Interaction of α-Lipoic Acid with the Human Na⁺/Multivitamin Transporter (hSMVT)∗

Britta Zehnpfennig†, Pattama Wiriyasermkul§, David A. Carlson‡, and Matthias Quick§‖

From the‡†Center for Molecular Recognition and §Department of Psychiatry, Columbia University College of Physicians and Surgeons, New York, New York 10032, ¶GeroNova Research, Inc., Richmond, California 94806, and †Division of Molecular Therapeutics, New York State Psychiatric Institute, New York, New York 10032

Background: Transport of α-lipoate by hSMVT and its stereospecificity have been elusive.

Results: Using hSMVT expressed in oocytes and in Pichia pastoris yielded detailed information about the stereospecificity of hSMVT-mediated lipoate transport and binding.

Conclusion: hSMVT can bind two molecules of R-(+)-α-lipoate, its physiological substrate.

Significance: In addition to biotin, pantothenate, and iodide, hSMVT mediates the transport of lipoate.

The human Na⁺/multivitamin transporter (hSMVT) has been suggested to transport α-lipoic acid (LA), a potent antioxidant and anti-inflammatory agent used in therapeutic applications, e.g. in the treatment of diabetic neuropathy and Alzheimer disease. However, the molecular basis of the cellular delivery of LA and in particular the stereospecificity of the transport process are not well understood. Here, we expressed recombinant hSMVT in Pichia pastoris and used affinity chromatography to purify the detergent-solubilized protein followed by reconstitution of hSMVT in lipid bilayers. Using a combined approach encompassing radiolabeled LA transport and equilibrium binding studies in conjunction with the stabilized R-(+)- and S-(−)-enantiomers and the R,S-(+/−) racemic mixture of LA or lipoamide, we identified the biologically active form of LA, R-LA, to be the physiological substrate of hSMVT. Interaction of R-LA with hSMVT is strictly dependent on Na⁺. Under equilibrium conditions, hSMVT can simultaneously bind ~2 molecules of R-LA in a biphatic binding isotherm with dissociation constants (Kd) of 0.9 and 7.4 μM. Transport of R-LA in the oocyte and reconstituted system is exclusively dependent on Na⁺ and exhibits an affinity of ~3 μM. Measuring transport with known amounts of protein in proteoliposomes containing hSMVT in outside-out orientation yielded a catalytic turnover number (kcat) of about 1 s⁻¹, a value that is well in agreement with other Na⁺-coupled transporters. Our data suggest that hSMVT-mediated transport is highly specific for R-LA at our tested concentration range, a finding with wide ramifications for the use of LA in therapeutic applications.

α-Lipoic acid (thiotic acid; 5-(1,2-dithiolan-3-yl)pentanoic acid; LA⁴) is a naturally occurring substance that is involved in the aerobic metabolism of all cells. LA is synthesized in small amounts in mitochondria of mammals and plants as well as in bacteria (1). The biologically active form of LA, the R-enantiomer, is endogenously synthesized de novo from octanoic acid (2), and is covalently bound to specific LA-dependent enzyme complexes (3). Most notably, R-LA serves as a cofactor in the pyruvate dehydrogenase and α-ketoglutarate dehydrogenase complexes (4), enzymes that catalyze central redox reactions related to energy production in the cell as well as in oxidative decarboxylations of α-keto acids and amino acids (5).

Because of the presence of two juxtaposed thiol groups, free LA (in its oxidized as well as in its reduced form as dihydrolipoic acid) possesses anti-inflammatory and antioxidant properties due to its ability to scavenge reactive oxygen species and reactive nitrogen species, regenerate endogenous antioxidants, and chelate toxic metals (6, 7). Synthetic LA, which predominantly exists as a racemic mixture of LA (R,S-LA), has been successfully used as a therapeutic agent for about 60 years in the treatment of, e.g. diabetic neuropathy, peripheral artery disease, and various skin and liver diseases (8–10). Recent studies indicate that LA may interfere with the pathogenesis and/or progression of Alzheimer disease (11), Parkinson disease (12), and multiple sclerosis (13). Both forms of LA, R-LA and S-LA, are widely available as dietary antioxidants (14, 15), and it is suggested that LA (in free form or as lipoyl protein) from foodstuff is absorbed in the intestine by the Na⁺/multivitamin transporter (SMVT) (16, 17) due to the fact that LA inhibited the uptake of the other two SMVT substrates, biotin and pantothenic acid, in concentration-dependent manner. However, evidence for the transport of lipoic acid or the (stereo)specificity of the interaction of lipoic acid with SMVT is rather limited (18) mostly due to the

* This work was supported, in whole or in part, by National Institutes of Health Grant DA017293 (to M. Q.). This work was also supported by National Science Foundation Grant MCB130730 (to M. Q.). The authors declare that they have no conflicts of interest with the contents of this article.

† Both authors contributed equally to this work.

‡ To whom correspondence may be addressed: GeroNova Research, Inc., 2600 Hilltop Dr., Bldg. B, Suite C120, Richmond, CA 94806. Tel.: 775-887-4907; E-mail: david@geronova.com.

§ To whom correspondence may be addressed: New York State Psychiatric Inst., 1051 Riverside Dr., Unit 25, New York, NY 10032. Tel.: 646-774-8604; E-mail: mq2102@cumc.columbia.edu.

¶ The abbreviations used are: LA, α-lipoic acid; (h)SMVT, (human) Na⁺/multivitamin transporter; RAMBA, R-(+)-α-methylbenzylamine; SAMBA; S-(−)-α-methylbenzylamine; LAM, lipoamide; DDM, n-dodecyl-β-maltopyranoside; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; POPG, 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1′-racemic-glycerol); SPA, scintillation proximity assay; kcat, catalytic turnover number; Kd, dissociation constant; Vmax, maximum velocity of transport.

© 2015 by The American Society for Biochemistry and Molecular Biology, Inc. Published in the U.S.A.
lack of suitable study systems that allow the detailed kinetic characterization of the target protein without interfering proteins found in cell or membrane preparations.

To shed light on the specifics of LA transport by the human SMVT (hSMVT), we have measured radiolabeled LA uptake in the established Xenopus oocytes system expressing hSMVT and tested the effect of the racemic or enantiomeric forms of LA or lipoamide (5-(1,2-dithiolan-3-yl)pentanamide) on the transport reaction. Furthermore, we have expressed hSMVT in Pichia pastoris cells and purified the recombinant protein from the membrane of the yeast in active form, which allows for the functional characterization of hSMVT in detergent-solubilized form and reconstituted in nanodiscs and proteoliposomes.

Experimental Procedures

Instrumentation for Chemical Syntheses—A Jasco Analytical Instruments Model P2000 digital polarimeter was used. For normal phase HPLC, an Agilent 1100 HPLC system with diode array detection was used for chiral chromatography in combination with a Daicel Chiralpak AD-H column (Table 1) and validated reference standards by a method developed at GeroNova Research, Inc. Melting points were performed on a nation with a Daicel Chiralpak AD-H column (Table 1) and normal phase HPLC, an Agilent 1100 HPLC system with diode

| TABLE 1 |
|-----------------------------------------------|
| HPLC conditions | Daicel, Chiralpak AD-H, 5 μm, 25 cm (L) × 4.6 mm (ID) |
| Guard column | Daicel, Chiralpak AD-H, 8.4 cm (ID) × 1 cm |
| Mobile phase | Hexanes:ethanol with 0.5% TFA (95:5) (premixed) |
| Column temperature (°C) | 25 |
| Detection wavelength (nm) | 215 |
| Flow rate (ml/min) | 1.0 |
| HPLC sample compartment | |
| Temperature (°C) | 4 |
| Injection volume (μl) | 10 |
| Run time (min) | 25 |

Syntheses of R-(+)-α-Lipoic Acid; S-(−)-α-Lipoic Acid; and R-(−), S-(−), R,S-(−/ +)-Lipoamide—R,S-La (Tohope Pharmaceuticals, Jiangsu, China) was recrystallized twice from cyclohexane/ethyl acetate. 206.33 g (1 mol) of R,S-La was dissolved at 30–35 °C in 2 liters of toluene. 0.54 mol of R-(+)-α-methylbenzylamine (RAMBA; obtained from BASF Corp.) and >99% enantiomerically pure) in 0.1 liter of toluene was added dropwise from a dropping funnel in a steady stream over the course of 5 min with vigorous overhead stirring, causing a slight exothermic reaction (T = 40–45 °C) without exceeding 45 °C. The reaction mixture was stirred for 30 min at 40–45 °C and gradually cooled to room temperature over the course of 2 h (cooling rate of ~0.17 °C/min). The precipitate was filtered and washed twice with 35 ml of previously chilled toluene. The moist salt pair was recrystallized three to five times from 400 ml of ethyl acetate and then dried in a vacuum at <45 °C, yielding 95 g of the R-LA-RAMBA diastereomic salt pair (R-LA-RAMBA = 95 g, melting point = 110–115 °C, [α]20 D = +74.8° (c = 1; ethanol)) (Fig. 1A).

The above process was repeated with fresh R,S-LA solutions and S-(−)-α-methylbenzylamine (SAMBA), yielding 92.5 g of the S-LA-SAMBA salt pair (S-LA-SAMBA = 92.5 g, melting point = 110–115 °C, [α]20 D = −73.4° (c = 1; ethanol)). The salt pair was suspended in 3 liters of water at 25 °C and 1 liter of cyclohexane with vigorous overhead stirring. The mixture was slowly adjusted to pH 3 by 1 N citric acid and to pH 1 with 5% sulfuric acid with ice cooling. The mixture was then allowed to warm to room temperature. The phases were separated, and the aqueous phase was re-extracted once with 300 ml of cyclohexane. The combined cyclohexane extracts were dried, filtered, and cooled to 5–10 °C. The cyclohexane was seeded with R-LA or S-LA crystals post-stirred at this temperature for 5 h for crystallization. The precipitate was filtered off, rewashed with 30 ml of cyclohexane, and dried at 25 °C in a vacuum. The filtered solutions were combined and concentrated with rotary evaporation for further processing. The toluene mother liquor was washed with dilute HCl and H2O and analyzed for the enantiomer composition, and appropriately adjusted amounts of RAMBA or SAMBA were added to isolate the crude and opposite enantiomer as in the initial resolution step. In each case, for the test compounds utilized in this study, only the initially isolated salt pairs were used after three to five recrystallizations. One mole of R,S-LA yielded 48.0 g of R-LA (23.6% based on R,S-LA, melting point = 44–46 °C (sinter), [α]20 D = +121° (c = 1; ethanol)) and 45.4 g of S-LA (22% based on R,S-LA, melting point = 43–45 °C (sinter), [α]20 D = −118° (c = 1; ethanol)) (Fig. 1A).

The three amides (R,S-(−/ +)-lipoamide (R,S-LAM), R-(−)-lipoamide (R-LAM), and S-(−)-lipoamide (S-LAM)) were made by a modification of the procedure originally patented by E. Merck, Darmstadt, Germany for preparation of R,S-lipoylpyrdoxamine (19) (Fig. 1B). 51.5 g (0.25 mol) of R-LA was dissolved in 200 ml of anhydrous THF and 34.9 ml of triethylamine (25.56 g, 0.25 mol, 99%) chilled to −25 °C with vigorous overhead stirring. 24.6 ml of prechilled ethyl chloroformate (28 g, 0.25 mol, 97%) in 50 ml of prechilled anhydrous THF was added dropwise with continued and vigorous stirring. Triethylamine HCl precipitated immediately. The mixture was stirred at −25 °C for 30 min, filtered under dry nitrogen in a closed funnel, and washed with dry THF into a second dry reaction vessel. The THF solution of the mixed R-lipoformyl anhydride was rechilled at −25 °C with vigorous overhead stirring while 200 ml of 28% NH4OH solution was added dropwise at −25 to −10 °C over the course of 1 h. The mixture was allowed to warm to room temperature over the course of another hour. The solvent was removed on a rotary evaporator under reduced
pressure with the bath temperature not exceeding 45 °C. The evaporation flask was cooled, and the crude LAMs were redisolved in ethyl acetate. The ethyl acetate solutions were washed once with H2O, extracted twice with dilute (5%) NaHCO3 solution to remove any unreacted LA, dried with Na2SO4, and filtered into a dry evaporating flask. The ethyl acetate solution was reduced to 25% of its initial volume and allowed to cool to room temperature. Crude LAM was filtered and rinsed with chilled, fresh ethyl acetate with the solvent collected into a dry evaporator flask. The process was repeated to reduce the volume by 25% and cooled. The crude LAMs from the two filtrations were combined and recrystallized once from ethanol and once from acetone, yielding sparkling yellow flakes. The crystals were filtered and dried under vacuum (Fig. 1B).

Expression of hSMVT in Xenopus laevis Oocytes and Functional Studies—The mRNA transcript for hSMVT was produced in vitro with the Ambion MegaScript kit using the T3 RNA polymerase and an RNA cap analog following NotI linearization of pMV1 (18). X. laevis oocytes were injected with 50 ng of mRNA transcript for hSMVT and subsequently incubated in Barth’s medium containing gentamicin (5 mg/ml) at 18 °C for 3–6 days.

Transport of R,S- or R-[3H]LA (both 12 Ci/mmol; American Radiolabeled Chemicals, Inc.) was performed in hSMVT-expressing oocytes or control oocytes in assay buffers composed of 10 mM HEPES/Tris, pH 7.4 (or 5.5 when indicated), 2 mM KCl, 1 mM MgCl2, 1 mM CaCl2, and either 100 mM NaCl, cholinel chloride, sodium gluconate, or lithium chloride (LiCl). Oocytes were removed from the assay buffer after the indicated period of time and subsequently washed with ice-cold assay buffer without radiotracer followed by scintillation counting after solubilization of individual oocytes with 100 μl of 5% SDS. All uptake experiments were performed at least in duplicate with oocytes from different donor frogs. Data are expressed as mean ± S.E. of 7 oocytes, kinetic constants were obtained by non-linear regression fitting of the data with Prism 5 (GraphPad), and errors indicate the S.E. of the fit.

Production of hSMVT in P. pastoris—For immunological detection and affinity chromatography, we engineered a recombinant hSMVT construct that carries a C-terminal FLAG epitope and a hexahistidine (His) tag by using standard PCR protocols with synthetic mutagenic oligonucleotides using pMV1 as a template. All modifications in the resulting plasmid pMV1-FH were verified by DNA sequencing, and activity measurements in oocytes revealed virtually indistinguishable activity between native and recombinant hSMVT. The cDNA of recombinant hSMVT was introduced into methanol-inducible pPICHOLI vector (MoBiTec, Inc.) and introduced into P. pastoris KM71H cells. Transformed cells were cultured by consecutive growth at 29 °C, and the expression of hSMVT was induced by replenishing the inducer methanol (at 0.5% (v/v)) every 12 h at 20 °C for 1.5 days in growth medium with 50% reduced yeast extract and Bacto™ tryptone (BD Biosciences). Purification of Recombinant hSMVT—P. pastoris cells expressing recombinant hSMVT were disrupted by a 5-fold passage through an EmulsiFlex-C3 homogenizer (Avestin) at
27,000 p.s.i., followed by a Bead Beater (BioSpec) (20). Unbroken cells and cell debris were removed by centrifugation at 10,000 × g for 10 min before membrane vesicles were collected at 292,000 × g for 45 min. Membrane vesicles were suspended at a final protein concentration of 20 mg/ml in 50 mM Tris/Mes, pH 7.5, 150 mM NaCl, 20% (v/v) glycerol, 1 mM tris(2-carboxyethyl)phosphine, and EDTA-free protease inhibitor mixture (Roche Applied Science) and mixed with an equal volume of 8 M urea for 20 min at 4 °C. Urea-treated membrane vesicles were collected after centrifugation at 369,879 × g for 2 h and solubilized with 1.5% (w/v) n-dodecyl-β-D-maltopyranoside (DDM) for 60 min at 4 °C. The insoluble fraction was removed by ultracentrifugation (250,000 × g), and the supernatant was bound to pre-equilibrated anti-FLAG M2 (α-FLAG) affinity gel resin (Sigma) with a gel:supernatant ratio of 1:10 (v/v) at 4 °C for 4 h with gentle shaking. Unbound protein was removed by washing with 10 column volumes of 150 mM Tris/Mes, pH 7.5, 50 mM NaCl, 20% (v/v) glycerol, 1 mM tris(2-carboxyethyl)phosphine, and 0.1% (w/v) DDM. hSMVT was eluted by competition with 150 μg/ml FLAG peptide.

Immunological Detection of hSMVT—Immunological detection of recombinant hSMVT in oocytes was performed with the mouse monoclonal anti-FLAG M2 IgG (Sigma) or as control with a rabbit polyclonal antibody (SMVT-11A; Alpha Diagnostic, San Antonio, TX; data not shown) after solubilization of the oocytes as described (18). Immunoreactive bands were visualized with suitable horseradish peroxidase-conjugated secondary antibodies in conjunction with the enhanced chemiluminescence (ECL) method (SuperSignal West Pico kit, Thermo Scientific). The monoclonal anti-FLAG M2 antibody was used to detect recombinant hSMVT in membrane vesicles of P. pastoris or in detergent-solubilized form during purification.

Reconstitution of hSMVT-containing Nanodiscs—MSP1E3D1 and lipids were prepared as described (21) with small modifications. Briefly, MSP1E3D1 was expressed in Escherichia coli BL21(DE3) containing the MSP1E3D1-expressing plasmid (Addgene). The protein was purified using the PrepdEase Histidine-tagged High Yield Purification Resin (Affymetrix) and dialyzed for 16 h in the presence of tobacco etch virus protease to remove the hexa-His tag from the purified protein. The sample was cleared from non-cleaved MSP1E3D1 and the hexa-His tag peptide by an additional Ni²⁺-based chromatography step. E. coli polar lipids, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1’-racemic-glycerol) (POPG) (both from Avanti), and cholesterol (Sigma) were dissolved at the indicated ratios in chloroform and dried under a stream of nitrogen gas. The lipid film was resuspended at a concentration of 20 mM in 10 mM Tris, pH 7.4, 100 mM NaCl, 0.5 mM EDTA, and 0.01% NaN₃, hSMVT was assembled into nanodiscs at a 0.1:1:500 molar ratio of hSMVT:MSP1E3D1:lipids. Detergent was removed by serial addition of Biobeads SM2 (Bio-Rad). The Biobeads were removed by filtration, and the hSMVT-assembled nanodiscs were resuspended in 100 mM Tris/Mes, pH 7.4 and 1 mM tris(2-carboxyethyl)phosphine after collection by ultracentrifugation at 365,000 × g for 1 h. Samples of the preparation (nanodisc-containing pellet and supernatant) were collected after the ultracentrifugation step and analyzed by silver staining of SDS-polyacrylamide gels. The protein concentration of all samples was measured with the Amido Black protein assay (22). The concentration of hSMVT in nanodiscs was quantified from silver-stained SDS-polyacrylamide gels with densitometry using NIH ImageJ and known amounts of purified hSMVT as reference.

Scintillation Proximity Assay (SPA)-based Binding Studies—50 ng of hSMVT in purified form or reconstituted in nanodiscs was bound to 250 μg of Cu²⁺-coated yttrium silicate SPA beads in a 100-μl assay volume. R-[³H]LA binding experiments were performed in 50 mM Tris/Mes, pH 7.5, 150 mM NaCl (or 150 mM Tris/Mes), 20% glycerol, 1 mM tris(2-carboxyethyl)phosphine, and 0.1% (w/v) DDM. Equilibrium binding was performed in the dark for 16 h at 4 °C with vigorous shaking on a vibrating platform, and the counts/minute (cpm) were determined in the SPA mode of a Wallac 1450 MicroBeta™ plate PMT counter. 800 mM imidazole (which prevents the interaction of the His-tagged protein with the Cu²⁺-coated SPA beads) was added to the samples to determine the non-proximity background signal. All experiments were performed at least in duplicate with replicates of ≥3, and data are expressed as mean ± S.E. Data fits of kinetic analyses were performed using non-linear regression algorithms in Prism 5 (GraphPad), and errors represent the S.E. of the fit.

R-[³H]LA Uptake in Proteoliposomes—Purified recombinant hSMVT was reconstituted at a 1:100 (w/w) ratio in preformed, Triton X-100 (0.12%, w/v)-destabilized liposomes that were prepared from E. coli polar lipid extract in the absence or presence of 10% (w/w) cholesterol in 100 mM potassium phosphate, pH 7.5 and 2 mM β-mercaptoethanol. Prior to the uptake measurements, frozen proteoliposomes were thawed at 23 °C and extruded through a 400-nm filter. The uptake reaction was initiated by the 20-fold dilution of the proteoliposome suspension (5 mg of lipid/ml) into assay buffer composed of 10 mM HEPES, pH 7.4, 100 mM NaCl (or 100 mM choline chloride), 1 mM CaCl₂, and 1 mM MgCl₂ plus the indicated concentrations of R-[³H]LA (1 Ci/mmol) at 23 °C. Reactions were stopped by the addition of ice-cold 100 mM potassium phosphate, pH 6.0 and 100 mM LiCl and filtered through Millipore 0.22-μm nitrocellulose filters. The radioactivity retained on the filters was determined with scintillation counting. Known amounts of radioactivity were used to convert cpm to mol.

The orientation of hSMVT in hSMVT-containing proteoliposomes was determined based on the accessibility of the hexa-His tag at the hSMVT C terminus to Ni²⁺ affinity resin. 0.01 g of PrepEase Histidine-tagged High Yield Purification Resin was mixed with 1.5 μg of hSMVT-containing intact proteoliposomes and incubated for 1 h at 4 °C. The resin/protein mixture was loaded onto a disposable column, and the flow-through was collected. The resin was then washed with binding buffer (100 mM KPi, pH 7.5), and bound sample was eluted from the column with 400 mM imidazole-containing binding buffer. All fractions were collected and solubilized with 1% DDM, and normalized volumes were subjected to SDS-PAGE followed by silver staining of the gel.
Interaction of Lipoic Acid with hSMVT

Results

Characterization of the Transport of LA by hSMVT in Oocytes—In the presence of Na\(^+\), lipoate elicits electrical currents in hSMVT-expressing oocytes, and uptake of radiolabeled pantethenic acid and biotin was shown to be inhibited by lipoate with a half-maximum effective concentration between 1 and 5 \(\mu\)M lipoate (16). Direct evidence for the hSMVT-mediated translocation of lipoic acid across the plasma membrane was recently demonstrated with \(R,S\)-[\(3\text{H}\)]LA, the racemic mixture of the \(S\) and \(R\)-enantiomers, in hSMVT-expressing oocytes (18). However, to obtain reproducible data with a suitable signal-to-noise ratio, a >10 \(\mu\)M concentration of racemic \(R,S\)-[\(3\text{H}\)]LA was chosen for these uptake studies (Fig. 2A). In contrast, performing the assay with 1.66 \(\mu\)M \(R\)-[\(3\text{H}\)]LA yielded a signal-to-noise ratio of \(\sim 1:10\), a concentration that corresponds approximately to the half-maximum effective concentration determined with indirect measurements (Fig. 2B). The time course of 1.66 \(\mu\)M \(R\)-[\(3\text{H}\)]LA uptake in the presence of 100 mM NaCl exhibited a linear rate of transport for the initial 15 min with a steady state of accumulation of about 4 pmol per oocyte after 60 min (Fig. 2C). Assuming the diameter of an oocyte to be 1 mm, its internal volume is 0.523 \(\mu\)l; at equilibrium conditions with 1.66 \(\mu\)M \(R\)-LA inside and outside of the oocyte, there would be 0.87 pmol accumulated in the oocyte. These results demonstrate that hSMVT uses the Na\(^+\) electrochemical gradient to drive the accumulation of \(R\)-LA against its concentration gradient, a key feature of Na\(^+\)-coupled symport reactions (23). Uptake of \(R\)-[\(3\text{H}\)]LA transport in hSMVT-expressing oocytes was saturable with a \(K_m\) of 4.0 \(\pm\) 0.5 \(\mu\)M and a maximum velocity of transport (\(V_{\text{max}}\)) of 15.5 \(\pm\) 0.6 pmol\(\cdot\)oocyte\(^{-1}\)\(\cdot\)min\(^{-1}\) (Fig. 2D). Removing Cl\(^-\) from the assay buffer had no significant effect, confirming the Cl\(^-\) independence of hSMVT-mediated substrate transport (Fig. 2E). Furthermore, the equimolar replacement of NaCl with LiCl or lowering the pH of the uptake assay from pH 7.4 to pH 5.5 (generation of a proton gradient across the oocyte membrane) resulted in \(R\)-[\(3\text{H}\)]LA accumulation in hSMVT-expressing oocytes that was indistinguishable from that observed in control oocytes (Fig. 2F). To test whether the low signal-to-noise ratio of \(R,S\)-LA uptake could be attributed to the specific effect of \(S\)-LA on \(R\)-LA uptake or whether the effective concentration of the transported enantiomer was reduced in the racemic mixture, we performed uptake of 1.66 \(\mu\)M \(R\)-[\(3\text{H}\)]LA in the presence of non-labeled LA and lipoamide in their \(R\), \(S\), and \(R,S\) forms. Whereas the \(S\)-enantiomer of LA as well as all stereoisomers of lipoamide failed to inhibit uptake of 1.66 \(\mu\)M \(R\)-[\(3\text{H}\)]LA more than 5%, uptake in the presence of 50 \(\mu\)M \(R\)-LA was reduced to values observed in control oocytes lacking hSMVT. 50 \(\mu\)M \(R,S\)-LA reduced uptake by about 50%, consistent with the notion that the racemic mixture contains equal amounts of the biologically active \(R\)-enantiomer and the inactive \(S\)-enantiomer (Fig. 2F). These results indicate that hSMVT possesses a high specificity for \(R\)-LA, the naturally occurring and biologically active LA enantiomer. 50 \(\mu\)M biotin or pantethenic acid, the other two organic substrates of hSMVT, inhibited the uptake of 1.66 \(\mu\)M \(R\)-[\(3\text{H}\)]LA by 82.5 \(\pm\) 2.1 and 31.1 \(\pm\) 4.1%, respectively (Fig. 2F).

Functional Expression of hSMVT in P. pastoris—To facilitate the immunological detection of hSMVT in suitable expression systems and its subsequent purification, we introduced a FLAG epitope and a hexa-His tag at the C terminus of hSMVT (hereafter referred to as recombinant hSMVT). As determined with \(R\)-[\(3\text{H}\)]LA uptake studies, recombinant and unmodified
hSMVT had virtually indistinguishable transport activity in oocytes (Fig. 3A) and can be immunologically detected with mononclonal anti-FLAG M2 IgG (Fig. 3B). Building on the availability of a fully functional recombinant hSMVT, we tested expression of hSMVT in *P. pastoris*, a eukaryotic host system that was previously successfully used for the expression of other mammalian (and human) recombinant membrane proteins (24). As judged by Western blot analysis using the anti-FLAG M2 IgG, inducing the expression of recombinant hSMVT from the methanol-inducible alcohol dehydrogenase promotor readily yielded full-length recombinant hSMVT in the plasma membrane of *P. pastoris* with a similar glycosylation pattern as that observed in oocytes (18). To confirm that the immunoreactive bands at ~65 and ~58 kDa corresponded to the differently glycosylated versions of hSMVT, we subjected the sample to peptidyl *N*-glycosidase F treatment. Complete peptidyl *N*-glycosidase F digestion of the detectable bands resulted in a single band, corresponding to non-glycosylated recombinant hSMVT, at ~55 kDa (Fig. 3C). In addition to the differently glycosylated forms of hSMVT at ~55, ~58, and ~65 kDa, we occasionally detected a faint immunoreactive band at ~40 kDa that may be a degradation product of recombinant hSMVT containing the C-terminal FLAG epitope. Recombinant hSMVT was purified by anti-FLAG immune affinity chromatography (25) after extracting the protein with DDM (Fig. 3D).

**Interaction of LA with Purified Recombinant hSMVT**—To test the activity of purified hSMVT in detergent-solubilized form, we assessed binding of *R*-[^3]H]LA with the scintillation proximity assay (Fig. 4). In this assay, purified recombinant hSMVT was immobilized on copper-coated scintillation beads (using the hexa-His tag in recombinant hSMVT) that emit light only when a radioactive ligand stably binds to the immobilized target protein (26). 0.83 μM *R*-[^3]H]LA yielded a robust specific SPA signal when 50 ng (about 0.73 pmol) of purified hSMVT was used per assay (Fig. 4A). Performing the assay in the absence of NaCl yielded a signal that was indistinguishable from the non-proximity (background) signal obtained in the presence of imidazole that competes with the hexa-His-tagged protein for binding to the copper-coated SPA beads, indicative of the strict Na⁺ dependence of the interaction of LA with hSMVT and extending previous direct and indirect studies on the Na⁺ requirement for hSMVT activity (Fig. 4A).

To test the interaction of stereoisomers of LA and its derivative, we performed binding of 1.66 μM *R*-[^3]H]LA in the presence of 50 μM stereoisomers of the LA derivatives tested in oocyte uptake experiments (Fig. 2F). Again, the *S*-enantiomers of LA and lipoamide at a concentration of 50 μM did not reduce binding of 0.4 μM *R*-[^3]H]LA to hSMVT by more than 5%, whereas the *R*,*S* racemic mixtures of LA inhibited binding by about 50%. Like the *S*-enantiomers, the three tested forms of lipoamide failed to noticeably inhibit *R*-LA binding under these experimental conditions. *R*-LA inhibited binding of 0.4 μM *R*-[^3]H]LA to about 10% (Fig. 4B).

We then measured equilibrium binding of increasing concentrations of *R*-[^3]H]LA to 50 ng of purified hSMVT in the SPA (Fig. 4C). Reminiscent of substrate binding by the related solute:sodium symporter members vSGLT and PutP (27), binding of increasing concentrations of *R*-[^3]H]LA by purified hSMVT revealed a biphasic saturation binding curve under equilibrium conditions. This curve was best fit with two separate one-site models (Fig. 4C), yielding a *K*_d of 1.84 ± 0.09 molecules of LA per molecule of hSMVT. Fitting the data of the first phase (data points between 0 and 5 μM) preferred a single site model with Hill slope when compared with a single site model without Hill slope (*p* < 0.0001) and yielded a *K*_d of *R*-LA binding to hSMVT of 0.9 ± 0.03 μM with a Hill slope of 1.99 ± 0.11. Data points for the second phase (data points between 5 and 25 μM) were fitted with a single site model, yielding a *K*_d of 7.38 ± 1.44 μM.

These data indicate that the solubilization and purification of hSMVT with DDM did not adversely affect its activity. We then tested the effect of lipids on the activity of the protein reconstituted into nanodiscs of defined lipid composition. Purified hSMVT was incorporated into nanodiscs composed of polar *E. coli* lipids supplemented with 0, 6, or 10% (w/w) cholesterol or those composed of POPC/POPG (at a 3:2 (w/w) ratio) supplemented with 6% cholesterol. Incorporation of hSMVT into nanodiscs was highest in those composed of *E. coli* polar lipids in the presence of 6% cholesterol > *E. coli* polar lipids with 10% cholesterol ≥ POPC/POPG with 6% cholesterol > *E. coli* polar lipids (Fig. 4D); however, the lipid composition had virtually no effect on the binding activity at a saturating concentration of *R*-[^3]H]LA by hSMVT when the samples were normalized with regard to the amount of incorporated hSMVT (Fig. 4E).
**Interaction of Lipoic Acid with hSMVT**

**FIGURE 4. Interaction of R-LA with purified hSMVT.** Binding of R-[3H]LA to 50 ng of purified hSMVT was measured with the SPA by immobilizing recombinant hSMVT via the engineered His tag to copper-coated yttrium silicate SPA beads. A, binding of 0.83 μM R-[3H]LA (12 Ci/mmol) was assayed in the presence of 150 mM NaCl or Tris/Mes. To determine the nonspecific background binding activity, 800 mM imidazole (imid) was added to the samples because imidazole competes with the His tag of recombinant hSMVT for binding to the copper-coated SPA beads. B, effect of stereoisomers of LA and derivatives on R-[3H]LA equilibrium binding. Binding of 0.4 μM R-[3H]LA was tested in 150 mM NaCl in the presence or absence of the indicated compounds at 50 μM. C, saturation binding of R-[3H]LA (0.1 Ci/mmol) to hSMVT in the presence of 150 mM NaCl. Saturation equilibrium binding was performed with increasing concentrations (0.2–25 μM) of R-[3H]LA. Data fits of the two phases were performed independently with one-site models. Fitting the data ranging between 0 and 5 μM R-[3H]LA yielded a Kᵦ of 0.9 ± 0.03 μM with a Hill coefficient of 1.99 ± 0.11, and fitting the data points between 5 and 25 μM R-[3H]LA yielded a Kᵦ of 7.38 ± 1.44 μM. The Bₘₐₓ was calculated to be 1.84 ± 0.09 molecules of LA bound per molecule of hSMVT. Kinetic constants represent the mean ± S.E. of the fit. D, silver-stained SDS-polyacrylamide gel of hSMVT-containing nanodiscs. hSMVT was assembled into nanodiscs of different lipid composition: E. coli polar lipid (ND1), E. coli polar lipid with 6% (ND2) or 10% cholesterol (ND3), and POPC/POPG (3:2) with 6% cholesterol (ND4). After reconstitution, samples of the nanodiscs preparations (ND) and the supernatant (sup) that correspond to the amount of hSMVT (hS) and the membrane scaffold protein (MSP) used for the reconstitution were analyzed by 14% SDS-PAGE. For comparison, the amount of purified MSP1E3D1 (lane MSP, open arrowhead) and hSMVT (lane hS; solid arrowhead) used for the reconstitution are shown. E, comparison of the binding activity of hSMVT in different lipid environments. Binding of 20 μM R-[3H]LA (0.1 Ci/mmol) was assessed with the SPA using 50 ng of hSMVT in purified, detergent-solubilized form or reconstituted into nanodiscs of different lipid composition (ND1–4; see D for details). hSMVT amounts in nanodiscs were calculated based on densitometry measurements in NIH ImageJ of hSMVT-containing nanodiscs subjected to SDS-PAGE followed by silver staining. Data in A, B, C, and E are from representatives experiments performed ≥2 times and are shown as mean ± S.E. (error bars) of triplicate determinations.

**hSMVT-mediated Uptake in Proteoliposomes—**Guided by the results of the binding experiments performed in nanodiscs, we reconstituted affinity chromatography-purified hSMVT (see Fig. 3D) in liposomes premade with polar E. coli lipids that contained 10% (w/w) cholesterol. Previous studies (28) revealed the location of the hSMVT C terminus in the cytoplasm. Assessing the accessibility of the His tag (located at the C terminus of hSMVT) in hSMVT-containing proteoliposomes with Ni²⁺ chelate affinity-based immobilization revealed that the transporter is unidirectionally oriented in outside-out orientation in the proteoliposome membrane (Fig. 5A). Uptake of 0.83 μM R-[3H]LA in hSMVT-containing proteoliposomes was strictly dependent on the presence of Na⁺ as the time course of uptake in the absence of NaCl was virtually indistinguishable from that observed in control liposomes lacking hSMVT (Fig. 5B). In the presence of 100 mM NaCl, the time course of R-[3H]LA uptake peaked at about 10 min and showed a decline to equilibrium conditions at ∼3 h. Using the initial linear portion of uptake for the determination of the kinetics of R-LA transport, we measured 1-min intervals of R-[3H]LA transport with varying concentrations ranging from 0.2 to 53 μM. hSMVT-mediated transport was concentration-dependent with a concentration constant (Kₘ) at 50% of the Vₘₐₓ of 3.9 ± 0.6 μM and a Vₘₐₓ of 983.8 ± 43.4 nmol × mg of hSMVT⁻¹ × min⁻¹ (Fig. 5C). Transforming this number revealed a kₗₘₐₓ of transport of 1.13 ± 0.05 s⁻¹. Given the orientation of hSMVT in the proteoliposome membrane (Fig. 5A), this result indicates that the determined kₗₘₐₓ reflects the activity of hSMVT under physiological conditions, i.e. mediating the intracellular delivery of lipoic acid from the external medium.

**Discussion**

R-LA is an essential cofactor in many biological enzymatic reactions. In particular, R-LA serves as cofactor in central redox reactions related to energy production in the cell (4, 7). Furthermore, free LA is an effective biological antioxidant due to the high energy content of the disulfide group in LA. The disulfide in LA is in a dithiolane, a five-membered ring, with a dihedral
angle of 35°. This constellation results in an energetically unfavorable interaction of the orbital of the lone p_e electron pairs of the sulfur atoms that in turn exerts a high energy content of the disulfide group of LA with oxidizing species (29). Because of this potent hydroxyl scavenger and chelating potential, LA has gained a salutary role as a food supplement and in the treatment of several clinical conditions such as Amanita mushroom poisoning (30), diabetic neuropathy (31), Alzheimer disease (11), Parkinson disease (12), and multiple sclerosis (13). However, although only R-LA has been identified as the biologically active form of LA (32), R,S-LA has been administered in therapeutical applications as well as in food supplements simply due to more cost-efficient achiral production processes that were introduced in the 1950s (33). Whereas S-LA (as free form or in R,S-LA) is considered non-toxic, there is evidence that S-LA can adversely affect the activity of R-LA (34). To test whether this effect can be attributed to the cellular delivery of LA by the Na⁺/multivitamin transporter, the transport system suggested to be central in the transport of LA (16), we characterized the interaction of LA with hSMVT using transport and binding studies.

hSMVT is a member of the SLC5 gene family (SLC5A6) (35) of the solute:sodium symporter family (36). hSMVT has been identified in essentially every tissue of the body (16) where it mediates the Na⁺/dependent transport of the two vitamins biotin and pantothenic acid and iodoide (I⁻) (16, 18). In analogy to Na⁺-coupled substrate uptake of related SLC5 members SGLT1 (the Na⁺/glucose transporter 1; SLC5A1) and the Na⁺/iodide symporter (SLC5A5) (for recent reviews, see Refs. 37 and 38)), hSMVT has been suggested to couple the flux of its substrates to the flux of Na⁺. By using radiolabeled LA, we recently showed that in addition to biotin, pantothenic acid (16), and iodide (18) hSMVT transports racemic LA (18). However, in contrast to the favorable signal-to-noise ratios observed for the transport of biotin, pantothenic acid, and I⁻ concentrations close to their reported (apparent) affinities, uptake of racemic R,S-LA exhibited a rather low signal-to-noise ratio (Fig. 2A). Using radiolabeled R-LA, the biologically active form of LA, the signal-to-noise ratio of the uptake measurements performed with hSMVT-expressing oocytes increased dramatically, thus suggesting that R-LA is indeed the physiological substrate of hSMVT. Competition experiments involving the S and R,S isomers of LA confirm this notion. Testing the effect of biotin and pantothenic acid, the other two organic substrates of hSMVT, on R-LA uptake revealed different levels of inhibition. This may indicate that biotin is competing with R-LA for the same site(s). Conversely, the relatively low inhibitory effect observed for pantothenic acid may indicate that different sites are used for R-LA and pantothenic acid transport. This complex interaction of hSMVT with its substrates requires a thorough analysis that is the subject of our ongoing investigations.

To assess the stereospecificity of the interaction of hSMVT with LA, hSMVT was successfully expressed in P. pastoris and purified in functional form. This allowed for the direct characterization of the LA-specific binding kinetics in purified, detergent-solubilized form as well as reconstituted in nanodiscs (21). Performing the reconstitution in this artificial membrane system of defined lipid composition showed that the addition of cholesterol increased the incorporation efficiency of hSMVT in the membrane lipid bilayer; however, the lipid composition had no significant effect on the activity of hSMVT per se. Guided by these results, we measured the LA uptake kinetics of hSMVT-reconstituted proteoliposomes containing 10% cholesterol. Transport of R-LA by hSMVT was Na⁺-dependent and yielded a $K_{m}$ of 3.9 ± 0.6 μM, a value virtually identical to that obtained in hSMVT-expressing oocytes (4.0 ± 0.5 μM). The catalytic turnover number of hSMVT-mediated transport (about 1 molecule of R-LA per s) is well in agreement with the turnover numbers described for other Na⁺-dependent solute transporters (26, 39–43). Furthermore, accessibility measurements of the His tag, which served as a reporter for the location of the C terminus (located in the cytoplasm in cellular systems), revealed that hSMVT is inserted in physiologically "correct" orientation; i.e. its cytoplasmic face is located in the lumen of the proteoliposomes. Thus, the $k_{cat}$ determined in our measurements can be considered as the catalytic turnover number of the uptake reaction that mediates the translocation of LA from the extracellular milieu into the cytoplasm. Taken together,
Interaction of Lipoic Acid with hSMVT

these results strongly suggest that the expression of hSMVT in _P. pastoris_, its subsequent detergent-based extraction from the _P. pastoris_ membrane, and purification maintain hSMVT in fully functional form. Note that the glycosylation pattern in oocytes is similar to that observed in the yeast.

Binding of R-LA (and _R,S_-LA; not shown) is strictly dependent on Na⁺. Assessing saturation binding of R-LA with the purified, detergent-solubilized protein yielded an R-LA-to-hSMVT stoichiometry of ~2 with a biphasic binding isotherm. This result is in line with our recent findings (27) according to which the related solute:sodium symporter members vSGLT and PutP possess two substrate binding sites that are important for substrate translocation by proteins with a LeuT fold (see below). These studies extend our earlier studies on LeuT that reveal the importance of two substrate binding sites for Na⁺-coupled substrate transport (44) and for the conformational changes that are associated with the translocation process (45).

Taking advantage of the availability of functional hSMVT and the stable chiral forms of LA and LAM (46), we were able to test the interaction of the different racemric and enantiomeric forms of the two compounds with hSMVT in transport and binding assays. First, LAM did not inhibit R-LA binding and transport, indicating that the free carboxyl group of LA is important for hSMVT-mediated transport and that LA as lipoyl protein is not transported by the transporter. Adumbrating that hSMVT may share an overall similar structure with LeuT (47), referred to as the common LeuT fold that was also identified for hSMVT may share an overall similar structure with LeuT (47), this notion is supported by the fact that, like in LeuT (45), replacing Na⁺ with Li⁺ impaired transport of R-LA. For example, in LeuT, a molecule of leucine is bound in the so-called central or primary substrate binding site located in the core of the protein and formed by a specific set of residues. In addition, its carbonyl oxygen is coordinated by (or coordinates) the Na⁺ in the proximal Na₁ site (47). Because our data highlight the significance of the hydroxyl oxygen of R-LA for its interaction with hSMVT, a similar molecular interaction of substrate and Na⁺ as observed for LeuT is conceivable. Second, the fact that _S_-LA did not inhibit hSMVT-mediated R-LA uptake and binding may indicate that R-LA is the physiological substrate of hSMVT. Consistent with this notion, the racemic mixture (at approximately equimolar ratio of _R_-LA and _S_-LA) shows partial inhibition of _R_-[^3]H-LA uptake and binding when compared with the inhibition caused by _R_-LA.

LA was found to be absorbed into the cells as the free form and later to be oxidized to dihydrolipoic acid or incorporated into enzymatic complexes (51). hSMVT may thus play an important role in the absorption of _R_-LA in the intestine and other tissues in the body (16). Studies in healthy volunteers showed _R_-LA in plasma and that its bioavailability was higher than _S_-LA after administration of racemic LA (52, 53), suggesting that hSMVT transports exclusively _R_-LA across epithelial cells. However, the detailed pharmacokinetics of LA in humans is not fully characterized. Because of its high polymerization tendency, _R_-LA is relatively unstable and is poorly dissolved in aqueous solutions, reasons that may lead to poor absorption and low bioavailability. Dietary supplements that typically range from 50 to 600 mg are the primary sources of LA in animal models or clinical studies (14). A recent study shows that the plasma _R_-LA levels in healthy humans after administration of 600 mg of _R_-LA peak at about 15 min at a concentration of about 15 μg/ml, which corresponds to a molar concentration of ~80 μM _R_-LA (15), which is about 10% of the total administered _R_-LA. This result corresponds to previous studies that revealed that ~80% of LA was renally excreted (14, 54).

Although the amount of _R_-LA absorption in plasma and tissues was higher than that of _S_-LA, both enantiomers were found in plasma at a similar time after oral administration of the racemic mixture to healthy volunteers (52, 53). Thus, the question remains of whether there are other/additional mechanisms involved in the absorption and secretion of LA at such high concentrations found in food supplements. Takaishi _et al._ (55) determined the transport of 500 μM [³⁵Cl]LA in human intestinal Caco-2 cells. The transport was Na⁺-independent and not inhibited by either biotin or pantothenic acid, thus suggesting that SMVT was not the main means of LA translocation in those studies. The authors proposed that the monocarboxylic acid transporter was probably involved in LA transport in the intestinal cell line (55). It may be possible that monocarboxylic acid transporter or another, yet unknown fatty acid transporter mediates the uptake of LA at concentrations ≥60 μM, the highest concentration used in our assays. However, although there is currently no robust evidence for the involvement of another transport mechanism associated with the intracellular delivery of LA, we cannot rule out that such (additional) mechanisms exist.

Acknowledgments—We thank Dr. Peter Geggier for expert help with the kinetic models, Fernanda Delmondes de Carvalho for helpful discussions, and Ashley Lee for technical assistance.

References

1. Carreau, J. P. (1979) Biosynthesis of lipoic acid via unsaturated fatty acids. *Methods Enzymol.* **62**, 152–158
2. Reed, L. J. (2001) A trail of research from lipoic acid to α-keto acid dehydrogenase complexes. *J. Biol. Chem.* **276**, 38329–38336
3. Zhao, X., Miller, J. R., Jiang, Y., Marletta, M. A., and Cronan, J. E. (2003) Assembly of the covalent linkage between lipoic acid and its cognate enzymes. *Chem. Biol.* **10**, 1293–1302
4. Milne, J. L., Wu, X., Borgia, M. J., Lengyel, J. S., Brooks, B. R., Shi, D., Perham, R. N., and Subramaniam, S. (2006) Molecular structure of a 9-MDa icosahedral pyruvate dehydrogenase subcomplex containing the E2 and E3 enzymes using cryoelectron microscopy. *J. Biol. Chem.* **281**, 4364–4370
5. Bustamante, J., Lodge, J. K., Marcocci, L., Tritschler, H. J., Packer, L., and Rihn, B. H. (1998) α-Lipoic acid in liver metabolism and disease. *Free Radic. Biol. Med.* **24**, 1023–1039
6. Smith, A. R., Shenvi, S. V., Widiyanski, M., Suh, J. H., and Hagen, T. M. (2004) Lipoic acid as a potential therapy for chronic diseases associated with oxidative stress. *Curr. Med. Chem.* **11**, 1135–1146
7. Packer, L., Witt, E. H., and Tritschler, H. J. (1995) α-Lipoic acid as a biological antioxidant. *Free Radic. Biol. Med.* **19**, 227–250
8. Packer, L., Tritschler, H. J., and Wessel, K. (1997) Neuroprotection by the metabolic antioxidant α-lipoic acid. *Free Radic. Biol. Med.* **22**, 359–378
9. Vincent, H. K., Bourguignon, C. M., Vincent, K. R., and Taylor, A. G. (2007) Effects of α-lipoic acid supplementation in peripheral arterial disease: a pilot study. *J. Altern. Complement. Med.* **13**, 577–584
Interation of Lipoic Acid with hSMVT

10. Foster, T. S. (2007) Efficacy and safety of α-lipoic acid supplementation in the treatment of symptomatic diabetic neuropathy. *Diabetes Educ. 33*, 111–117

11. Maczurek, A., Hager, K., Kenklies, M., Sharran, M., Martins, R., Engel, I., Carlson, D. A., and Münch, G. (2008) Lipoic acid as an anti-inflammatory and neuroprotective treatment for Alzheimer’s disease. *Adv. Drug Deliv. Rev. 60*, 1463–1470

12. Bharat, S., Cochran, B. C., Hsu, M., Liu, J., Ames, B. N., and Andersen, J. K. (2002) Pre-treatment with R-lipoic acid alleviates the effects of GSH depletion in PC12 cells: implications for Parkinson’s disease therapy. *Neurotoxology 23*, 479–486

13. Schreibelt, G., Musters, R. J., Reijerkerk, A., de Groot, L. R., van der Pol, S. M., Hendrix, E. M., Oppe, E. D., Dijkstra, C. D., Drukarch, B., and De Vries, H. E. (2006) Lipoic acid affects cellular migration into the central nervous system and stabilizes blood-brain barrier integrity. *J. Immunol. 177*, 2630–2637

14. Shay, K. P., Moreau, R. F., Smith, E. J., Smith, A. R., and Hagen, T. M. (2009) α-Lipoic acid as a dietary supplement: molecular mechanisms and therapeutic potential. *Biochim. Biophys. Acta 1790*, 1149–1160

15. Carlson, D. A., Smith, A. R., Fischer, S. J., Young, K. L., and Packer, L. (2007) The plasma pharmacokinetics of R- (+)-lipoic acid administered as sodium R- (+)-lipoate to healthy human subjects. *Altern. Med. Rev. 12*, 343–351

16. Wang, H., Huang, W., Fei, Y. J., Xia, H., Yang-Feng, T. L., Leibach, F. H., Devoe, L. D., Ganapathy, V., and Prasad, P. D. (1999) Human placental Na”-dependent multivitamin transporter. Cloning, functional expression, gene structure, and chromosomal localization. *J. Biol. Chem. 274*, 14875–14883

17. Balamurugan, K., Ortiz, A., and Said, H. M. (2003) Biotin uptake by human intestinal and liver epithelial cells: role of the SMVT system. *Am. J. Physiol. Gastrointest. Liver Physiol. 285*, G73–G77

18. de Carvalho, F. D., and Quick, M. (2011) Surprising substrate versatility in SLC5A6: Na”- coupled I” transport by the human Na”-multivitamin transporter (hSMVT). *J. Biol. Chem. 286*, 131–137

19. Schmidt, U. (November 29, 1966) U. S. Patent US5288797 A

20. Zehnpfennig, B., Urbatsch, I. L., and Galla, H. J. (2009) Functional reconstitution of human ABCC3 into proteoliposomes reveals a transport mechanism with positive cooperativity. *Biochemistry 48*, 4423–4430

21. Nasr, M. L., and Singh, S. K. (2014) Radioligand binding to nanodisc-reconstituted membrane transporters assessed by the scintillation proximity assay. *Biochemistry 53*, 4–6

22. Schaffner, W., and Weissmann, C. (1973) A rapid, sensitive, and specific method for the determination of protein in dilute solution. *Anal. Biochem. 56*, 502–514

23. Patlak, C. S. (1957) Contributions to the theory of active transport: II. The gate type non-carrier mechanism and generalizations concerning tracer flow, efficiency, and measurement of energy expenditure. *Bull. Math. Bio. 19*, 209–235

24. Licata, L., Haase, W., Echkhardt-Strelau, L., and Parcej, D. N. (2006) Overexpression of a mammalian small conductance calcium-activated K”+ channel in Pichia pastoris: effects of trafficking signals and subunit fusions. *Protein Expr. Purif. 47*, 171–178

25. Quick, M., and Wright, E. M. (2002) Employing *Escherichia coli* to functionally express, purify, and characterize a human transporter. *Proc. Natl. Acad. Sci. U.S.A. 99*, 8597–8601

26. Quick, M., and Javitch, J. A. (2007) Monitoring the function of membrane transport proteins in detergent-solubilized form. *Proc. Natl. Acad. Sci. U.S.A. 104*, 3603–3608

27. Li, Z., Lee, A. S., Bracher, S., Jung, H., Paz, A., Kumar, J. P., Abramson, J., Quick, M., and Shi, L. (2015) Identification of a second substrate-binding site in solute-sodium symporters. *J. Biol. Chem. 290*, 127–141

28. Subramanian, V. S., Marchant, J. S., Bouwlaire, M. J., Ma, T. Y., and Said, H. M. (2009) Membrane targeting and intracellular trafficking of the human sodium-dependent multivitamin transporter in polarized epithelial cells. *Am. J. Physiol. Cell Physiol. 296*, C663–C671

29. Bast, A., and Haenen, G. R. (2003) Lipoic acid: a multifunctional antioxidant. *BioFactors 17*, 207–213

30. Berkson, B. M. (1979) Thiotic acid in treatment of hepatotoxic mush-
Interaction of Lipoic Acid with hSMVT

52. Hermann, R., Niebch, G., Borbe, H. O., Fieger-Bueschges, H., Ruus, P., Nowak, H., Riethmueller-Winzen, H., Peukert, M., and Blume, H. (1996) Enantioselective pharmacokinetics and bioavailability of different racemic \( \alpha \)-lipoic acid formulations in healthy volunteers. *Eur. J. Pharm. Sci.* **4**, 167–174

53. Breithaupt-Grögler, K., Niebch, G., Schneider, E., Erb, K., Hermann, R., Blume, H. H., Schug, B. S., and Belz, G. G. (1999) Dose-proportionality of oral thiocic acid—coincidence of assessments via pooled plasma and individual data. *Eur. J. Pharm. Sci.* **8**, 57–65

54. Harrison, E. H., and McCormick, D. B. (1974) The metabolism of dl-(1,6-\(^{14}\)C)lipoic acid in the rat. *Arch. Biochem. Biophys.* **160**, 514–522

55. Takaishi, N., Yoshida, K., Satsu, H., and Shimizu, M. (2007) Transepithelial transport of \( \alpha \)-lipoic acid across human intestinal Caco-2 cell monolayers. *J. Agric. Food Chem.* **55**, 5253–5259