Structure and mechanism of the mammalian fructose transporter GLUT5

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The altered activity of the fructose transporter GLUT5, an isoform of the facilitated-diffusion glucose transporter family, has been linked to disorders such as type 2 diabetes and obesity. GLUT5 is also overexpressed in certain tumour cells, and inhibitors are potential drugs for these conditions. Here we describe the crystal structures of GLUT5 from Rattus norvegicus and Bos taurus in open outward- and open inward-facing conformations, respectively. GLUT5 has a major facilitator superfamily fold like other homologous monosaccharide transporters. On the basis of a comparison of the inward-facing structures of GLUT5 and human GLUT1, a ubiquitous glucose transporter, we show that a single point mutation is enough to switch the substrate-binding preference of GLUT5 from fructose to glucose. A comparison of the substrate-free structures of GLUT5 with occluded substrate-bound structures of Escherichia coli XylE suggests that, in addition to global rocker-switch-like re-orientation of the bundles, local asymmetric rearrangements of carboxy-terminal transmembrane bundle helices TM7 and TM10 underlie a ‘gated-pore’ transport mechanism in such monosaccharide transporters.

GLUT transporters belong to the solute carrier 2 family (SLC2) and, so far, 14 different isoforms (GLUT1–GLUT14) have been identified1,2. Except for GLUT1, GLUT transporters are uniporters, which facilitate the diffusion of monosaccharides like glucose and fructose across the cell membrane in a concentration-dependent manner1,2. Each GLUT transporter shows a distinct pattern of tissue distribution, gene regulation, substrate preference and kinetic properties1,2. For example, GLUT1 is distributed in a wide range of tissues, including the blood–brain barrier, and is essential for glucose transport into the brain3, whereas GLUT4 is mostly localized to skeletal muscles and adipose tissue, and is the major insulin-stimulated glucose transporter1-4. GLUT5 is the only member specific to fructose5,6, and together with GLUT2, which transports fructose in addition to glucose, they make up the major fructose transporters in the body7,8. GLUT5 is primarily expressed in the small intestine, but lower levels are also expressed in brain, adipose tissue, kidney, testes and skeletal muscle9,10. GLUT activity is also associated with various diseases9-11. For instance, increased GLUT5 expression has been linked to several metabolic disorders12,13 and several types of cancers such as breast cancer14, because of the higher energy demand of cancer cells stimulating sugar uptake, the so-called Warburg effect15. GLUT transporters belong to the larger major facilitator superfamily (MFS), the members of which have a fold consisting of two symmetrical six transmembrane helix (TM) bundles16,17. Within the MFS they belong to a subfamily of sugar porter transporters18,19, whose members are found in all domains of life and are important targets for industrial and biomedical applications20. Recently, an open inward-facing structure of human GLUT1 was reported with a bound sugar from a detergent head-group in the substrate-binding site and compared to previous structures of the related Escherichia coli d-xylose:H+ symporter XylE in the outward- and inward-occluded conformations, suggesting a ‘rotor-switch’-type transport mechanism21-23. However, as little is known about the molecular basis of substrate binding and release in GLUT transporters, their alternating-access mechanism is yet to be fully understood.

**Open outward and inward GLUT5 structures**

Rat and bovine GLUT5 (rGLUT5 and bGLUT5, respectively) that share ~81% sequence identity to human GLUT5 were selected and optimized for structural studies using fluorescence-based screening methods (Methods). rGLUT5 was crystallized in complex with an Fv antibody fragment (rGLUT5–Fv; see Methods). The rGLUT5–Fv and bGLUT5 structures were solved by molecular replacement (MR) and refined against data extending up to 3.3 Å and 3.2/4.0 Å (anisotropic贝footnote), respectively (Extended Data Tables 1 and 2, Extended Data Fig. 1 and Methods). The GLUT5 structure shows the typical MFS fold, plus five additional helices on the intracellular side, one at the C terminus (ICH5) and the other four, ICH1–ICH4, located between the amino- and C-terminal TM bundles (Fig. 1), bGLUT5 crystallized in an open inward-facing conformation (Fig. 1), and although human GLUT1 (hGLUT1) and bGLUT5 share only 43% sequence identity, their inward-facing structures superimpose well, with a root mean squared deviation (r.m.s.d.) of 1.12 Å for 364 pairs of C atoms (Methods and Extended Data Fig. 2a). The rGLUT5–Fv structure

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**Central fructose-binding site of GLUT5**

The GLUT5 substrate-binding site is closely related to those of hGLUT1 and *E. coli* XyIE (refs 21, 22) (Fig. 2a and Extended Data Fig. 2b). Many of the residues lining the central cavity are conserved between GLUT5 and hGLUT1, and include Ile169, Ile173, Gln166, Gln287, Gln288, Asn324 and Trp419 (Fig. 2a and Extended Data Fig. 4). In GLUT5, Trp419 is the only tryptophan positioned in the substrate-binding site (Fig. 2a and Extended Data Fig. 5a), and it is essential for transport26. Consistent with rGLUT5 transport activity (Extended Data Fig. 6a), strong quenching of tryptophan fluorescence could be observed with the addition of D-fructose, but not with the addition of L-fructose or known GLUT1 substrates like D-glucose, D-galactose or D-mannose (Extended Data Fig. 5). Using this assay, the affinity of rGLUT5 for D-fructose was measured to have a dissociation constant ($K_d$) around 6–9 mM (Fig. 2b and Extended Data Fig. 6b), which is similar to that reported for human GLUT5 (refs 5, 8). Throughout this study tryptophan fluorescence quenching of rGLUT5 was henceforth used to assess substrate binding.

Gln166 is the only conserved residue in the substrate-binding site that is positioned differently between the hGLUT1 and GLUT5 structures (Fig. 2a). In hGLUT1 the equivalent glutamine (Gln161) points that is positioned differently between the hGLUT1 and GLUT5 structures, as viewed within the plane of the membrane, which highlight the accessibility of the sugar to the central cavity (shown as a dotted ellipse). c, Ribbon diagrams of GLUT5 viewed from the cytoplasm in the open outward-facing (left) and inward-facing (right) conformations.

**Figure 1 | Structures of rat GLUT5 in the open outward-facing conformation and bovine GLUT5 in the open inward-facing conformation.**

a, Ribbon representation of open outward-facing rat GLUT5 (left) and open inward-facing bovine GLUT5 (right) structures, viewed in the plane of the membrane. TM1 and TM4, and TM2, TM3, TM5 and TM6 in the N-terminal TM bundle are coloured in blue and light-blue, respectively. TM7 and TM10, and TM8, TM9, TM11 and TM12 in the C-terminal TM bundle are coloured in red and yellow–brown, respectively. The intracellular domain helices ICH1 to ICH5 are shown in grey. b, Slab through the surface electrostatic potential of the open outward-facing (left) and open inward-facing (right) GLUT5 structures, as viewed within the plane of the membrane, which highlight the accessibility of the sugar to the central cavity (shown as a dotted ellipse). c, Ribbon diagrams of GLUT5 viewed from the cytoplasm in the open outward-facing (left) and inward-facing (right) conformations.

shows an open outward-facing conformation, which is a state that has not been observed previously in any of the related sugar porter structures22–25 (Fig. 1). The open outward-facing conformation is possibly stabilized by the Fv fragment, which binds to the ICHs (Extended Data Fig. 3).

**Figure 2 | The fructose-binding site of GLUT5.**

a, The substrate-binding site in the inward-facing hGLUT5 structure (left panel; coloured as in Fig. 1) is very similar to the inward-facing hGLUT1 structure (right panel; light grey). To facilitate comparison to rGLUT5, bGLUT5 residues are labelled with rGLUT5 numbering. For hGLUT1, only Q161 and all other residues that are different in hGLUT5 are labelled. The D-glucopyranoside moiety of bound n-onyethyl-$\beta$-D-glucopyranoside in hGLUT1 is shown in stick representation. b, D-fructose binding to GLUT5 as measured by tryptophan (Trp) fluorescence quenching (excitation 295 nm; emission 338 nm) after addition of increasing concentrations of D-fructose to wild-type (WT; black squares) and to wild-type protein that had been previously incubated with the GLUT inhibitor HgCl$_2$ (open circles). c, Trp fluorescence quenching for purified substrate-binding site mutants after addition of 40 mM D-fructose (open bars) relative to wild type (filled bar). d, Trp fluorescence quenching after addition of either 40 mM D-fructose or D-glucose to purified wild type (filled bars) or Q166E (open bars); pre-incubation with the inhibitor HgCl$_2$ is indicated. e, Trp fluorescence quenching after addition of increasing concentrations of D-fructose to either purified Q166E (filled squares), or Q166E previously incubated with HgCl$_2$ (open circles) or wild type (open triangles). In all experiments errors bars indicate s.e.m.; n = 3.
forming the salt bridges are labelled and are shown as sticks. To facilitate comparison to rGLUT5, bGLUT5 residues are labelled with rGLUT5 numbering. Breakage of conserved salt bridges as seen here for GLUT5 has also been predicted for XyIE\(^{23}\), b, Superimposition of the N- and C-terminal 6-TM bundles. Strictly conserved and pseudo-symmetry-related charged residues forming the salt bridges are labelled and are shown as sticks. c, D-fructose binding as measured by Trp fluorescence quenching after incubation with 40 mM D-fructose for purified wild-type GLUT5 (filled bar) and single alanine mutations of key acidic residues E400 and E151 that form inter-bundle salt bridges, and G389, which is located in the hinge point of TM10 critical for TM10 conformational change (open bars). Trp fluorescence quenching by D-fructose for these mutants is displayed as a percentage of wild-type binding.

**Figure 3** | Inter-TM salt bridges form between bundle cytoplasmic ends in the outward-facing conformation. a, Cartoon representation of GLUT5 as viewed from the cytoplasm in the outward-facing (left) and inward-facing (right) conformations. ICHs are not shown for clarity. TMs are coloured as in Fig. 1a, and residues forming salt bridges are shown as sticks. To facilitate comparison to rGLUT5, bGLUT5 residues are labelled with rGLUT5 numbering. **Figure 4** | Substrate-induced gates are predominantly formed by TM7 and TM10 in the C-terminal bundle. a, Superposition of GLUT5 open outward- and inward-facing (*) structures, as viewed from the extracellular (left) and intracellular (right) side of the membrane. TMs are coloured as in Fig. 1a, except inward-facing helices TM1*, TM4* shown in orange and TM7*, TM10* shown in cyan. ICHs have been removed for clarity. b, Superimposition of the GLUT5 open outward- and inward-facing structures as viewed in the plane of the membrane. For clarity, TM5, TM5*, TM8 and TM8* are not shown. Cavity-closing contacts are mostly formed by TM1* and TM7* on the extracellular side in the inward-facing conformation and by TM4 and TM10 on the intracellular side in the outward-facing conformation. These TMs are the first TMs in each of the four 3-TM repeats of the MFS fold\(^{16,22}\), p-xylene, as it is in the occluded-outward-facing XyIE structure (PDB 4GBY), is shown in stick representation. With the inward movement of TM7, conserved tyrosine residues are likely to occlude the substrate from exiting, as seen for the equivalently located tyrosine residues in the substrate-occluded XyIE structure\(^{22}\) and as supported by D-fructose binding data (Extended Data Fig. 6d). The opening movement of TM10 to enable cytosolic substrate release has been described previously for XyIE\(^{23}\) and other unrelated MFS transporters\(^{23,24}\). c, Interactions between hydrophobic residues between TM7 and TM10 in the outward-facing conformation (left) are lost in the inward-facing conformation (right). To facilitate comparison to rGLUT5, bGLUT5 residues are labelled with rGLUT5 numbering.

**Salt bridges stabilize the outward conformation**

A common feature observed in recent structures of MFS transporters is the inter-TM salt bridges that break and form near the central cavity during transport\(^{16,29,30}\). In H\(^+\)-coupled transporters, it is thought that the protonation state of these salt bridges is coupled to substrate binding and further to structural transitions\(^{16,29,30}\). In GLUT5, no salt bridges are observed near the central cavity in either conformation. Instead, inter-TM bundle salt bridges are observed only in the outward-facing conformation and far from the central cavity, linking the cytoplasmic ends of TM3, TM4 and TM5 in the N-terminal TM bundle to those of TM9, TM10 and TM11 in the C-terminal TM bundle (Fig. 3a). Specifically, Glu151 (TM4) forms a salt bridge to both Arg97 (TM3) and Arg407 (TM11), and similarly Glu400 (TM10) forms a salt bridge to both Arg158 (TM5) and Arg340, which is essential for D-glucose transport\(^{28}\).
ICH1–ICH3 interact with the C-terminal bundle via a salt bridge (refs 21, 22). In GLUT5, ICH5 lacks charged residues that could be to provide additional stabilization of the outward-facing conformation, as suggested previously21.

**Figure 5 | Alternating-access transport mechanism in GLUT5.** Schematic representation of the rocker-switch-type movement of the N- and C-terminal TM bundles of the local, gating conformational changes of TM7 and TM10 supporting a gated-pore-type transport mechanism in GLUT5.

(TM9). Owing to their strict conservation these residues make up the well-described sugar porter signatures18,19 (Extended Data Fig. 4), and are related by a pseudo-two-fold symmetry axis running through the centre of the transporter and perpendicular to the membrane plane (Figs 1a and 3b). In the inward-facing conformation the inter-TM bundle salt-bridge pairs Glu400–Arg158 and Glu151–Arg407 are separated by some ~7 Å and 13 Å, respectively (Fig. 3a). In the inward-facing conformation, no inter-TM bundle salt bridges are formed on the extracellular side, indicating that in the absence of substrate GLUT proteins may favour the outward-facing conformation. Consistently, the substitution of Glu400, Arg407 and Glu336 equivalent residues in GLUT4 to neutral amino acids arrests the transporter in an inward-facing conformation31. Moreover, in the outward-facing conformation Glu336 (TM8) in the C-terminal TM bundle forms a salt bridge to Arg340, which is connected to the inter-TM bundle Glu400–Arg158 salt bridge (Fig. 3a). The equivalent glutamate to Glu336 in hGLUT1 (Glu329) was substituted to glutamine to stabilize the inward-facing hGLUT1 structure31. The salt bridges formed between inter-TM bundles seem to be coupled also to substrate binding in GLUT5, because substituting Glu151 and Glu400 with alanine shows significantly reduced D-fructose binding (Fig. 3c). Additionally, from the inward-occluded XylE conformation23,24, the extracellular half of TM7 shows the largest shift towards the substrate-binding site (Extended Data Fig. 8a). Similarly, from the inward-occluded XylE conformation23 to the inward-open GLUT5 conformation, the intracellular half of TM10 shows the largest shift away from the substrate-binding site (Extended Data Fig. 8b, c); this TM10 movement has also been described previously for XylE23,24. In GLUT5 the observed conformational changes in TM7 and TM10 occur at hinge points that contain glycine residues (Fig. 4b, c and Extended Data Fig. 4). Tyr382 in TM10 interacts tightly with TM7 residues Ile295 and Val292 in the outward-facing state and these interactions do not take place in the inward-facing conformation (Fig. 4c). The interactions between TM7 and TM10 seem important to coordinate their conformational changes during transport, because mutation of the Ile295 equivalent residue in hGLUT5 to valine or alanine abolishes D-fructose transport34. Consistently, similar interface-perturbing mutations of Ile295 significantly reduce D-fructose binding in rGLUT5 (Extended Data Fig. 6d). Furthermore, in hGLUT1 the equivalent residue to Val292 (Ile287) was substituted to every other amino acid, and each mutant shows markedly different D-glucose affinities and transport kinetics35. Therefore, it suggests that the interactions between TM7 and TM10 are important and tuned with respect to substrate affinity and transport kinetics.

**Conclusions**

Symmetrical substrate binding and rigid-body movements of the N- and C-terminal TM bundles around a centrally located substrate-binding site form the structural basis of the ‘rocker-switch’ mechanism in MFS transporters36. However, here we have described asymmetrical rearrangements in the MFS subfamily of sugar porters, consistent with the asymmetrical binding mode of the sugar reported previously in XylE and hGLUT1 structures23,25. Therefore, we conclude that transport in GLUT5 is not only controlled by the rocker-switch-type movement21 of the N- and C-terminal TM bundles, but also by a gated-pore mechanism, in the form of local, gating movements by TM7 and TM10 in the C-terminal TM bundle that are coupled to substrate binding and release (Fig. 5 and Supplementary Videos 1, 2 and 3). TM10 is also part of the inter-TM bundle salt-bridge network, indicating how local gating and global rocker-switch-type movements are coupled. Importantly, a deeper understanding of GLUT5 structure and

The ICH domain is below this cytoplasmic salt-bridge network, and positioned similarly, with respect to the N- and C-terminal TM bundles, in both outward-open GLUT5 and outward-occluded XylE structures23 (Extended Data Fig. 7a). ICH1, ICH2 and ICH3 are linked together by salt bridges, as previously shown for XylE and GLUT1 (refs 21, 22). In GLUT5, ICH5 lacks charged residues that could interact with ICH1–ICH3 (Extended Data Fig. 7b), as it does in the outward-occluded XylE structure23. Rather, the N-terminal helices ICH1–ICH3 interact with the C-terminal bundle via a salt bridge formed between Glu252 in ICH3 and Arg407 in TM11; thus linking the ICH domain to a TM involved in the inter-bundle salt-bridge network. In inward-facing GLUT5, these interactions are broken (Extended Data Fig. 7c) and, as observed in the inward-facing hGLUT1 and XylE structures23,25–27, ICH5 could not be built (Extended Data Fig. 2a). Therefore, the role of the ICH domain might be to provide additional stabilization of the outward-facing conformation, as suggested previously21.

**TM7 and TM10 form substrate–induced gates**

In GLUT5, the N- and C-terminal TM bundles undergo a small rotation of ~15° between the open outward- and inward-facing conformations (Fig. 1). As observed in other MFS transporter structures16, cavity-closing contacts in GLUT5 form mostly between TM1 and TM7 on the outside, and between TM4 and TM10 on the inside (Figs 1c and 4a). Among these helices, however, TM7 and TM10 in the C-terminal TM bundle seem to have the most prominent roles in occluding the substrate-binding site from the outside and inside, respectively (Fig. 4b). Comparisons of the substrate-free open GLUT5 structures with the substrate-occluded XylE structures23,24 highlight the central role of TM7 and TM10 in gating (Extended Data Fig. 8). Inverted-symmetry-related TM7 and TM10 make up highly conserved sugar transporter signatures2, and they also form a large fraction of the substrate-binding site in hGLUT1 (ref. 21) and XylE (refs 21, 22), in agreement with previous functional data26,32,33. Between the outward-open GLUT5 conformation and outward-occluded XylE conformation23, the extracellular half of TM7 shows the largest shift towards the substrate-binding site (Extended Data Fig. 8a). Similarly, from the inward-occluded XylE conformation23 to the inward-open GLUT5 conformation, the intracellular half of TM10 shows the largest shift away from the substrate-binding site (Extended Data Fig. 8b, c); this TM10 movement has also been described previously for XylE23,24. In GLUT5 the observed conformational changes in TM7 and TM10 occur at hinge points that contain glycine residues (Fig. 4b, c and Extended Data Fig. 4). Tyr382 in TM10 interacts tightly with TM7 residues Ile295 and Val292 in the outward-facing state and these interactions do not take place in the inward-facing conformation (Fig. 4c). The interactions between TM7 and TM10 seem important to coordinate their conformational changes during transport, because mutation of the Ile295 equivalent residue in hGLUT5 to valine or alanine abolishes D-fructose transport34. Consistently, similar interface-perturbing mutations of Ile295 significantly reduce D-fructose binding in rGLUT5 (Extended Data Fig. 6d). Furthermore, in hGLUT1 the equivalent residue to Val292 (Ile287) was substituted to every other amino acid, and each mutant shows markedly different D-glucose affinities and transport kinetics35. Therefore, it suggests that the interactions between TM7 and TM10 are important and tuned with respect to substrate affinity and transport kinetics.
transport mechanism, as described here, should facilitate the structure-based design of novel inhibitors with therapeutic potential.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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No statistical methods were used to predetermine sample size.

Rattus norvegicus GLUT5 cloned full-length sequence (UniProt accession number P43442); NIH3T3 cells were harvested and overexpression levels assessed by additional C-terminal residues retained after TEV cleavage are shown in italic (see next section for cloning details): MEPQDPVKREGRLTPVIVLATYGYNVAAVNSPSEFMQQFYG;

Target identification using fluorescence-based screening methods. GLUT5 homologues were cloned into the GAL1 inducible TEV cleavable GFP–His6 2μ vector pDDGP2, transformed into the Saccharomyces cerevisiae strain FY217 (MATa, ura3–52, lys2A201, and pep4a) and overexpressed as described previously44,45. In brief, 10 ml S. cerevisiae FY217 cell cultures in − URA media and 0.1% glucose were grown at 30 °C and expression was induced by the addition of 2% (v/v) β-galactose at an OD600 of 0.6. After ~22 h, cells were harvested by centrifugation and membranes were prepared by lysis with a French press and overexpression levels were re-assessed by whole-cell GFP fluorescence44,45. Fusions with detectable expression levels were re-grown in larger 2 l culture volumes and membranes subsequently isolated. The monodispersity of expressed fusions was confirmed in crude dodecyl-β-D-maltopyranoside (DDM), decyl-β-D-maltopyranoside (DDM), nonyl-β-D-maltopyranoside (NM) and n-dodecyl-N,N-dimethylamino-N-oxide (LDAO) solubilized membranes by fluorescence-detection size exclusion chromatography (FSEC) as described previously46. Out of the GLUT5 homologues screened, rat and bovine GLUT5 showed the sharpest monodispersity profiles and was the most stable after purification in detergent47.

Large-scale production and purification of rat and bovine GLUT5. For rat GLUT5, cells were harvested from 10 l. cerevisiae cultures, resuspended in buffer containing 50 mM Tris-HCl pH 7.6, 1 mM EDTA, 0.6 M sorbitol, and lysed by mechanical disruption as described previously48. Membranes were isolated by ultracentrifugation at 195,000 g for 3 h, homogenized in 20 mM Tris-HCl pH 7.5, 0.3 M sucrose, 0.1 mM CaCl2, frozen in liquid nitrogen and stored at −80 °C. Rat GLUT5 membranes were solubilized with 1% DDM in equilibration buffer (EB) consisting of 1× PBS, 10 mM imidazole, 150 mM NaCl, 10% glycerol. After ultracentrifugation at 195,000 g for 45 min, the supernatant was incubated with 10 ml of Ni2+–nitrilotriacetic affinity resin (Ni-NTA; Qiagen) for 2 h at 4 °C. The resin was washed with 100 ml of EB containing 0.05% DDM and 30 mM imidazole, and the protein eluted in 25 ml of EB containing 0.05% DDM and 250 mM imidazole. The eluted protein was incubated with TEV–His6 tag and dialysed against 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 10% glycerol. 0.03% DDM. Dialysed sample was then loaded onto a 5 ml HiTrap column (GE Healthcare) equilibrated in dialysis buffer, and the flow-through was collected and concentrated. The protein was further purified by size-exclusion chromatography (SEC) using a Superdex 200 column (GE Healthcare) in buffer consisting of 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.02% DDM. All rat GLUT5 mutants were generated with a standard PCR-based strategy and were sub-cloned, overexpressed and purified as described for wild type.

Protein complexes were prepared by incubation of the HisTrap-purified GLUT5 with the SEC-purified Fv fragment (supplemented with 0.02% (w/v) DDM) at a molar ratio of 1:1.5 for 1 h on ice. The complex was subjected to SEC (Superdex 200, GE Healthcare). The SEC step was repeated twice to ensure removal of crystals. Proteins containing rat GLUT5–Fv complex were concentrated to ~10 mg ml−1 by ultrafiltration (Millipore, MWCO 50 kDa), and immediately used for crystallization experiments.

For bovine GLUT5, membranes were isolated from 40 l S. cerevisiae cultures. After membrane solubilization and ultracentrifugation (as described for rat GLUT5), the supernatant was supplemented with 55 mM imidazole, and incubated with 30 ml of Ni-NTA resin for 2 h at 4 °C. The resin was washed with 600 ml of EB supplemented with 55 mM imidazole and 0.03% DDM, and the protein was eluted using 125 ml of EB containing 250 mM imidazole and 0.03% DDM. After cleavage of Fv–His6 cleavage by TEV–His6, the protein was re-loaded onto the same 30 ml Ni-NTA resin column (no imidazole), the flow-through was concentrated and passed through a 1 ml Ni-NTA column for further clean up. The untagged protein was further purified by SEC in buffer containing 10 mM Tris–HCl pH 7.5, 150 mM NaCl, and 0.09% undecyl-β-maltopyranoside (UDM), and then concentrated to ~4 mg ml−1 for crystallization experiments.

Generation of single-chain Fv (scFv) fragments. Animal experiments conformed to the guidelines outlined in the Guide for the Care and Use of Laboratory Animals and were approved by the University of Tokyo Animal Care Committee (approval no. RAC07101). Rat GLUT5-specific scFv fragment were generated essentially as previously described45. In brief, purified GLUT5 was reconstituted into liposomes containing chicken egg yolk phosphatidylycholine (egg PC; Avanti) and adipant lipid A (Sigma–Aldrich) by detergent removal method. Small unilamellar proteoliposomes were prepared by sonication. MRL/lpr mice were infected with the proteoliposome antigen three times at two-week intervals. Immunized mice were killed, and RNA in their splenocytes was isolated and converted into cDNA via reverse-transcription PCR. The Vι and Vιι regions were assembled via an 18-amino-acid flexible linker and cloned into the phage display vector pComb3XSS. Biotinylated proteoliposomes were prepared by reconstituting GLUT5 with a mixture of egg PC and 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine–N-(cap biotinyl) (160 biotinyl CapPE; Avanti), and used as binding targets for scFv-phage selection. The targets were immobilized onto streptavidin-coated paramagnetic beads (Dynabeads) or streptavidin-coated microplates (Nunc). After four rounds of biopanning, proteoliposome-targeted enzyme-linked immunosorbent assays (‘liposome ELISAs’) were performed on periplasmic extracts of individual colonies. Positive clones were collected and evaluated using a Biacore T100 (GE Healthcare).

Expression and purification of Fv fragment. Antibody fragments in the scFv format are undesirable for use as crystallization chaperones because they are able to intermolecularly form domain-swapped dimers, and dimer-monomer equilibrium may increase structural heterogeneity. Therefore, we used Fv fragments for crystallization trials. The Fv fragment was expressed in Brevibacillus choshunensis as a secreted His6-tagged protein and purified from culture medium. The cells were grown at 30 °C with 200 rpm in 25Y medium (sosyn 40 g l−1, yeast extract 5 g l−1, glucose 20 g l−1, CaCl2 0.15 g l−1) supplemented with 50 mg l−1 neomycin. The cells expressing the Vι and Vιι regions were grown initially as separate overnight precultures. The pre-cultures were then combined and diluted in 25Y medium to give OD600 of 0.02 of each strain and grown for further 65–70 h. The cells were removed by centrifugation at 6000 g for 15 min. The recovered culture supernatant was adjusted to a final ammonium sulfate concentration of 60% saturation, and the precipitate was pelleted, dissolved in TBS buffer (10 mM Tris–HCl, pH 7.5, 150 mM NaCl) and dialysed overnight against the same buffer. dialysed sample was mixed with Ni-NTA resin equilibrated with Fv1 buffer (10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 250 mM imidazole), mixed with TEV–His6, and dialysed overnight against TBS buffer. Cleaved His6 tag and TEV–His6, were removed by binding to a HisTrap column equilibrated with Fv1 buffer. The tag-free Fv fragment was concentrated and loaded onto a HiLoad16/60 Superdex 75 column (GE Healthcare) equilibrated with TBS buffer. Peak fractions were pooled, concentrated, flash frozen in liquid nitrogen, and stored at −80 °C.

Transport activity of reconstituted GLUT5. The proteoliposome pH-rectose uptake assay was as previously described49. In brief, purified GLUT5 was reconstituted with bovine liver and sonicated to make unilamellar liposomes. 500 μl of a mixture containing ~10 μg of purified GLUT5 and 20 mg of liposomes in 10 mM Tris–HCl (pH 7.5) was flash frozen and
thawed at room temperature. Large, unilamellar proteoliposomes were prepared by extrusion (LiposoFast, Avetstein; membrane pore size, 400 nm). For each time point, 10 μl proteoliposomes (0.4 mg lipid; 0.2 μg GLUT5) was added to 10 μl transport buffer containing 10 mM Tris pH 7.5 and 2 mM MgSO4 (pH 7.5) with or without the addition of 0.5 mM HgCl2. Time course of 0.1 mM [3H]-D-glucopyranose transport was measured at 25°C at the indicated time intervals and stopped by the addition of cold buffer containing 10 mM TrisSO4, 2 mM MgSO4 (pH 7.5) and 0.5 mM HgCl2 and immediately filtered. Non-specific uptake was estimated with 0.1 mM [3H]-D-glucopyranose. The radioactivity corresponding to the internalized substrate was measured by scintillation counting. Each experiment was performed in triplicate and data points shown indicate average values of two technical replicates.

**Substrate specificity.** Unless otherwise stated the rat GLUT5 deglycosylation mutant (N50Y) used for structure determination is referred to as wild-type-like protein (WT). Only rat GLUT5 wild type and mutants showing a monodisperse peak in DDM during gel filtration were assessed for their ability to bind sugar by tryptophan fluorescence. In all experiments, either purified rat GLUT5 wild type or mutants were diluted to 0.06 mg/ml in purification buffer containing 150 mM NaCl, 20 mM Tris pH 7.5, 0.03% DDM, D-fructose, D-glucose, D-mannose, D-xylene and D-galactose stocks, that were freshly prepared in purification buffer, were added to the diluted protein to reach a final concentration of 40 mM. After each sugar addition the sample was incubated at room temperature for 2 min before measuring tryptophan fluorescence. Measurements were performed using a Cary Eclipse Fluorescence Spectrophotometer (Agilent Technologies), with an excitation wavelength of 295 nm and the emission spectra recorded in the range of 300–400 nm with a 5 nm excitation slit in a High Precision Cell Quartz Suprasil 10 × 2 mm cuvette (Hellma Analytics). The emission peak of 338 nm was taken as the average of the four wavelengths 335.72, 337.96, 339.06 and 341.64 nm. Each experiment was carried out in triplicate in the absence and presence of the well-known GLUT inhibitor HgCl2 (5 mM).47 To compare mutant binding to D-fructose, nonspecific tryptophan fluorescence quenching of 2.5% (as measured with 40 mM L-fructose) was first subtracted from both wild-type and mutant quenching levels before calculating the final percentage of quenching relative to wild type.

**Binding analysis.** D-fructose or D-glucose stocks, that were freshly prepared in purification buffer, were sequentially added to the diluted protein to a final concentration of 40 mM. After each sugar addition the sample was incubated at room temperature for 2 min before measuring. Tryptophan fluorescence measurements were performed as above. Each experiment was carried out in triplicate in the absence and presence of the known GLUT inhibitor HgCl2 (5 mM). The rat GLUT5 curves were fitted by nonlinear regression using the software Prism. The bovine GLUT5 construct used for structural studies binds D-fructose with similar affinity as rat GLUT5 with a Kd of 5.5 ± 0.6 mM.

**Crystallization.** The structure of the Fv fragment was used as a search model for the molecular replacement. Crystals of the Fv fragment appeared in the well buffer at 20°C containing 0.1 M CAPS-NaOH (pH 10.5), 1.6 M (NH4)2SO4, 0.1 M Li2SO4, 6.5 mM n-nonyl-β-D-glucopyranoside (DDM), and 4% glycerol. The crystals were equilibrated against a 2:1 DDM reservoir (1:1), and placed over the well solution diluted (4:1) and then the protein solution (1:1), and placed over the well solution diluted (4:1) before sealing. For freezing in liquid nitrogen, crystals were soaked for 1 min in the mother liquor supplemented with ~15% PEG 300.

**Data collection, structure determination and analysis.** For the Fv fragment, diffraction data were collected at 100 K at SPring-8 beamline BL41XU (Japan) and processed using HKL2000 packages46 and the CCP4 suite48. The crystal belonged to the space group C2 with two molecules per asymmetric unit. The structure was determined by molecular replacement with Molrep9 using the Fv portion of an antibody structure (PDB code 1G5T) as an initial model. Refinement was performed till the Rfree value decreased to ~22% with REFMACS. For rat GLUT5–Fv complex, diffraction data were collected at 100 K at SPring-8 beamline BL41XU (Japan) and processed using HKL2000 packages46 and the CCP4 suite48. The data set used for structure determination and refinement was generated by the combination of 4 data sets from 4 independent crystals. The space group was determined to be P21, with two rat GLUT5–Fv complexes per asymmetric unit. The structure was determined by molecular replacement with Molrep9 using two search models (polyalamine of the transmembrane region in the outward-facing conformation of FucP (PDB code 3OTQ), and the separately determined 1.5 Å structure of the Fv fragment used in this study). Refinement was performed with PHENIX49 followed by manual examination and rebuilding of the refined coordinates using COOT48. Recently determined structures of Xyfe (PDB code 4GCO) and human GLUT1 (PDB code 4PYP) helped with the modelling. The 6 N-terminal residues (Met1–Gln6), 22 C-terminal residues (Ser481–Gln502), and 22 residues (Asn39–Asn60) in molecule A and 12 residues (Met45–Arg56) in molecule B in TM2 are not included in the structure as they did not have interpretable densities. For bovine GLUT5, data were collected on frozen crystals at beamline I02, Diamond Light Source (UK). The data set used for structure determination and refinement was generated using HKL2000 by processing and scaling together two data sets collected on two different parts of the same crystal, and by correcting for anisotropy (UCLA server, http://services.mbi.ucla.edu/anisoscale/) (Extended Data Tables 1 and 2 and Extended Data Fig 1). The structure was solved by molecular replacement using the N-terminal and C-terminal bundles of rat GLUT5 as independent search models in PHASER, and refined using REFMACS and BUSTER against data up to 3.0 Å resolution after anisotropy correction48, with rounds of re-building in COOT48. Final refinement was performed using PHENIXX at 3.2 Å resolution (Extended Data Fig 1). Structural alignments were performed using the align command of PYMOL software using Cx coordinates.

**Homology modelling.** Models for the outward-occluded and inward-occluded conformations were based on corresponding Xyfe crystal structures (4GBY and 4JA3, respectively) and were produced using Modeller 9v12 (ref. 54). Multiple alignment of GLUT5 homologues (Homo sapiens, Canis lupus, Rattus norvegicus, Felis catus, Ecaecilia glutinosa, and Sympetrum viride) sequences were combined with multiple alignments from the Xyfe structures and the GLUT5 structures using expresso mode of t-coffee55. The multiple alignment in combination with helical restraints corresponding to the TM5s of bovine GLUT5 structure was used as an input for Modeller. For each conformation, 20 models were generated with loop optimization. The DOPE scoring function was used to select the final model. Outward-facing bovine GLUT5 was modelled with the same protocol based on the rat outward-facing GLUT5 structure and an alignment of GLUT5 sequences. The four conformations (outward-open, outward-occluded, inward-occluded and inward-open) were morphed and rendered using PYMOL (http://www.pymol.org).

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Your data has SEVERE anisotropy based on the spread in values of the three principle components: 73.43 Å^2.

The principle components are the exponential scale factors used to correct for anisotropy. They may be regarded as B factors applied to the three principle directions of the data set. Larger values indicate stronger anisotropy.

In decreasing order the components are:

33.89 Å^2
5.64 Å^2
-39.53 Å^2

The recommended resolution limits along \(a^*, b^*, c^*\) are 4.0 Å, 3.0 Å, 4.0 Å.

These are the resolutions at which \(F_{\text{sigma}}\) drops below an arbitrary cutoff of 3.0.

24,188 reflections were in the initial data set. 10,997 were discarded because they fell outside the specified ellipsoid with dimensions 1/4.0, 1/3.0, 1/4.0 Å along \(a^*, b^*, c^*\), respectively. Among these, 3,701 reflections remain after ellipsoidal truncation. Anisotropic scale factors were then applied to remove anisotropy from the data set. Lastly, an isotropic B of 61.00 Å^2 was applied to restore the magnitude of the high resolution reflections distorted by anisotropic scaling. The following pseudo-precession images illustrate the individual steps.
Extended Data Figure 1 | Anisotropy descriptors of bGLUT5 data reported by the UCLA-MBI Diffraction Anisotropy Server and 2Fo – Fc electron density maps for the bovine and rat GLUT5 structures. a, Degree of anisotropy of bGLUT5 data, resolution limits for the 3 principal axes (left), and panel illustrating steps along correction of bGLUT5 data for anisotropy (right).
b, Representative portions of the electron density map (1.5σ) for bGLUT5 overall model (left) and a close-up of the substrate binding site (right); residues highlighted are numbered based on rGLUT5 for the sake of clarity. c, Electron density (1.0σ) for rGLUT5 showing one of the inter-bundle salt-bridge clusters that form in the open outward-facing conformation.
Extended Data Figure 2 | Superimposition of open inward-facing bGLUT5 and hGLUT1 structures, and comparison of the substrate-binding site in bGLUT5 and inward-facing XylE

**a.** Ribbon representation of inward-facing bGLUT5 (coloured as in Fig. 1a) and inward-facing hGLUT1 (light grey) structures, as viewed in the plane of the membrane. The d-glucopyranoside moiety of the detergent molecule bound to GLUT1 (n-nonyl-β-D-glucopyranoside (β-NG)) is shown in stick representation. Density for ICH5 at the C terminus is missing in both hGLUT1 and bGLUT5 inward-facing structures and highlighted with the dotted ellipse. The beginning of TM1 kinks further outwards in the bGLUT5 structure compared to hGLUT1 and residues 1–18 could not be built. The r.m.s.d. (root mean square deviation) after superposition of the two structures is 1.12 Å for 364 pairs of Cα atoms (see Methods).

**b.** The substrate-binding in the inward-facing bGLUT5 structure (coloured as in Fig. 1) is very similar to that seen in inward-facing XylE (4JA4) structure (shown in light grey). Only non-conserved residues and the equivalent glutamine to Q166 are labelled for XylE.
Extended Data Figure 3 | Structure of the rat GLUT5–Fv complex.

a, Cartoon representation of the complex between rGLUT5 (grey) and 4D111Fv (heavy-chain variable region (V_H) is in blue; light-chain variable region (V_L) is in red). 4D111Fv binds to the cytoplasmic domain of GLUT5, including ICH2 (residues 226, 230, 234), the loop between ICH2 and ICH3 (residues 238, 240, 241), and ICH3 (residue 243), with ~848 Å² of buried surface area at the interface.

b, Packing of the rat GLUT5–Fv complex molecules in the crystal. The unit cell is represented as green lines.
Extended Data Figure 4 | Sequence alignment of rat GLUT5 (rGLUT5), bovine GLUT5 (bGLUT5), human GLUT5 and GLUT7 (hGLUT5 and hGLUT7), human GLUT1–4 (hGLUT1–4), Saccharomyces cerevisiae HXT7, Plasmodium falciparum PfHT), Arabidopsis thaliana GlcT and Escherichia coli XylE. Structure elements of rat GLUT5 are indicated above the alignment, and coloured as in Fig. 1a. Strictly conserved residues are highlighted in black-filled boxes, and highly conserved residues are shaded in grey. Green boxes highlight central cavity residues that are specific to GLUT5 and red boxes highlight those that are conserved among GLUTs. Purple boxes highlight residues forming the salt bridges between cytosolic TM segments. A blue box (TM5) highlights Gln166, whose mutation to glutamic acid, as present in GLUT7, weakens D-fructose binding but supports strong D-glucose binding in rGLUT5. The brown box (TM8) highlights Glu336 that is conserved across all the GLUTs and replaced with glutamic acid in XylE. Red bars underneath the alignment indicate the sugar porter (SP) family motifs18,19. Note that because bGLUT5 and hGLUT5 have an additional amino acid at position 8, their numbering differs from rGLUT5 by 1 amino acid. For clarity, bGLUT5 residues are labelled using rGLUT5 numbering.
Extended Data Figure 5 | D-fructose binding monitored by tryptophan fluorescence quenching.  

**a**, Cartoon representation of the outward-facing rGLUT5 structure, as viewed from the plane of the membrane with the colouring as shown in Fig. 1a. Atoms in all tryptophan residues are shown as spheres and tryptophan W419, whose fluorescence is quenched by substrate, is labelled. **b**, Emission fluorescence spectra for purified deglycosylated rGLUT5 wild-type-like mutant N50Y (referred to as WT), shown in the range of 320–360 nm with an excitation wavelength of 295 nm after the addition of 40 mM D-fructose (top), and 40 mM L-fructose (bottom). Emission fluorescence spectra for purified wild-type protein that had been previously incubated with the inhibitor HgCl₂ is also shown for D-fructose (open bars). Tryptophan fluorescence quenching for purified wild-type protein that had been previously incubated with the inhibitor HgCl₂ is also shown for D-fructose (open bar). **c**, Tryptophan fluorescence quenching (excitation 295 nm; emission 338 nm) after incubation of purified rGLUT5 N50Y with either 40 mM D-fructose (filled bar) or L-fructose, D-glucose, D-mannose, D-xylose or D-galactose as labelled (open bars). Tryptophan fluorescence quenching for purified wild-type protein that had been previously incubated with the inhibitor HgCl₂ is also shown for D-fructose (open bar). **d**, As in c, but for rGLUT5 with a single tryptophan residue (W419), which contains the following mutations: N50Y, W70F, W191F, W239F, W265F, W275F, W338F and W370F. No tryptophan quenching was observed for D-fructose (5 mM HgCl₂), L-fructose, D-glucose or D-galactose. In all experiments errors bars indicate s.e.m.; n = 3.
Extended Data Figure 6 | Substrate specificity in GLUT5.  

**a.** Time-dependent uptake of D-[14C]-fructose by rGLUT5 wild type (open squares and triangles) and the deglycosylated mutant N50Y (filled squares and triangles) in proteoliposomes incubated with or without the inhibitor HgCl$_2$ as labelled. Non-specific uptake was estimated with 0.1 mM L-[14C]-glucose for wild type (filled circles) and the N50Y mutant (open circles). In all experiments errors bars represent a spread of duplicates. Inset shows SDS–PAGE analysis of the purified rat GLUT5 wild type and the deglycosylated N50Y mutant. 

**b.** Tryptophan fluorescence quenching (excitation 295 nm; emission 338 nm), after incubation of purified rat GLUT5 mutant (N50Y, W70F, W191F, W239F, W265F, W275F, W338F, W370F) that contains one single tryptophan residue, W419, with increasing concentrations of D-fructose (filled squares) and to the protein previously incubated with the inhibitor mercury chloride (open circles). 

**c.** Slab through the surface of the outward-facing rGLUT5 structure as viewed in the plane of membrane. The structure of substrate-bound XyIE structure was further superimposed onto rGLUT5 and is shown here as a grey ribbon. In XyIE, Trp392 (Trp388 in hGLUT1) is located at the bottom of the cavity (spheres; magenta) and coordinates D-xylose (stick form; yellow). In GLUT5, the equivalent residue is an alanine, making the cavity deeper. 

**d.** D-fructose binding as measured by tryptophan fluorescence quenching (excitation 295 nm; emission 338 nm) after incubation with 40 mM D-fructose for wild type (open bar), and TM7 mutations of Ile295 (interacts with TM10 residues) and Tyr296 and Tyr297 residues. Equivalently located tyrosine residues in XyIE occlude the sugar-binding site from the outside. 

Fluorescence quenching for the mutants are displayed as a percentage of total wild-type binding. In all experiments errors bars indicate s.e.m.; n = 3.
Extended Data Figure 7 | The intracellular helical domain (ICH).

a, Cytoplasmic view of the ICH domain after superposition of the open, outward-facing rGLUT5–sFv (grey) and outward-facing occluded \textit{E. coli} XylE (teal) (4GBY) structures. b, In the outward-facing GLUT5 structure ICH1–ICH3 are linked together by several salt bridges (side chains are labelled and shown as sticks in yellow). In contrast, no polar interactions are formed between ICH5 and either ICH1–ICH3 or cytoplasmic ends of N-terminal TM bundle helices. A salt bridge forms (dotted line in magenta), however, between Glu225 in ICH3 and Arg407 in TM11, which also forms part of the inter-bundle salt-bridge network (side chains are labelled and shown as sticks in cyan). c, In the inward-facing GLUT5 structure, this inter-bundle salt-bridge network is not formed, because the cytoplasmic ends of the N- and C-terminal bundle have moved apart; consistently, the ICH domain functional role is proposed to act as a scaffold domain that further helps to stabilize the outward-facing conformation\textsuperscript{21}.
a  outward-open GLUT5 and outward-occluded XyIE (grey)

b  inward-open GLUT5 and inward-occluded XyIE (grey)

c  Inward-open GLUT5 and inward-open XyIE (grey)
Extended Data Figure 8 | Access to the central cavity and substrate-binding site is gated by TM7 on the outside and TM10 on the inside. a, Superposition of outward-facing open GLUT5 and outward-facing occluded *E. coli* XylE (4GBY) structures. The TM numbering for outward-facing occluded XylE has an additional asterisk. The inward-facing GLUT5 structure is coloured as in Fig. 1a and that of XylE in grey. The bound D-xylose is shown in stick representation in green. The r.m.s.d. is 1.38 Å for 290 pairs of Cα atoms (see Methods). b, Superposition of inward-open GLUT5 and inward-occluded *E. coli* XylE structure (4JA3) with colouring and annotation as described in a. The r.m.s.d. is 1.80 Å for 274 pairs of Cα atoms (see Methods). The bound D-xylose in 4GBY is represented in stick form in green. The ICH domain is not shown for clarity. c, Superposition of inward-facing open GLUT5 and inward-facing open XylE (4JA4) structures as viewed from the cytoplasmic side with colouring and annotation as described in a. The ICH domain is not shown for clarity. The r.m.s.d. is 1.70 Å for 273 pairs of Cα atoms (see Methods).
### Extended Data Table 1 | Crystallographic data collection and refinement statistics

|                      | rat GLUT5-Fv | bovine GLUT5 * |
|----------------------|--------------|---------------|
| **Data collection**  |              |               |
| Space group          | $P2_1$       | $P2_1\cdot2_1\cdot2_1$ |
| Cell dimensions      |              |               |
| $a$, $b$, $c$ (Å)    | 76.78, 151.54, 106.40 | 74.61, 112.15, 139.57 |
| $\alpha$, $\beta$, $\gamma$ (°) | 90.00, 97.25, 90.00 | 90.00, 90.00, 90.00 |
| Resolution (Å)       | 50.3.27 (3.39-3.27) | 100 - 3.00 (3.11 - 3.01)* |
| $R_{	ext{sym}}$ or $R_{	ext{merge}}$ | 21.5 (>100) | 10.06 (>100) |
| $R_{	ext{col}}$     | 11.6 (1.5)   | 11.21 (0.86)  |
| Completeness (%)     | 100 (100.0)  | 99.4 (93.9)   |
| Redundancy           | 13.0 (13.0)  | 10.2 (8.6)    |

### Refinement

|                      |              |               |
| Resolution (Å)       | 50.3.27 (3.5-3.27) | 33.3 - 3.2 (3.31 - 3.20) |
| No. reflections (Rfree set) | 38017 (3355) | 13346 (1331) |
| $R_{	ext{cryst}}$, $R_{	ext{free}}$ | 24.2/28.8 (35.4/37.5) | 23.6/25.8 (32.8/36.5) |
| No. atoms            |              |               |
| Protein              | 10667        | 3382          |
| B-factors            |              |               |
| Protein              | 157.0        | 149.2         |
| R.m.s deviations     |              |               |
| Bond lengths (Å)     | 0.004        | 0.003         |
| Bond angles (°)      | 0.97         | 0.90          |
| Ramachandran plot (%)|              |               |
| Favored              | 98.0         | 94.5          |
| Outliers             | 0            | 0             |

*Data were obtained by scaling together two datasets collected on the same crystal

*Highest resolution shell used in the final refinement is shown in parenthesis.
**Extended Data Table 2 | Completeness of bovine GLUT5 data per resolution shell after correction for anisotropy**

| Resolution range (Å) | Completeness (%) |
|----------------------|------------------|
| 100.0 - 8.10         | 96.5             |
| 8.10 - 6.43          | 100.0            |
| 6.43 - 5.62          | 99.9             |
| 5.62 - 5.10          | 99.9             |
| 5.10 - 4.74          | 100.0            |
| 4.74 - 4.46          | 99.7             |
| 4.46 - 4.24          | 99.6             |
| 4.24 - 4.05          | 100.0            |
| 4.05 - 3.90          | 78.4             |
| 3.90 - 3.76          | 53.4             |
| 3.76 - 3.64          | 40.6             |
| 3.64 - 3.54          | 33.4             |
| 3.54 - 3.45          | 26.3             |
| 3.45 - 3.36          | 21.2             |
| 3.36 - 3.29          | 15.4             |
| 3.29 - 3.22          | 13.1             |
| 3.22 - 3.15          | 9.4              |
| 3.15 - 3.09          | 6.0              |
| 3.09 - 3.04          | 3.5              |
| 3.04 - 3.00          | 1.7              |