HHS Public Access

Author manuscript

Nat Struct Mol Biol. Author manuscript; available in PMC 2012 August 01.

Published in final edited form as:

Nat Struct Mol Biol. ; 19(2): 207–211. doi:10.1038/nsmb.2197.

Experimental conditions can obscure the second high-affinity site in LeuT

Matthias Quick[1,2,3], **Lei Shi**[4,5], **Britta Zehnpfennig**[1,2], **Harel Weinstein**[4,5], and **Jonathan A. Javitch**[1,2,3,6]

[1]Center for Molecular Recognition, Columbia University College of Physicians and Surgeons, 630 W. 168th, New York, New York 10032, USA

[2]Department of Psychiatry, Columbia University College of Physicians and Surgeons, 630 W. 168th, New York, New York 10032, USA

[3]Division of Molecular Therapeutics, New York State Psychiatric Institute, New York, NY 10032, USA

[4]Department of Physiology and Biophysics, Weill Medical College of Cornell University, 1300 York Avenue, New York, NY 10021, USA

[5]HRH Prince Alwaleed Bin Talal Bin Abdulaziz Alsaud Institute for Computational Biomedicine, Weill Cornell Medical College, Cornell University, 1300 York Avenue, New York, NY 10021, USA

[6]Department of Pharmacology, Columbia University College of Physicians and Surgeons, 630 W. 168th, New York, New York 10032, USA

Abstract

Neurotransmitter:Na^+ Symporters (NSSs), the targets of antidepressants and psychostimulants, recapture neurotransmitters from the synapse in a Na^+-dependent symport mechanism. The crystal structure of the NSS homologue LeuT from *Aquifex aeolicus* revealed one leucine substrate in an occluded centrally-located (S1) binding site next to two Na^+. Computational studies combined with binding and flux experiments identified a second substrate (S2) site and a novel molecular mechanism of Na^+/substrate symport that depends upon the allosteric interaction of substrate molecules in the two high-affinity sites. Here we show that the S2 site, which has not yet been identified by crystallographic approaches, can be blocked during preparation of detergent-solubilized LeuT, thereby obscuring its crucial role in Na^+-coupled symport. This finding brings to light the caution needed in the selection of experimental environments in which the properties and mechanistic features of membrane proteins can be delineated.

Jonathan A. Javitch, M.D., Ph.D., Columbia University College of Physicians and Surgeons, Center for Molecular Recognition, 630 West 168th Street, P&S 11-401, New York, NY 10032, USA; Phone: 212-305-3974; Facsimile: 212-305-5594; jaj2@columbia.edu.

AUTHOR CONTRIBUTIONS

M.Q. designed, carried out, and analyzed the functional characterization of LeuT. B.Z. expressed, purified, and helped with the preparation of LeuT. J.A.J. helped design the functional characterization, and, with L.S. and H.W., helped to interpret the data. All the authors participated in writing and editing the manuscript.

According to the first reported LeuT structure[1] (pdb 2A65) one leucine (Leu) substrate is bound in an occluded centrally-located binding site, termed the primary substrate (S1) binding site, next to two Na^+ ions. Subsequent LeuT structures revealed other amino acid substrates in place of Leu in the S1 site with an essentially identical protein structure[2]. Although density in an extracellular vestibule ~11 A above the Leu-bound S1 site was originally interpreted as water molecules by Singh et al.[2] (see Fig. S7 in ref. 2), this structure was refined to show a molecule of n-octyl-β-D-glucopyranoside (OG) detergent in this position (pdb 3F3E, with an all-Cα RMSD of 0.17 against pdb 2A65)[2], in agreement with another structure that resolved OG in this site (pdb 3GJC)[3]. In other LeuT structures, tricyclic antidepressants (TCAs) were bound in this same extracellular vestibule above the substrate-bound S1 site[4–5], again within a structure nearly identical to the original LeuT.

In our studies of the molecular mechanism of Na^+-coupled substrate transport we identified a second high-affinity substrate (S2) binding site in the extracellular vestibule using computational molecular dynamics simulations in conjunction with radiotracer binding and flux experiments[6]. The position and functional role of the substrate in the S2 site led us to propose a mechanistic model of Na^+-coupled symport in which intracellular release of Na^+ and S1 substrate is triggered by the binding of a second substrate molecule in the S2 site[6]. In functional studies, this mechanism was shown to be blocked if the S2 site is disrupted by mutations[6] or by its occupation with TCAs[5–6] or OG[3], the detergent used for all LeuT crystallization. The disruption of the S2 site by these means abolishes transport of alanine (Ala), a well transported LeuT-substrate, and eliminates the characteristic dynamics of the Ala-induced intracellular gating process we observed with single-molecule imaging[7]. A compelling feature of the dynamic model proposed from these studies, in which long range allosteric effects determine functional processes, is that it explains experimental evidence obtained for other transporters that are known to share the LeuT-like fold, even when details are different, such as for ApcT[8], and that it agrees with data for the homologous eukaryotic NSSs for which structures are not yet available (e.g., see ref. 9,10).

The role of two substrate binding sites in this model has been challenged by a recent study reporting on a variety of binding measurements that seemed to lead to the conclusion that LeuT has only a single high-affinity substrate site[11]. As described here, we have sought to eliminate the possibility that this discrepancy is due to methodological limitations. To this end we performed equilibrium dialysis studies to measure the molar Leu-to-LeuT ratio using LeuT-WT, and LeuT mutants with either an impaired S1 site (LeuT-F253A)[7,12] or S2 site (LeuT-L400S)[3,6–7]. We found the stoichiometry of LeuT-WT to be 2, whereas disruption of either binding site led to a stoichiometry of 1, identical to our results using scintillation proximity analysis[6]. However, we now show that Leu binding in the S2 site can be impaired by the protein-preparation procedures used for crystallography and for some functional studies, which explains the apparent discrepancy in reported stoichiometry. When these protein preparation procedures are used, the apparent stoichiometry is 1 and the functional mechanism that depends on the interplay between the substrate effects in the doubly occupied transporter is masked.

RESULTS

LeuT has two high-affinity substrate binding sites

Consistent with our previous findings, the equilibrium dialysis experiments performed in order to determine the molar Leu-to-LeuT ratio showed that one molecule of LeuT-WT binds 1.94±0.05 (n=4) molecules of ^{3}H-Leu with a dissociation constant (K_d) of 57.3±5.8 nM (Fig. 1a), whereas one molecule of LeuT-L400S or -F253A binds only 1.1±0.04 (n=2) or 1.01±0.04 (n=2) molecule of Leu, with a K_d of 103.2±12.2 nM or 110.2±17.5 nM, respectively (Fig. 1b & c). Notably, all three constructs exhibit similar affinities for Leu, supporting our previous conclusion that the two binding sites exhibit comparable high-affinity Leu binding[3,6]. Furthermore, we observed inhibition of ^{3}H-Leu binding by clomipramine (CMI), a TCA that was shown to bind in the extracellular vestibule[4] and to inhibit about 50% of ^{3}H-Leu binding by LeuT-WT[6], again in contrast to Piscitelli et al.[11]. Equilibrium dialysis showed, as our previous studies have, that 1 mM CMI inhibited ^{3}H-Leu binding by LeuT-WT to 45.2±4.7% (n=3) of binding in the absence of CMI. (Fig. 1d), consistent with the notion that CMI blocks Leu binding to the S2 site, as would be expected based on its localization in the S2 site in the crystal structure[4–5]. Moreover, CMI abolished almost completely the binding of Leu by the S1 site mutant LeutT-F253A (5.8% residual binding compared to that in the absence of CMI; n=2) (Fig. 1d) that can bind Leu only in the S2 site[7,12]. In contrast, 1 mM CMI did not affect ^{3}H-Leu binding by the S2 site mutant LeuT-L400S (95.6% residual binding compared to that in the absence of CMI; n=2) (Fig. 1d). The stark disagreement between the results of our equilibrium dialysis binding experiments and those reported by Piscitelli et al.[11] raised the interesting question about the experimental conditions that could lead to such contrasting findings about the behavior of these membrane protein systems.

The S2 site can be impaired by concentrating the protein

All of our binding experiments described above were performed with protein that was used after immobilized metal chelate chromatography (IMAC). Surprisingly, we found that concentrating LeuT-WT about 10-fold by centrifugal filtration reduced the binding stoichiometry of Leu-to-LeuT from about 2 to 1 (Fig. 2a). In contrast, the binding stoichiometry of the S2 site mutant LeuT-L400S remained ~1, regardless of whether or not the protein had been previously concentrated (Fig. 2b), whereas binding to LeuT-F253A, in which the mutation eliminates binding to the S1 site, was almost abolished by concentrating the protein prior to the assay (Fig. 2c). This behavior was observed when the binding assay was performed by SPA (Fig. 2a–c) or by equilibrium dialysis (Fig. 2d). Since only Leu binding in the S2 site was impaired by concentrating the protein, we conclude that concentrating LeuT can obscure the S2 site, mimicking the effect of the S2 site blocker CMI or the detergent OG[3]. Consistent with this inference, when previously concentrated LeuT-WT was used, addition of CMI had no appreciable effect on Leu binding activity (Supplementary Fig. 1).

Detergent affects LeuT activity

Because the elucidation of functional mechanisms of this transporter, and indeed membrane proteins in general, is strongly dependent on the type of biochemical experiments

represented here, it is essential to understand why concentrating the protein blocks Leu binding to the functionally important S2 site. We have previously shown that the detergent OG, like CMI, binds to the S2 site[3,7], and that competition for S2 binding blocks the dynamic cooperativity between substrate bound in the S2 and S1 sites[3,7] that is required for the Na^+-coupled symport model we proposed. Therefore, we hypothesized that raising the concentration of n-dodecyl-β-D-maltopyranoside (DDM), in which the binding studies described here were performed, might also impair binding to the S2 site. Indeed, concentrating LeuT by centrifugal filtration led to an essentially proportional increase in DDM concentration (Supplementary Fig. 2). The loss of Leu binding with increase in DDM concentration was found to be progressive (Fig. 3a), but much slower than that produced by OG. Thus, addition of 1.17% OG rapidly reduced binding of 500 nM ^{3}H-Leu by LeuT-WT to about 50% of that observed in 0.1% DDM. The half time of binding loss observed when increasing the DDM concentration in the assay buffer to 0.3% was ~250 hours, with a stable plateau at half of the original binding. Further, consistent with our hypothesis that the S2 site is specifically impaired by detergent, Leu binding to the S2-site mutant LeuT-L400S was unaffected either by addition of OG[3] or by raising the DDM concentration to 0.3% (Fig. 3b). In contrast, when binding is limited to the S2 site alone by the F253A mutation, OG or elevated DDM nearly eliminated Leu binding with half-time constants similar to those for WT (Fig. 3c). All these data show that the increased concentration of DDM causes a specific, time-dependent elimination of Leu binding in the S2 site. Notably, Chae et al.[13] recently demonstrated that following an increase in the final concentration of DDM from ~0.05 (w/v)% to ~0.21 (w/v)%, ^{3}H-Leu binding to LeuT was reduced by about 50% over a period of 12 days. These authors attributed the loss of binding to instability and denaturation of LeuT in DDM, but we have found that Leu binding by LeuT is stable for at least several weeks in the presence of 0.1 (w/v)% DDM[3,6], suggesting that Chae et al.[13] were observing a slow loss of S2 binding induced by the increase in DDM concentration. Likewise, Piscitelli et al. used protein that was concentrated by centrifugal filtration[11], apparently leading to loss of Leu binding to the S2 site.

The integrity of the S2 site can be preserved by immediate dilution of concentrated LeuT in 0.1% DDM-containing assay buffer[3]. However, when the protein was kept in 0.3% DDM in the absence of Leu, and then assayed in 0.1% DDM, LeuT-WT exhibited a rapid loss of ^{3}H-Leu binding, reaching 50% binding with a half time of only ~0.4 h (Fig. 3d). This is in contrast to the much slower loss of ^{3}H-Leu binding in 0.3% DDM observed in the presence of 500 nM Leu (Fig. 3a), showing that the presence of Leu in the S2 site slows by nearly 3-orders of magnitude the loss of Leu binding to the S2 site caused by increasing the concentration of DDM. To determine the concentration dependence of the inhibitory effect of DDM on the ‘unprotected’ S2 site, we measured binding of ^{3}H-Leu by LeuT-WT that had been pre-incubated in the presence of increasing concentrations of DDM for 2 h. The threshold-like effect between 0.15% and 0.175% DDM (Supplementary Fig. 3) - far above the critical micelle concentration (CMC) - seems inconsistent with a simple bi-molecular binding reaction and invokes the abrupt transitions in the lipid-water phase diagram.

Na^+/substrate symport requires S1 and S2 binding

As expected, the elimination of the S2 site by elevated DDM affects the mechanistic picture of LeuT-mediated Na^+-coupled transport. The interaction between substrate molecules bound simultaneously in the S1 and S2 sites was demonstrated previously by trapping ^{3}H-Leu in the S1 site, emptying the S2 site, and then rebinding non-labeled Leu in the S2 site in the absence of Na^+, which triggers the inward release of Leu from the S1 site (Fig. 4a)[3,6]. LeuT-WT that was subjected to extended treatment with 0.3% DDM still traps ^{3}H-Leu in the S1 site, but adding Leu in the absence of Na^+ fails to induce the release of ^{3}H-Leu from the S1 site (Fig. 4a), consistent with the loss of Leu binding to the S2 site where it must act as a symport effector to enable transport.

Specific lipid requirements have been demonstrated before for transporters and channels[14–15]. Because we found for LeuT that the increase in DDM concentration was accompanied by a reduction in the lipid-to-protein ratio (Supplementary Fig. 2), we tested the addition of polar *E. coli* lipids at a concentration typically used for subsequent reconstitution into proteoliposomes, but this failed to prevent or restore the loss of Leu binding to the S2 site observed in the presence of 0.3% DDM (Supplementary Fig. 4). The possibility of restoring the S2-related functional mechanism by reconstitution of concentrated LeuT into liposomes made of polar *E. coli* lipids was tested by adding Biobeads to remove the detergent[16–18]. Indeed, we found that reconstitution of LeuT-WT and -F253A into proteoliposomes restored S2 binding, as we observed essentially identical activity patterns for WT and the mutants regardless of whether we used non-concentrated or concentrated material for the reconstitution (Fig. 4b). Because Ser substitution of Leu at position 400 abrogates binding to the S2 site, ^{3}H-Leu binding by LeuT-L400S was comparable in detergent-solubilized form or reconstituted into proteoliposomes, regardless of whether or not the material had been concentrated (Fig. 4b). This is consistent with the notion that concentrating the protein affects the S2 site specifically. In contrast, restoration of the S2 site by reconstitution led to a binding stoichiometry for LeuT-WT twice that of LeuT-L400S, consistent with recovery of binding to the S2 site. Moreover, whereas binding to the S1-site mutant LeuT-253A was negligible after pre-incubation in 0.3% DDM, binding to the S2 site was restored after reconstitution. Not surprisingly, the efficiency of reconstitution was not perfect, and we measured a stoichiometry of ~1.5 molecules of Leu per molecule of LeuT-WT (at 500 nM ^{3}H-Leu), while the variants with impaired S2 or S1 sites (LeuT-L400S and -F253A, respectively) bound ~0.8 Leu/LeuT after their reconstitution into proteoliposomes (Fig. 4b). Further consistent with the mechanistic proposal that intact S1 and S2 sites are required for Na^+-coupled transport, reconstituted LeuT-WT mediated robust Ala uptake activity, regardless of whether concentrated or non-concentrated material was used (Fig. 4c). In contrast, loss of a functional S2 or S1 site (by mutation of L400S or F253A, respectively), resulted in an impaired transport phenotype that was indistinguishable from that observed in control liposomes without LeuT (Fig. 4c).

DISCUSSION

The breakthrough structural context from the first high-resolution structure of LeuT[1] informed computational studies combined with binding and flux experiments[6] that revealed

a second high-affinity substrate (S2) binding site in an extracellular vestibule also shown to bind tricyclic antidepressants (TCAs)[4–5]. We proposed a model of Na^+-coupled symport in which intracellular release of Na^+ and S1 substrate is triggered by the binding of a second substrate in the S2 site. The transport mechanism was shown to be blocked if the S2 site is disrupted by mutations[6] or by its occupation with TCAs[4–5] or octylglucoside[3]. Single molecule imaging also demonstrated that disrupting the S2 site prevents not only transport but also the enhancement of intracellular gate dynamics induced by a well-transported substrate, Ala[7]. Thus, substrate binding in the S2 site acts cooperatively with the S1 site to control the Na^+-coupled symport mechanism through intracellular gating more than 30 Å away.

We show here that the S2 site can be blocked during preparation of detergent-solubilized LeuT, thereby obscuring Na^+-coupled symport. This brings to light the need for appropriate attention to the experimental environment in delineating the properties and mechanistic features of membrane proteins. The S1 or S2 site mutants studied here (F253A and L400C) have an ‘all-or-nothing’ effect on Leu binding to the targeted site, presumably because the mutation has sufficiently impaired binding affinity to render Leu binding to the targeted site undetectable under our experimental conditions. In a search for an intermediate phenotype we discovered that the construct with a Cys instead of Asp404 in the S2 site[6] demonstrates the complex allosteric connection between the two binding sites. A mutagenesis study of the aligned Glu in SERT has implicated it in forming part of an external gate[19]. Leu binding to LeuT-D404C in 0.1% DDM (Supplementary Fig. 5a) follows a complex binding isotherm, best fit by a 2-site model with one high affinity site of 34.1±5.4 nM and a stoichiometry of 1 (constrained), and a second lower affinity site with an affinity of 238.2±121 nM and a stoichiometry of 0.47±0.07. Given the position of the mutation in the S2 site, these data provide evidence for partially disrupted binding of Leu in the S2 site. By contrast, pre-incubation of the purified protein in 0.3% DDM yielded a saturation curve that was well fit with a single-site equation with a Leu-to-LeuT stoichiometry of 1.01±0.05 and a dissociation constant of 76.8±10.8 nM (Supplementary Fig. 5b) consistent with binding to the S1 site alone. Thus, the effect of 0.3% DDM is to eliminate completely binding to the S2 site, leaving normal residual S1 binding. That impaired binding in the S2 site of the D404C mutant is accompanied by a partially-uncoupled interaction between the S1 and S2 sites in D404C, is supported by the impaired transport phenotype of this mutant, which is only 15–20% of that observed for LeuT-WT (Supplementary Fig. 5c). In contrast, as discussed above, transport is completely disrupted in F253A and L400C (Fig. 4c), which have lost the ability to bind substrate in the S1 or S2 sites, respectively (Fig. 4b). The structural underpinning of the altered Leu-binding and transport behavior of LeuT-D404C may relate to the observation that Asp404 forms salt-bridge/cation-π interactions with Arg30 and Phe253, the latter of which functions as the “gate” to the S1 site. Asp404 also forms an H-bond with Tyr107, at the bottom of the S2 site. From our *in silico* mutagenesis (with alchemical free energy perturbation/molecular dynamics (FEP/MD)) we find that the mutation of Asp404 and the associated loss of the Asp404-Tyr107 H-bond alter the dynamics of the aromatic cluster between the S1 and S2 sites[12], and thereby disrupt efficient formation of the S2 site.

The findings described here highlight a dramatic and unexpected example of the profound effects that common protein-preparation procedures can have on the observation and measurement of properties and mechanism of LeuT function. Indeed, here they are shown to obscure the nature and extent of ligand binding and dynamic interactions between the S1 and S2 sites of LeuT. These findings reaffirm our previous observations that Na^+-coupled substrate transport requires the high-affinity binding of substrate in both the S1 and S2 sites of LeuT to produce the long-range conformational gating dynamics suggested from the computational simulations, and measured in the EPR and smFRET experiments[6,7,12]. It is clear, however, that the preparation procedure can impair Leu binding to the S2 site, and thus the allosteric transport mechanism. Because the communication between the sites in the doubly occupied transporter is essential, the mechanism will not work when one site is occluded by experimental conditions. Given the widely recognized role of allosteric mechanisms in the function of membrane proteins[20–24], and the great interest in biophysical experimentation to reveal functional mechanisms, our results inform about important considerations regarding effects that detergent and lipids can have on mechanistic inferences. We show that the mechanistic investigation of the rapidly increasing number of membrane protein structures that are likely to use the same type of mechanisms, in which long range allosteric effects determine functional processes, requires specific attention to the effects of preparations that can dramatically alter protein function and lead to misinterpretation of structural data. It is thus imperative to develop new methods, and/or combine existing ones, to delineate the key mechanistic features with appropriate attention to specific requirements for the properties of the environment in which these protein systems are studied, both experimentally and computationally.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank MiEstrella Miller-Cruz for the preparation of membranes and Sebastian Stolzenberg for help with the FEP/MD simulations. This work was supported in part by National Institutes of Health Grants DA17293 and DA022413 (J.A.J.), U54GM087519 (H.W. & J.A.J.), DA12408 (H.W.), and DA023694 (L.S.).

References

1. Yamashita A, Singh SK, Kawate T, Jin Y, Gouaux E. Crystal structure of a bacterial homologue of Na^+/Cl^--dependent neurotransmitter transporters. Nature. 2005; 437:215–223. [PubMed: 16041361]
2. Singh SK, Piscitelli CL, Yamashita A, Gouaux E. A competitive inhibitor traps LeuT in an open-to-out conformation. Science. 2008; 322:1655–1661. [PubMed: 19074341]
3. Quick M, et al. Binding of an octylglucoside detergent molecule in the second substrate (S2) site of LeuT establishes an inhibitor-bound conformation. Proc Natl Acad Sci U S A. 2009; 106:5563–5568. [PubMed: 19307590]
4. Singh SK, Yamashita A, Gouaux E. Antidepressant binding site in a bacterial homologue of neurotransmitter transporters. Nature. 2007; 448:952–956. [PubMed: 17687333]
5. Zhou Z, et al. LeuT-desipramine structure reveals how antidepressants block neurotransmitter reuptake. Science. 2007; 317:1390–1393. [PubMed: 17690258]

6. Shi L, Quick M, Zhao Y, Weinstein H, Javitch JA. The mechanism of a neurotransmitter:sodium symporter – inward release of Na^+ and substrate is triggered by substrate in a second binding site. Mol Cell. 2008; 30:667–677. [PubMed: 18570870]
7. Zhao Y, et al. Substrate-modulated gating dynamics in a Na^+-coupled neurotransmitter transporter homologue. Nature. 2011; 474:109–113. [PubMed: 21516104]
8. Shi L, Weinstein H. Conformational rearrangements to the intracellular open states of the LeuT and ApcT transporters are modulated by common mechanisms. Biophys J. 2010; 99:L103–105. [PubMed: 21156121]
9. Kniazeff J, et al. An intracellular interaction network regulates conformational transitions in the dopamine transporter. J Biol Chem. 2008; 283:17691–17701. [PubMed: 18426798]
10. Shan J, Javitch JA, Shi L, Weinstein H. The substrate-driven transition to an inward-facing conformation in the functional mechanism of the dopamine transporter. PLoS One. 2011; 6:e16350. [PubMed: 21298009]
11. Piscitelli CL, Krishnamurthy H, Gouaux E. Neurotransmitter/sodium symporter orthologue LeuT has a single high-affinity substrate site. Nature. 2010; 468:1129–1132. [PubMed: 21179170]
12. Claxton DP, et al. Ion/substrate-dependent conformational dynamics of a bacterial homolog of neurotransmitter:sodium symporters. Nat Struct Mol Biol. 2010; 17:822–829. [PubMed: 20562855]
13. Chae PS, et al. Tandem facial amphiphiles for membrane protein stabilization. J Am Chem Soc. 2010; 132:16750–16752. [PubMed: 21049926]
14. Robertson JL, Kolmakova-Partensky L, Miller C. Design, function and structure of a monomeric ClC transporter. Nature. 2010; 468:844–847. [PubMed: 21048711]
15. Bogdanov M, Heacock P, Guan Z, Dowhan W. Plasticity of lipid-protein interactions in the function and topogenesis of the membrane protein lactose permease from Escherichia coli. Proc Natl Acad Sci U S A. 2010; 107:15057–15062. [PubMed: 20696931]
16. Rigaud JL, Pitard B, Levy D. Reconstitution of membrane proteins into liposomes: application to energy-transducing membrane proteins. Biochim Biophys Acta. 1995; 1231:223–246. [PubMed: 7578213]
17. Quick M, Javitch JA. Monitoring the function of membrane transport proteins in detergent-solubilized form. Proc Natl Acad Sci U S A. 2007; 104:3603–3608. [PubMed: 17360689]
18. Quick M, Wright EM. Employing *Escherichia coli* to functionally express, purify, and characterize a human transporter. Proc Natl Acad Sci U S A. 2002; 99:8597–8601. [PubMed: 12077304]
19. Cao Y, Li M, Mager S, Lester HA. Amino acid residues that control pH modulation of transport-associated current in mammalian serotonin transporters. J Neurosci. 1998; 18:7739–7749. [PubMed: 9742144]
20. Barrondo S, Salles J. Allosteric modulation of 5-HT(1A) receptors by zinc: Binding studies. Neuropharmacology. 2009; 56:455–462. [PubMed: 18951909]
21. Lacroix J, et al. Kinetic analysis of the regulation of the Na^+/H^+ exchanger NHE-1 by osmotic shocks. Biochemistry. 2008; 47:13674–13685. [PubMed: 19035652]
22. Kuszak AJ, et al. Purification and functional reconstitution of monomeric mu-opioid receptors: allosteric modulation of agonist binding by Gi2. J Biol Chem. 2009; 284:26732–26741. [PubMed: 19542234]
23. Bai F, et al. Conformational spread as a mechanism for cooperativity in the bacterial flagellar switch. Science. 2010; 327:685–689. [PubMed: 20133571]
24. Stahl E, Ellis J. Novel allosteric effects of amiodarone at the muscarinic M5 receptor. J Pharmacol Exp Ther. 2010; 334:214–222. [PubMed: 20348203]

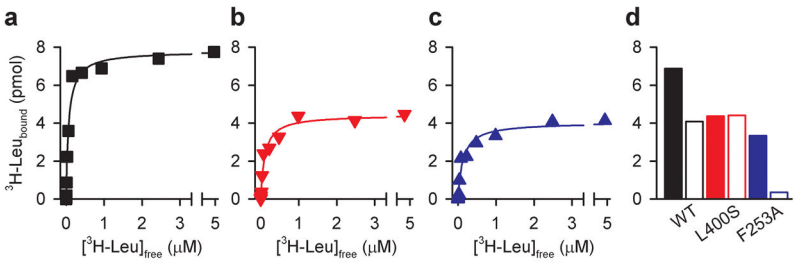

Figure 1.

LeuT has two high affinity Leu binding sites. Equilibrium dialysis was performed in 150 mM Tris/Mes, pH 7.5, 50 mM NaCl, 1 mM TCEP, 0.1 (w/v)% n-dodecyl-β, D-maltopyranoside (DDM) using 4 pmol of LeuT-WT (**a**), -L400S (**b**), and -F253A (**c**) in the presence of increasing concentrations of ^{3}H-Leu ranging from 0.5 nM to 5 μM. Dissociation constants (K_d) and molar binding ratios were determined by fitting the data with a hyperbolic non-linear regression model and shown in the text. (**d**) Clomipramine inhibits Leu binding to the S2 site. Equilibrium dialysis using LeuT-WT (black), -L400S (red), or -F253A (blue) was performed at 1 μM ^{3}H-Leu in the presence (open bars) or absence (solid bars) of 1 mM clomipramine (CMI). Data shown are from representative experiments that were repeated ≥2 times.

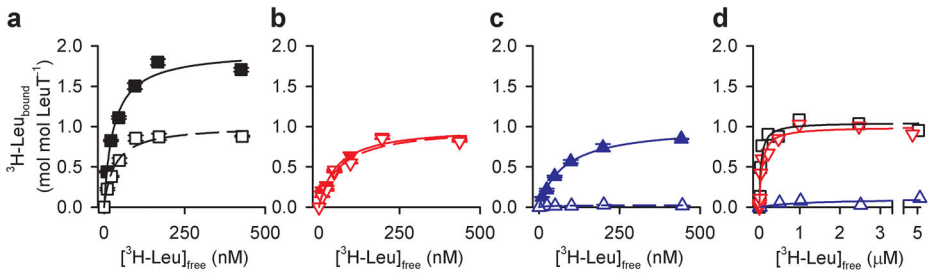

Figure 2.

Impairment of Leu binding to the S2 site in LeuT. Equilibrium binding was performed by means of the scintillation proximity assay (SPA) with 0.4 pmol LeuT-WT (**a**), -L400S (**b**), or -F253A (**c**) in the presence of increasing concentrations of ^{3}H-Leu in 0.1 (w/v)% n-dodecyl-β, D-maltopyranoside (DDM). The solid symbols represent protein that was assayed without further treatment after IMAC purification, whereas the open symbols indicate protein samples that were concentrated 10-fold 72 h prior to performing the SPA. Data are from a representative experiment and error bars indicate the SEM of triplicate determinations. To determine the kinetic constants, data of independent experiments (n≥2) were subjected to one-site binding global fitting, yielding stoichiometries of 1.95±0.06 and 1.02±0.04 for non-concentrated and concentrated LeuT-WT, respectively, with a K_d of 29.8±3.4 nM and 33.3±4.8 nM. For LeuT-L400S the stoichiometry and K_ds of the non-concentrated and concentrated sample, were 1.0±0.05 and 0.99±0.05, and 54.6±11.6 nM and 64.9±11.1 nM, respectively, whereas the stoichiometry and K_d for non-concentrated LeuT-F253A was 1.01±0.03 and 77.8±3.3 nM. Concentrating LeuT-F253A greatly impaired Leu binding, thereby precluding meaningful data fits. (**d**) Representative equilibrium dialysis experiment using concentrated LeuT-WT (□), -L400S (▽), or -F253A (△). Whereas LeuT-F253A exhibit only marginal Leu binding activity (n=2), LeuT-WT and -L400S revealed stoichiometries of 1.03±0.03 (n=3) and 1.05±0.04 (n=2) with a K_d of 45.8±10.7 nM and 67.8±14.3 nM, respectively. The composition of the assay buffer used for equilibrium dialysis was identical to that used for the experiments shown in Fig. 1.

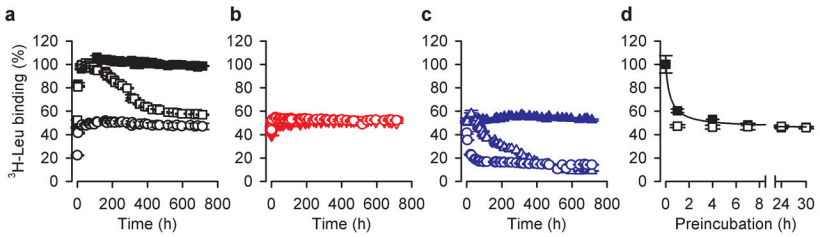

Figure 3.

Effect of detergent on LeuT binding activity. 500 nM ^{3}H-Leu binding by 0.4 pmol LeuT-WT (**a**), -L400S (**c**), or -F253A (**c**) was assayed with the SPA after IMAC in the presence of 0.1 (w/v)% DDM (■, ▼, ▲, respectively), 0.3 (w/v)% DDM (□, ▽, △), or 1.17 (w/v)% OG (○,○,○) and plotted as a function of time. (**d**) Effect of DDM on Leu equilibrium binding. IMAC-purified LeuT-WT (in 0.1 (w/v)% DDM) was pre-incubated with 0.3 (w/v)% DDM for the indicated periods of time and subjected to SPA-mediated binding of 500 nM ^{3}H-Leu in 0.1 (w/v)% DDM (■) or 0.3 (w/v)% DDM (□) for 16 h (equilibrium). Error bars in all panels are the SEM of triplicate determinations from representative experiments that were repeated ≥2 times.

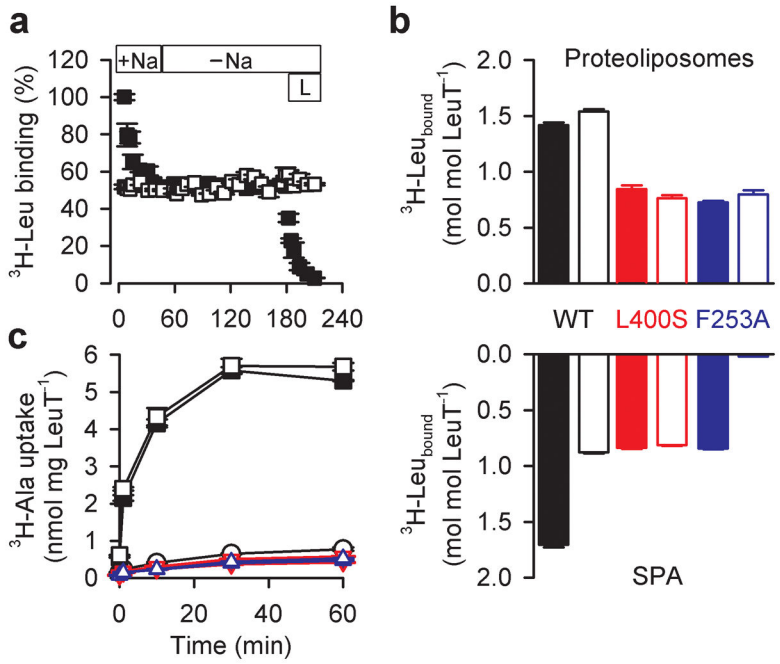

Figure 4.

Intact S1 and S2 sites are required for Na^+-coupled transport by LeuT. (**a**) Dissociation of 1 μM ^{3}H-Leu from LeuT-WT by dilution into 50 mM Na^+ (+Na) and into Na^+-free (−Na) media. Samples were pre-incubated in the presence of 0.1 (w/v)% DDM (■) or 0.3 (w/v)% DDM (□) for ~500 h. Release of ^{3}H-Leu trapped in the S1 site of LeuT assayed in 0.1 (w/v) % DDM was achieved by the addition of 2.5 μM Leu (L). (**b**) Binding of 500 nM ^{3}H-Leu to 0.4 pmol LeuT-WT (black), -L400S (red), or -F253A (blue) in 0.1 (w/v)% DDM measured by SPA (lower panel) for 16 h using non-concentrated (solid bars) or previously concentrated (open bars) material. Binding of 500 nM ^{3}H-Leu to 0.4 pmol of protein was also assayed after reconstitution of previously concentrated or non-concentrated LeuT-WT, -L400S, or -F253A into proteoliposomes (upper panel). Equilibrium binding in proteoliposomes was performed for 4 h in the presence of 25 μg gramicidin/mL (5-min pre-treatment) to dissipate the Na^+ electrochemical gradient, followed by capture of LeuT-containing proteoliposomes onto 0.22 μm nitrocellulose filters and subsequent scintillation counting. (**c**) Time course of Na^+-coupled uptake of 1 μM ^{3}H-Ala in proteoliposomes reconstituted with LeuT-WT, - L400S, or -F253A from non-concentrated (■,▼, ▲, respectively) or concentrated (□, ▽, △) material or in control liposomes (○). Error bars in all panels are the SEM of triplicate determinations from representative experiments that were repeated ≥2 times.