamics of the L-fucose/H\(^+\) symporter revealed by fluorescence spectroscopy

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Master of Science in Integrative Biology and Physiology

University of California, Los Angeles, 2012

Professor Alan Grinnell, Co-Chair

Professor H. Ronald Kaback, Chair

The transmembrane protein FucP of *Escherichia coli* is a symporter that catalyzes the cotransport of L-fucose and H\(^+\) across cytoplasmic membrane, and a crystal structure in an outward-facing conformation has been reported. However, nothing is known about FucP conformational dynamics. Here, it is shown that addition of L-fucose to purified FucP in detergent induces ~20% quenching of tryptophan (Trp) fluorescence in a concentration-dependent manner without a shift in \(\lambda_{\text{max}}\). Quenching is...
essentially abolished when both Trp38 and Trp278, which are positioned on opposing faces of the outward-facing cavity walls, are replaced with tyrosine (Tyr) or phenylalanine (Phe), and reduced quenching is observed when either Trp is mutated. Therefore, both Trp residues are involved in the phenomenon. Furthermore, replacement of either Trp38 or Trp278, predominantly Trp38, causes decreased quenching, decreased apparent affinity for L-fucose and significant inhibition of active L-fucose transport, indicating that the two residues are likely involved directly in sugar binding. It is proposed that sugar binding induces a conformational change in which the outward-facing cavity in FucP closes, thereby bringing Trp38 and Trp278 into close proximity around the bound sugar to form an ‘occluded’ intermediate. The unique location of these two Trp residues provides a novel method for analyzing structural dynamics in FucP.
The thesis of Junichi Sugihara is approved.

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2012
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ACKNOWLEDGEMENTS

First and foremost, I would like to express my deepest gratitude to my mentor, Dr. H. Ronald Kaback for his support and guidance in the completion of this project. Dr. Kaback has been very helpful, patient and encouraging during my graduate studies at University of California, Los Angeles.

I would like to thank my committee members, Dr. Alan Grinnell and Dr. Gordon Fain, for being on the committee and for taking the time to read my project report. I would like to acknowledge Dr. Vladimir Kasho and Dr. Irina Kasho for their support and encouragement during my graduate studies.

I would also like to thank Dr. Nieng Yan and Linfeng Sun for providing a plasmid encoding FucP and for helpful discussion and Dr. M. Gregor Madej for his artistic input in preparing the figure of FucP crystal structure.

This work was supported by NIH Grants DK51131, DK069463, and GM074929 to Dr. H. Ronald Kaback. Also, this work has been submitted to Proceedings of the National Academy of Sciences of the United States of America and accepted for publication.
1. INTRODUCTION

L-Fucose (6-deoxy-L-galactose) is a naturally occurring methylpentose found in mammalian cells as a major component of N- and O-linked glycans and glycolipids (1, 2). In addition, L-fucose, which is present in bacterial polysaccharides, is needed for adhesion and localization and is also utilized by a variety of microorganisms as a carbon and energy source (3, 4). In *Escherichia coli*, transport of L-fucose is mediated by the transmembrane protein FucP, which is encoded by *fucP*, one of the genes of the *fuc* regulon that is responsible for the catabolism of L-fucose (5-7). FucP consists of 438 amino acids (47,773 Da) and is an L-fucose/H⁺ symporter belonging to the fucose-galactose-glucose/H⁺ symporter (FGHS) subfamily of the major facilitator superfamily (MFS) (8-10). FucP catalyzes symport (cotransport) of L-fucose and H⁺ across the cytoplasmic membrane by transducing free energy stored in the electrochemical H⁺ gradient into a substrate concentration gradient (5, 8, 9).

FucP shares limited sequence homology with other sugar/H⁺ symporters (9), but the x-ray crystal structure reveals many characteristics observed in other MFS transporters (11). Thus, FucP contains 12 transmembrane α-helical domains arranged into two pseudo-symmetrical 6-helix bundles surrounding a deep water-filled cavity with the N and C termini on the cytoplasmic side of the membrane. However, unlike other MFS transporters, FucP is in an outward-facing conformation; the cavity is open on the periplasmic side and tightly sealed on the cytoplasmic side. Although it is likely that FucP functions by an alternating access mechanism like the lactose permease of *E. coli* (LacY) (reviewed in 12), nothing is known about its functional dynamics.
Out of a total of six Trp residues, FucP contains two---Trp38 (helix I) and Trp278 (helix VII)---that are on opposing walls of the open periplasmic cavity ~16 Å across from each other (Fig. 1). If FucP functions by an alternating access mechanism, sugar binding might cause closure of the periplasmic cavity. In order to test this notion, Trp fluorescence of purified FucP in the detergent dodecyl-β-D-maltopyranoside (DDM) was studied. Remarkably, binding of L-fucose and D-arabinose, which are substrates for FucP, induces ~20% quenching of Trp fluorescence, and it is demonstrated that these two Trp residues are responsible for the effect. Moreover, evidence is presented indicating that Trp38 (helix I) and Trp278 (helix VII) play a role in the transport mechanism, possibly as components of the sugar-binding site.

**Figure 1. X-ray crystal structure of FucP.** The two Trp residues on the surface of cavity walls, W38 (helix I) and W278 (helix VII), are shown as pink spheres, and the distance between them is approximately 16 Å. The other 4 Trp residues are shown as blue spheres. Two acidic residues, D46 (helix I) and E135 (helix IV), are shown as green spheres. Helices I and VII are shown in gold, and water-accessible surface of cavity is shown in light blue. The figure was prepared using UCSF Chimera and the water-accessible surface was calculated using CASTp webtool with a probe size of 1.4 Å.
2. MATERIALS AND METHODS

Materials. L-Fucose, D-arabinose, and D-galactose were obtained from Sigma-Aldrich (St. Louis, MO). L-Arabinose was from Gold Biotechnology (St. Louis, MO). L-[5,6-3H]Fucose was from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Restriction enzymes NotI and HindIII and T4 DNA ligase were from Fermentas (Glen Burnie, MD). All nucleotides were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). QuickChange II kits were obtained from Stratagene (La Jolla, CA), and DNA plasmid purification kits and the penta-His antibody–horseradish peroxidase (HRP) conjugate were from Qiagen (Valencia, CA). Supersignal West Pico Chemiluminescent substrate kit was from Pierce Inc. (Rockford, IL). Dodecyl-β-D-maltopyranoside (DDM) was obtained from Anatrace (Maumee, OH) and Talon superflow resin was from BD Clontech (Palo Alto, CA). All other materials were of reagent grade obtained from commercial sources.

Plasmid Construction. The fucP gene encoding wild-type FucP was isolated from pET21b-FucP using Ncol and HindIII restriction sites introduced by PCR at the 5’ and 3’ ends respectively. The isolated fucP gene fragment was subcloned into the expression vector pBAD using T4 DNA ligase. The codon for Thr304 in FucP was modified from ACC to ACG in order to delete a native Ncol restriction site at this position.

FucP mutants were constructed by site-specific mutagenesis using a QuickChange II site-directed mutagenesis kit and plasmid pBAD/FucP wild-type (WT) as a template. Thirty- to 40-bp direct and reverse primers bearing the mutated triplet in the middle of the primer were designed using the Vector NTI 10 Suit program (Invitrogen, Carlsbad, CA). Mutagenic constructs were sequenced over the entire gene and through
the restriction sites to confirm the mutations introduced and to discard unwanted mutations. All constructs were engineered with a C-terminal His-tag to enable protein purification by metal affinity chromatography.

**FucP Purification.** FucP wild-type and given mutants were purified from *E. coli* XL1-Blue cells (Stratagene, La Jolla, CA) transformed with pBAD plasmids harboring appropriate genes by using Co(II) affinity chromatography as described (13) with the following modifications. Overexpression of WT FucP and mutants was induced with 0.02% L-arabinose. The urea wash step in the protocol was omitted. Proteins eluted from the Co(II)-Talon column were concentrated and washed with 50 mM sodium phosphate (NaPi; pH 7.5)/0.01% DDM on an Amicon Ultra-15 concentrator with a 30K cut-off (Millipore, Billerica, MA). All protein preparations were at least 95% pure as judged by silver staining after SDS polyacrylamide gel electrophoresis. Samples with protein concentrations at 5–10 mg/ml in 50 mM NaPi buffer (pH 7.5)/0.01% DDM were frozen in liquid nitrogen and stored at -80°C.

**Fluorescence Measurements.** Steady-state fluorescence was monitored at room temperature using SLM-Aminco 8100 spectrofluorimeter (Urbana, IL) modified by OLIS, Inc. (Bogart, GA) as described (14) with excitation and emission wavelengths at 295 nm and 310-400 nm, respectively. The purified proteins used for fluorescence measurements were adjusted to a concentration at which an approximately equal level of Trp fluorescence was observed in the absence of sugar (approximately 0.3 µM, final concentration). Titrations were recorded after sequential addition of a given sugar. Titration data were corrected for dilution. Data fitting was carried out by using SigmaPlot 10 (Systat Software Inc., Richmond, CA).
**Transport Assays.** *E. coli* T184 [lacI\(^{-}\)O\(^{+}\)Z\(^{-}\)Y\(^{+}\)(A), rspL, met\(^{-}\), thr\(^{-}\), recA, hsdM, hsdR/F\(^{-}\) lacI\(^{q}\)OZ\(^{D118}\)(Y\(^{+}\)A\(^{+}\))] was transformed with the appropriate plasmids and grown aerobically overnight at 37 °C in Luria-Bertani culture medium containing 100 μg/ml of ampicillin. A ten-fold dilution of the culture was grown for 2 h before induction with 0.02% L-arabinose. Following induction, growth was continued for further 2 h, after which the cells were harvested by centrifugation, washed with 100 mM potassium phosphate (KP\(_{i}\); pH 7.0) and 10 mM MgSO\(_{4}\), and adjusted to an absorbance at 420 nm (A\(_{420}\)) of 15 (approximately 1.0 mg/ml of protein) for transport measurements. Transport was carried out with L-[5,6-\(^{3}\)H]fucose (10 mCi/mm) at a final concentration of 40 μM at room temperature. Transport was initiated by addition of 2 μl radiolabeled L-fucose to 50 μl aliquots of cells containing 50 μg of total protein and terminated by dilution followed by rapid filtration (15). WT FucP and the mutants were expressed to similar levels in the membrane of *E. coli* as detected by using penta-His HRP-conjugated antibody and Supersignal West Pico Chemiluminescent substrate.
3. RESULTS

3.1 Trp fluorescence

Emission spectra of purified WT FucP with excitation at 295 nm in the absence or presence of different sugars are shown in Fig. 2. Addition of L-fucose clearly induces quenching without shifting the $\lambda_{\text{max}}$ at 327 nm, and the magnitude of the effect is dependent on the L-fucose concentration (Fig. 2A). Trp quenching is also observed upon the addition of D-arabinose, a substrate for FucP, although the effect of sugar concentration is less dramatic (Figure 2B). In contrast, D-galactose, L-arabinose, sucrose and lactose exhibit a relatively small, non-specific effect (Fig. 2C & D; Fig. 3). The results are consistent with the substrate specificity of FucP (5, 9, 11).

Figure 2. Fluorescence emission of WT FucP. Emission spectra of purified, detergent-solubilized WT FucP was measured in the absence or presence of L-fucose (A), D-arabinose (B), D-galactose (C), and L-arabinose (D) at different concentrations (beginning at the top of the traces: 0, 0.5, 1, 2, 3, 5, 10, and 15 mM final concentration) as described in Materials and methods. The $\lambda_{\text{exc}}$ was 295 nm.
3.2 Sugar affinities

The apparent affinity of FucP for substrate (\(K_{d}^{\text{app}}\)) was determined from the concentration of sugar that produces half-maximum quenching of Trp fluorescence (Fig. 3). WT FucP exhibits a \(K_{d}^{\text{app}}\) of 0.20 ± 0.01 mM for L-fucose, close to the \(K_{d}\) obtained by isothermal calorimetry (11), and a \(K_{d}^{\text{app}}\) of 1.85 ± 0.27 mM for D-arabinose. In contrast, the ~5% quenching observed with L-arabinose, D-galactose, sucrose and lactose is clearly non-specific, as these sugars are not substrates for FucP.

![Figure 3. Trp quenching with different sugars.](image)

**Figure 3. Trp quenching with different sugars.** Changes in Trp fluorescence (\(\lambda_{\text{max}}\) at 327 nm) in response to addition of L-fucose (●), D-arabinose (▲), D-galactose (▼), L-arabinose (■), sucrose or lactose (○) are plotted as a function of sugar concentration. The curves represent the average of two independent measurements. The \(K_{d}^{\text{app}}\) values of WT FucP for L-fucose and D-arabinose observed in this experiment are 0.20 ± 0.01 and 1.85 ± 0.27 mM respectively. The change in Trp fluorescence observed in the presence of D-galactose, L-arabinose, sucrose and lactose is due to a nonspecific effect of these sugars, which are not substrates for FucP.
3.3 Role of Trp38 (helix I) and Trp278 (helix VII) in Quenching

FucP contains six Trp residues, and in order to identify which are involved in sugar-induced quenching, the two located on the walls of the outward-facing cavity--Trp38 and Trp278--were replaced with Tyr or Phe by site-directed mutagenesis, and the effect of L-fucose on quenching was monitored (Fig. 4). As described above, quenching induced by L-fucose is dramatic with WT FucP ($K_{d}\text{app}$ of 0.20 ± 0.01 mM), but markedly reduced with mutant W38Y, which manifests a $K_{d}\text{app}$ of 6.2 ± 1.1 mM. With mutant W38F, quenching is almost abrogated, but a $K_{d}\text{app}$ of 15 ± 4.5 mM can be approximated (i.e., the mutant binds L-fucose with very low affinity). Trp quenching is less severely reduced with mutant W278Y, which exhibits a $K_{d}\text{app}$ of 4.7 ± 0.8 mM, and with W278F a $K_{d}\text{app}$ of 0.31 ± 0.04 mM is obtained, which is comparable to WT FucP. Thus, Trp38 is clearly the major player in the quenching phenomenon. In any case, when Trp38 and Trp278 are both replaced with Tyr or Phe, almost no significant quenching is induced by L-fucose. The results demonstrate that both Trp residues--but predominantly Trp38--are involved in sugar-induced quenching, while the remaining four Trp residues in FucP play little or no part in the phenomenon. It is also notable that each of the mutants shows a marked decrease in apparent affinity for L-fucose.
Figure 4. Trp quenching by Trp38 and Trp278 mutants. Trp fluorescence of purified Trp38 or Trp278 mutants was measured at a $\lambda_{em}$ of 327 nm in the absence or presence of L-fucose at given concentrations as in Fig. 3 and plotted after correction for fluorescence quenching for the non-specific quenching by L-arabinose. The curves represent the average of two independent measurements. (○) WT; (▲) W38Y; (▼) W38F; (■) W278Y; (◆) W278F; (▲) W38YW278Y; (▽) W38F/W278F.

3.4 Active Transport (Uphill L-Fucose/H$^+$ Symport)

*E. coli* T184 expressing WT FucP catalyzes uphill L-fucose/H$^+$ at a rapid rate for ~20 s to a steady-state level of ~7 nmol/mg of protein within 30 s (Fig. 5). In contrast, mutants W38Y, W38F and W38I exhibit decreased rates of L-fucose transport to steady-state levels of 20-50% of WT (Fig. 5A). Mutant W278I transports ~70% as well as WT, while mutants W278Y and W278F transport L-fucose at reduced rates to essentially the same steady state as WT (Fig. 5B). Finally, replacing both Trp residues at position 38 and 278 with Tyr, Phe or Ile appears to approximate the averaged activity of the individual Trp38 and Trp278 mutants (Fig. 5C).
Figure 5. L-Fucose transport. T184 cells transformed with plasmid encoding WT FucP or mutants were grown and assayed as described in Materials and Methods. (A) Time courses of accumulation of 40 μM L-fucose by cells expressing WT (●), W38Y (▲), W38F (▼), W38I (■), or no permease (○). (B) Time courses of accumulation of 40 μM L-fucose by cells expressing WT (●), W278Y (▲), W278F (▼), W278I (■), or no permease (○). (C) Time courses of accumulation of 40 μM L-fucose by cells expressing WT (●), W38Y/W278Y (▲), W38F/W278F (▼), W38I/W278I (■), or no permease (○).

3.5 Asp46 and Glu135

Based on mutagenesis studies, Dang et al. (11) suggested that both Asp46 and Glu135 are involved in H⁺ translocation. Therefore, the effect of mutations at these positions on Trp quenching was tested (Fig. 6). Since Glu135 mutants do not bind L-fucose (11), it is not surprising that mutants E135Q or E135D exhibit little or no L-fucose-induced quenching. In contrast, Asp46 is apparently an ‘uncoupled’ mutant that binds L-fucose well and catalyzes counterflow, but is unable to catalyze uphill L-fucose/H⁺
symport. Mutant D46E quenches Trp fluorescence like WT with a $K_d^{\text{app}}$ of $0.50 \pm 0.05$ mM (i.e., ~twice the $K_d^{\text{app}}$ of WT) and mutant D46N is somewhat less effective, but has a $K_d^{\text{app}}$ of $0.43 \pm 0.06$ mM, close to the $K_d^{\text{app}}$ of WT FucP. Clearly, Asp46 is not involved in the conformational change that leads to quenching of Trp fluorescence.

![Trp quenching by Asp46 or Glu135 mutants](image)

**Figure 6. Trp quenching by Asp46 or Glu135 mutants.** Trp fluorescence of purified Asp46 or Glu135 mutants was measured at a $\lambda_{\text{em}}$ of 327 nm in the absence or presence of L-fucose at given concentrations as in Fig. 3. The curves represent the average of two independent measurements. (●) WT; (▲) D46E; (▼) D46N; (■) E135D; (♦) E135Q. The $K_d^{\text{app}}$ values for D46E and D46N are $0.50 \pm 0.05$ or $0.43 \pm 0.06$ mM, respectively. Titration of Trp fluorescence of WT FucP with L-arabinose (○) is shown as a control.
4. DISCUSSION

According to the alternating access model of transport, studied most extensively with LacY (reviewed in 12), co-substrate-binding sites for sugar and H\(^+\) in the middle of FucP become exposed alternatively to either side of the membrane due to reciprocal closing and opening of periplasmic and cytoplasmic cavities, respectively. The x-ray crystal structures of LacY (16-19) exhibit a tightly closed periplasmic aspect with a cavernous, cytoplasmic opening, and site-directed alkylation studies indicate that the protein has a similar structure in the membrane (20, 21). In contrast, the X-ray structure of FucP reveals the opposite—a wide open periplasmic side with a tightly sealed cytoplasmic face (11). However, little or nothing is known about the structure of FucP in the membrane or about its dynamics.

In polytopic membrane protein structures, Trp residues are preferentially localized at interfacial regions due to the favorable interaction of aromatic groups with the bilayer interface (22). But less frequently, Trp residues are found in the interior of membrane proteins near carbohydrate binding sites, for example, where they interact hydrophobically with sugar substrates (see 14, 23, 24). Fluorescence spectroscopy of endogenous Trp residues located in a substrate translocation pathway may be useful for studying structural dynamics since the indole rings of Trp residues can be utilized as an intrinsic fluorophore. The amino acid sequence of FucP contains six Trp residues, two of which (Trp38 and Trp278) are located on opposing faces of the outward-facing hydrophilic cavity (Fig. 1). In this study, it is demonstrated that the fluorescence of these two Trp residues is quenched specifically upon binding of L-fucose or D-arabinose, known substrates of this symporter.
The emission spectrum of purified wild-type FucP exhibits significant quenching of Trp fluorescence upon addition of L-fucose in a concentration-dependent manner with no change in $\lambda_{\text{max}}$ (Fig. 2). Therefore, it is unlikely that the quenching phenomenon observed is due to a change in the local environment of the Trp residues. The phenomenon also mirrors the substrate specificity of FucP since L-fucose and D-arabinose, sugars known to be transport substrates of FucP (5, 9, 25), quench Trp fluorescence while other sugars, which are not substrates, have no effect (Fig. 3).

Sugar-induced quenching specifically involves the two Trp residues at positions 38 and 278 on opposing faces of the cavity walls. Replacing either residue--particularly Trp38--with Tyr or Phe leads to decreased fluorescence quenching, and negligible fluorescence quenching is observed when both residues are replaced (Fig. 4). Thus, only the Trp residues at positions 38 and 278 are important, and other Trp residues located at interfacial regions are not involved in the phenomenon. Both Trp residues are located in the approximate middle of FucP ~16 Å apart from each other in outward-facing conformation (Fig. 1). Importantly, during the conformational change from outward-facing to occluded, helices I and VII are predicted to come together (11), and in all likelihood, it is these two Trp residues coming into closer opposition as the outward-facing cavity closes that is responsible for sugar-induced quenching in FucP. In this regard, although the experiments presented here were carried out with purified FucP in detergent the findings are consistent with the X-ray structure of FucP, which exhibits and outward-facing conformation devoid of substrate.

It is also highly likely that Trp38 and Trp278 comprise part of the sugar-binding site in FucP. Thus, replacement of either with Tyr or Phe causes a decrease in L-fucose-induced quenching with a significant decrease in affinity (i.e., an increase in $K_{d,\text{app}}$), and
simultaneous replacement of both Trp residues abolishes sugar-induced quenching (Fig. 4). In addition, replacing either or both Trp residues with Tyr, Phe or Ile significantly inhibits L-fucose transport (Fig. 5) as expected if these residues are directly involved in binding. Given a scenario in which Trp38 and Trp278 are components of the sugar-binding site and sugar binding induces quenching because the two Trp residues come into close opposition, it seems reasonable to propose that the two Trp may sandwich the bound L-fucose. By this means, the interaction between the bound pyranose ring of L-fucose and the indole rings of the Trp residues, as well as the close proximity of the two Trp residues might be primarily responsible for quenching.

Isothermal calorimetry measurements provide evidence that Glu135 is directly involved in binding, and as demonstrated here (Fig. 6), mutants E135D or E135Q exhibit essentially no L-fucose-induced Trp quenching, which is consistent with this interpretation. However, there is no evidence for the proposal that Glu135 plays a role in H⁺ translocation. In contrast, mutants D46E and D46N exhibit L-fucose-induced quenching with near-WT binding, and the D46A mutant binds well calorimetrically. Moreover, although mutant D46A does not catalyze uphill L-fucose/H⁺ symport, the mutant catalyzes counterflow (11). In this respect, Asp46 has some of the main characteristics of Glu325 LacY mutants. Mutants with neutral replacements for Glu325 are unable to catalyze all translocation reaction that involve H⁺ transport, but catalyze equilibrium exchange and counterflow. This and additional evidence (reviewed in 26, 27-29) indicate that Glu325 is directly involved in deprotonation of LacY. Furthermore, exchange and counterflow involve the global conformational change central to the alternating access mechanism. Therefore, Asp46 mutants in FucP are expected to exhibit L-fucose-induced quenching.
In conclusion, the experimental results presented here demonstrate a direct interaction of FucP with its sugar substrates by utilizing Trp fluorescence spectroscopy. Thus, sugar binding evokes quenching of Trp residues at positions 38 and 278 in a manner that clearly reflects a conformational change in FucP. Moreover, it is likely that this structural change is primarily closure of the outer-facing cavity to form an occluded state that may represent the high-energy conformation of the protein, as suggested for LacY (30).

The findings presented here have been submitted to Proceedings of the National Academy of Sciences of the United States of America and accepted for publication (31).
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