Helix 8 and Helix 10 Are Involved in Substrate Recognition in the Rat Monocarboxylate Transporter MCT1[†]

Basim Rahman,[‡] Hans-Peter Schneider,[§] Angelika Bröer,[‡] Joachim W. Deitmer,[§] and Stefan Bröer*,[‡]

Physiologisches Institut der Universität, Gmelinstrasse 5, D-72076 Tübingen, Germany, and Fachbereich Biologie, Universität Kaiserslautern, Postfach 3049, D-67653 Kaiserslautern, Germany

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ABSTRACT: Transport of lactate, pyruvate, and the ketone bodies, acetoacetate and β-hydroxybutyrate, is mediated in many mammalian cells by the monocarboxylate transporter MCT1. To be accepted as a substrate, a carboxyl group and an unpolar side chain are necessary. Site-directed mutagenesis of the rat MCT1 was used to identify residues which are involved in substrate recognition. Helices 8 and 10 but not helix 9 were found to contain critical residues for substrate recognition. Mutation of arginine 306 to threonine in helix 8 resulted in strongly reduced transport activity. Concomitantly, saturable transport was transformed into a nonsaturable dependence of transport activity on lactate concentration, suggesting that binding of the substrate was strongly impaired. Furthermore, proton translocation in the mutant was partially uncoupled from monocarboxylate transport. Mutation of phenylalanine 360 to cysteine in helix 10 resulted in an altered substrate side chain recognition. In contrast to the wild-type transporter, monocarboxylates with more bulky and polar side chains were recognized by the mutated MCT1. Mutation of selected residues in helix 9 and helix 11 (C336A, H337Q, and E391Q) did not cause alterations of the transport properties of MCT1. It is suggested that substrate binding occurs in the carboxy-terminal half of MCT1 and that helices 8 and 10 are involved in the recognition of different parts of the substrate.

Transport of lactate, pyruvate, and the ketone bodies, acetoacetate and β-hydroxybutyrate, is very physiologically important in almost all cells (*1*). Transport of this class of substrates is mediated by a family of monocarboxylate transporters (MCTs), members of which can be found from bacteria to humans (*2*−*5*). The kinetic properties of MCT1 have been investigated in detail due to its presence in erythrocytes and cultured cell lines (*6*−*8*). The cloned transporter has been investigated in MCT1-expressing *Xenopus laevis* oocytes (*9*, *10*). The membrane topology of MCT1 was probed by using antibodies generated against peptides thought to be present in terminal or loop regions of the transporter (*11*). In general, it was found that MCT1 was highly stable against proteolytic digestion in intact erythrocytes, suggesting that the small extracellular loops are probably not exposed (*11*). It could also be demonstrated that the carboxy terminus has a cytoplasmic location, and that the large putatively cytoplasmic loop between helices 6 and 7 is highly sensitive to proteolytic degradation in membrane preparations but not in intact erythrocytes. These findings confirmed the predicted two-dimensional topology of the transporter.

The substrate specificities of MCT1, -2, and -4[1] have been investigated in cultured cells and cRNA-expressing oocytes (*8*, *10*, *12*, *13*). In contrast, nothing is known about the function and substrate specificity of the other members of the family. Identification of critical residues for substrate binding in the prototypical transporter MCT1, combined with sequence homology studies, could facilitate the characterization of the other members of this family.

The monocarboxylate transporter MCT1 can be expressed at very high levels in *X. laevis* oocytes (*10*). It mediates the concerted translocation of $1H^+$ and a monocarboxylate$^-$ substrate by an ordered mechanism in which H^+ binding has to precede monocarboxylate binding (*7*). Lactate transport can be followed by using labeled lactate, and the proton translocation can be monitored as changes of the intracellular pH value measured with pH-sensitive microelectrodes (*10*). We assumed that cationic side chains might be involved in monocarboxylate binding, whereas protonated residues of any kind could be involved in H^+ translocation. The K_m values of 2-oxoacids for MCT1 are consistently fixed at about 1 mM, whereas 2-hydroxyacids, in the L-configuration, have 5-fold higher K_m values (*1*, *8*, *10*), suggesting that hydrogen bonding to the substituent in the α-position is involved in substrate recognition. The amino acid sequence of MCT1 contains only a small number of charged residues in putative transmembrane regions (Figure 1). These residues are concentrated in helices 8−11. We therefore assumed that this region could be critical for the activity of the transporter. Moreover, it is known that a spontaneously occurring mutant of phenylalanine 360 in helix 10 allows mevalonate to be

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* To whom correspondence should be addressed: Physiologisches Institut der Universität, Gmelinstrasse 5, D-72076 Tübingen, Germany. Telephone: +49-7071-2977362. Fax: +49-7071-293073. E-mail: stefan.broeer@uni-tuebingen.de.

[‡] Physiologisches Institut der Universität.

[§] Universität Kaiserslautern.

[1] As proposed in ref *13*, MCT3 of ref *5* has recently been renamed MCT4, because it is different from MCT3 of ref *4*. Accordingly, MCT4−7 of ref *5* have been renamed MCT5−8, respectively.

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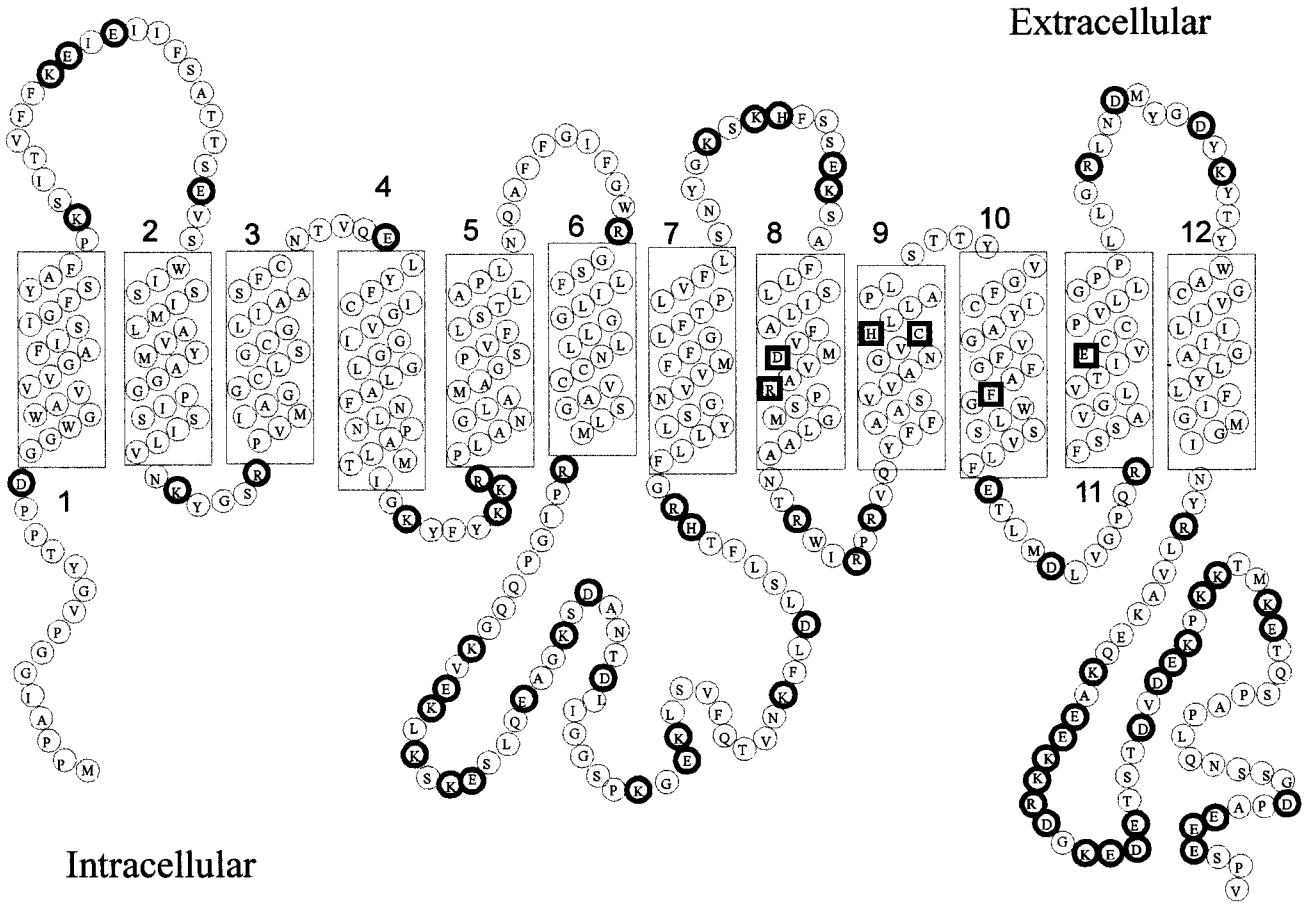

FIGURE 1: Topological model of the monocarboxylate transporter MCT1. The hydropathy plot of MCT1 was analyzed, and sufficiently long hydrophobic regions are depicted as transmembrane helices (denoted with the numbers 1−12). Confirmation of this model was derived from studies using peptide-specific antibodies in conjunction with limited proteolysis (*11*). Charged residues are denoted with bold circles, and mutated residues are denoted with squares.

translocated by MCT1 (*14*). An example for lactate binding can be found in the active center of the lactate dehydrogenase. Structure analysis at high resolution shows that an arginine residue binds to the carboxyl group and that a histidine residue forms a hydrogen bond with the hydroxyl group of lactate (*15*). A similar arrangement might be present in monocarboxylate transporters. Both arginine and histidine can indeed be found in MCT1 in transmembrane regions 8 and 9. The arginine residue in helix 8 is conserved in many members of the monocarboxylate transporter family and has been proposed to act as a substrate binding site in MCTs (*5*).

In this study, we have investigated for the first time the significance of helices 8−10 in substrate binding in the monocarboxylate transporter MCT1 by kinetic analysis of site-specific mutations. The results indicate that helices 8 and 10 recognize different parts of monocarboxylate substrates.

EXPERIMENTAL PROCEDURES

Materials. L-[U-^{14}C]Lactate (5.62 GBq/mmol) was purchased from Amersham Buchler (Braunschweig, Germany). Sodium L-lactate, α-ketoisocaproate, DL-β-hydroxybutyrate, α-cyano-4-hydroxycinnamate, α-ketoisovalerate, mevalonate, and acetoacetate were ordered from Sigma/Fluka (Deisenhofen, Germany). Mevalonate is delivered as mevalonic acid lactone, which slowly equilibrates with the free acid form when diluted in water. To facilitate equilibration, the liquid lactone was dissolved into equimolar amounts of 1 M NaOH. The solution was titrated to pH 7.0 and subsequently diluted to the final concentration. The cap analogue m^{7}G(5′)ppp(5′)G was obtained from New England Biolabs (Schwalbach, Germany). Collagenase (EC 3.4.24.3, 0.6−0.8 unit/mg) was from Boehringer (Ingelheim, Germany). All other chemicals were analytical grade and were purchased from E. Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany), and Boehringer (Mannheim, Germany).

Site-Directed Mutagenesis. For mutagenesis and transport studies, plasmid pGHJMCT1 was used. It contains the *Eco*RI fragment of rat MCT1 cloned into the oocyte vector pGEM-He (*9*). Site-directed mutagenesis was performed without subcloning by using the QuikChange site-directed mutagenesis kit (Stratagene, Heidelberg, Germany). Briefly, two complementary primers were constructed in which the desired mutation was flanked by 15 nucleotides corresponding to the MCT1 cDNA sequence. The primers were used to amplify the complete cDNA-containing vector by 12 PCR cycles. Subsequently, the template DNA was removed by digestion with *Dpn*I. The plasmid was isolated from transformed bacteria and sequenced to verify the mutation. To avoid second site mutations which might have been introduced during the amplification reaction, the transport activity of three independent clones was determined.

The following oligonucleotides were used (only the sense sequence is shown in the 5′ to 3′ direction, with the mutated

base given in lowercase letters, and the numbering of the amino acids corresponding to the rat MCT1 sequence (*16*); mutants are designated wild-type amino acid/position/mutant amino acid): D302N, ATT TTG GCT TTT GTT aAT ATG GTG GCC AGA CCG; D302E, ATT TTG GCT TTT GTT GAa ATG GTG GCC AGA CCG; R306T, GTT GAT ATG GTG GCC AcA CCG TCC ATG GGT CTT; C336A, GTT GCG AAT GGA GTG gcC CAT TTG CTG GCA CCT; H337Q, GCG AAT GGA GTG TGC CAg TTG CTG GCA CCT TTG; F360C, GTC TTT GGA TTT GCC TgT GGT TGG CTC AGC TCC; and E391Q, TTG GTG ACC ATT GTG cAA TGT TGT CCT GTC CTC.

Oocytes and Injections. X. laevis females were purchased from the South African Xenopus facility (Knysna, Republic of South Africa). Oocytes (stages V and VI) were isolated by collagenase treatment as described previously (*17*) and allowed to recover overnight before injection.

Plasmid DNA was linearized with *Not*I and transcribed in vitro with T7 RNA polymerase (Life Technologies, Eggenstein, Germany) in the presence of the cap analogue $m^7G(5')ppp(5')G$ at a concentration of 1 mM. The template plasmid was removed by digestion with RNase-free DNase I. The complementary RNA (cRNA) was purified by phenol/chloroform extraction followed by precipitation with $^1/_2$ of a volume of 7.5 M ammonium acetate and 2.5 volumes of ethanol to remove unincorporated nucleotides. The integrity of the transcript was checked by denaturing agarose gel electrophoresis. Oocytes were microinjected with 12.5 nL of MCT1 cRNA in water at a concentration of 1 μg/μl, by using a microinjection device (Bachofer, Reutlingen, Germany).

Cell Surface Localization of MCT1 Mutants. The cell surface expression of wild-type and mutated MCT1 was tested as described by Pajor et al. (*18*). Briefly, surface proteins were biotinylated with the membrane-impermeant reagent Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL). Subsequently, membrane proteins were solubilized by incubation in Triton X-100 containing buffer. Biotinylated proteins were bound to ImmunoPure immobilized streptavidin (Pierce), washed three times, and then dissolved in SDS−PAGE[2] sample buffer. Proteins were separated by SDS−PAGE and subsequently blotted on nitrocellulose membranes (Immobilon-NC Transfer Membrane, Millipore, Bedford, MA) with a constant voltage of 100 V and 250 mA for 1 h. The nitrocellulose membranes were blocked for a mimimum of 1 h in PBS (pH 7.4)/0.05% Tween 20/5% instant dried milk powder. Afterward, Western blots were incubated in a PBS solution containing the primary antibody for 2 h [kindly provided by P. J. Magistretti, University of Lausanne, Lausanne, Switzerland; the antibody is directed against the 16 carboxy-terminal amino acids of mouse MCT1 and was raised in rabbits, at a dilution of 1:200 in PBS (pH 7.4)/0.05% Tween 20]. After several washing steps, the alkaline phosphatase-coupled secondary antibody [sheep anti-rabbit IgG from Boehringer, diluted 1:5000 in PBS (pH 7.4)/0.05% Tween 20] was applied for 1 h. Western blots were developed by staining with nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate. The reaction was terminated by incubation of the membranes in 20 mM EDTA solution.

[2] Abbreviations: HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; KIC, α-ketoisocaproic acid (2-oxoisohexanoate); PBS, phosphate-buffered saline; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

Recording of Intracellular pH Values with Electrodes. Double-barrelled pH-sensitive microelectrodes for measuring the intracellular pH (pH_i) and membrane potential in *Xenopus* oocytes were prepared as previously described (*19*). Briefly, the electrodes were pulled in two stages and silanized by adding a drop of 5% tri-*N*-butylchlorosilane in 99.9% pure carbon tetrachloride into the prospective ion-selective barrel and then baking the pipet on a hot plate at 475 °C for 4.5−5 min.

For pH-selective microelectrodes, a small amount of H^+ cocktail (Fluka catalog no. 95291) was backfilled into the tip of the silanized barrel and the remainder filled with 0.1 M sodium citrate (pH 6.0). The reference barrel was filled with 2 M potassium acetate. Electrodes were accepted for experiments if their response exceeded 50 mV per unit change in pH and if they reacted faster in the bath during calibration before and after each experiment than the fastest pH_i changes recorded upon addition of lactate. On average, electrodes responded with a change of 54 mV to a change in pH of 1 unit.

The recording arrangement was the same as that described previously (*19*, *20*). The central and reference barrels were connected by chlorided silver wires to the headstages of an electrometer amplifier (Axoclamp-2B, Axon Instruments, Foster City, CA). The intracellular pH and membrane potential of the oocytes were displayed on a slow chart recorder and also stored on a personal computer using homemade data acquisition software.

As described previously (*10*), optimal pH changes were detected when the electrode was located near the inner surface of the plasma membrane. This was achieved by carefully rotating the oocyte with the impaled electrode. All the experiments were carried out at room temperature (22−25 °C). Only oocytes with a membrane potential below −25 mV were used for experiments.

Flux Measurements. For each determination, groups of seven cRNA or native oocytes were washed twice with 4 mL of OR2+ (82.5 mM NaCl, 2.5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM Na_2HPO_4, and 5 mM HEPES, titrated with NaOH to pH 7.0) before incubation at room temperature in a 5 mL polypropylene tube containing 70 μL of the same buffer supplemented with 5 kBq of [U-^{14}C]lactate and different amounts of unlabeled substrate. Transport was stopped after different intervals by washing oocytes three times with 4 mL of ice-cold OR2+ buffer. Repeated washing steps did not result in the leakage of labeled lactate (*10*). For efflux, equal volumes of [^{14}C]lactate (7.4 MBq/mL, 5.7 GBq/mmol) and 200 mM lactate (pH 7.3) were mixed and subsequently 50 nL of the mixture was injected into oocytes, which had been chilled to 4 °C. Efflux was initiated immediately by washing seven oocytes with buffer equilibrated to room temperature. After the final washing step, 1 mL of OR2+ was added and 100 μL aliqouts were taken for each time point.

Calculations. For the determination of kinetic parameters, nonlinear regression algorithms of Microcal Origin software (Microcal Software, Inc., Northampton, MA) were used. The initial slopes of pH transients from single oocytes were first fitted to a logistic equation, and then normalized by calculation of v/V_{max}. The final curve was then calculated from v/V_{max} values of the indicated number of experiments. K_m values were determined by nonlinear regression of values

Table 1: Transport Activity and Kinetic Constants of Mutated MCT1 Transporters[a]

mutant	transport activity (%) (0.1 mM lactate)	K_m (mM)	pH_{50}
MCT1	100 ($V_{max} = 0.69 \pm 0.03$ nmol/2.5 min)	4.5[b] (lactate at pH 7.0) 1.0[b] (pyruvate at pH 7.0) 1.5 ± 0.2 (lactate at pH 6.0)	6.4[b] (1 mM lactate)
D302N	-5 ± 2, -5 ± 3	not detectable	not detectable
D302E	12 ± 3, 7 ± 4	not detectable	not detectable
R306T	0 ± 9, 2 ± 2, 3 ± 3	not saturable	not determined
C336A	108 ± 17, 103 ± 9	not determined	not determined
H337Q	96 ± 3, 117 ± 17, 115 ± 9	not determined	6.5 ± 0.1 (1 mM lactate)
F360C	14 ± 4, 16 ± 3	0.6 ± 0.6 (lactate at pH 6.0)	not determined
E391Q	72 ± 7, 53 ± 5, 61 ± 8 ($V_{max} = 0.55 \pm 0.03$ nmol/2.5 min)	1.2 ± 0.2 (pyruvate at pH 7.0) 4.5 ± 0.9 (lactate at pH 7.0)	7.1 ± 0.1 (1 mM lactate)

[a] Oocytes were injected with 12.5 ng of mutated or wild-type cRNA, followed by an expression period of 3−5 days. Basal transport activity was determined at a substrate concentration of 0.1 mM for 10 min. The transport activity of two or three individual mutants is shown. Kinetic constants were determined as described in the Results. [b] Taken from ref *10*.

to the equation $v/V_{max} = [S]/(K_m + [S])$, and the pH dependence of lactate transport was fit by nonlinear regression to the equation $J/J_{max} = 10^{-h\times pH}/(10^{-h\times pK} + 10^{-h\times pH})$. The flux J is used in this equation to indicate that the maximum flux rate under the conditions of the experiment was used and not V_{max} which would require saturating substrate concentrations; h is the Hill coefficient. The number of investigated oocytes (n) is given in the text. When using pH-sensitive microelectrodes, single oocytes could be superfused with complete sets of concentrations (e.g., pH dependence or concentration dependence). For radioactive flux measurements, each individual data point represents the difference between the mean ± standard error uptake activity of n MCT1-expressing and n native oocytes (usually $n = 7$). The standard error of the difference was calculated by Gauss' law of error propagation.

RESULTS

Transport Acitivity of Mutants. Selected residues in helices 8−11 were exchanged which could be involved in either monocarboxylate binding or proton cotransport, and these mutations were D302N, D302E, R306T, C336A, H337Q, F360C, and E391Q. As a first characterization, the transport activity of these mutants was determined at a lactate concentration of 0.1 mM after expression in *X. laevis* oocytes (Table 1). Lactate uptake activity was strongly reduced in mutants D302N, D302E, R306T, and F360C. Replacement of E391 by glutamine resulted in the reduction of the transport activity by 30−40%, whereas mutation of H337 to glutamine and C336 to alanine did not affect uptake at all.

Surface Localization of Analyzed Mutants. A reduced transport activity might result from disturbances in the transport mechanism or the inadequate translocation of the protein to the plasma membrane. The surface localization of mutated transporters was probed by biotinylation of lysine residues of MCT1 by using a biotin-conjugated succinimide ester. Lactate transport via MCT1 can be inhibited by covalent binding of 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS; *21*), which indicates that lysine residues are exposed at the extracellular surface of the transporter. In agreement with this finding, MCT1 could be biotinylated by a biotin-conjugated *N*-hydroxysulfosuccinimide ester and was subsequently detected by Western blotting (Figure 2). MCT1 was visible as a distinct band with a molecular mass of 44 kDa. A number of other proteins were recognized by the MCT1 antibody as well, which might include the oocyte endogenous monocarboxylate transporter, because distinct bands were also visible in native oocytes. Analysis of the strongly impaired mutants D302N, D302E, R306T, and F360C revealed that only the D302N mutant was not properly translocated into the plasma membrane, whereas all other mutants, including E391Q (not shown), were present in the plasma membrane in similar amounts (Figure 2). The low activity of the D302E mutant, in contrast, must have its origin in a disturbance of the transport mechanism.

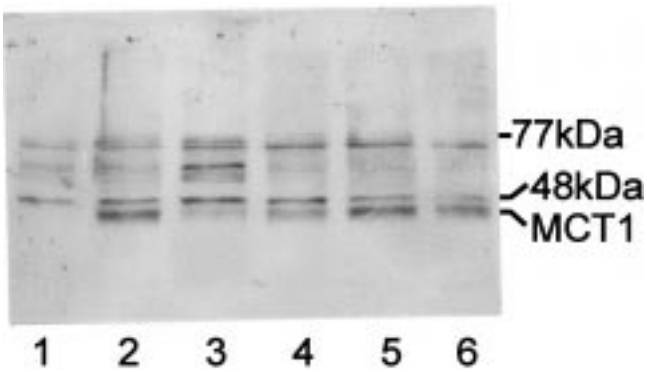

FIGURE 2: Surface localization of MCT1 wild type and mutants. Plasma membrane proteins of oocytes expressing MCT1 or mutants of it were biotinylated. Subsequently, oocyte membranes were solubilized and biotinylated proteins bound to streptavidin-coupled agarose. Samples were washed and dissolved in SDS−PAGE sample buffer. After separation by SDS−PAGE, proteins were blotted onto nitrocellulose and incubated with MCT1-specific antibodies. Subsequently, protein bands were visualized by using alkaline phosphatase-conjugated secondary antibodies and the chromogenic substrate NBT/BCIP. Oocytes expressed the following proteins: lane 1, none; lane 2, wild-type MCT1; lane 3, D302N; lane 4, R306T; lane 5, F360C; and lane 6, D302E. The positions of two molecular mass markers and the MCT1-specific band are denoted in the margin.

Kinetic Analysis of MCT1 Mutants. The velocity of H^+−monocarboxylate$^-$ transport via MCT1 strongly increases with increasing proton concentrations. The pH dependence resembles a titration curve with an apparent pK value varying between 6.0 and 7.0, depending on the substrate concentration. MCT1 contains only one histidine residue (H337) in transmembrane helix 9. Besides its possible role in proton translocation, this residue could also be involved in substrate recognition. However, transport activity and the pH dependence of lactate uptake in a H337Q mutant were indistinguishable from the activities of the wild type (data not shown). An exchange of the neighboring cysteine 336 with alanine similarly did not result in any changes in the transport activity (data not shown). Although the pK values of the

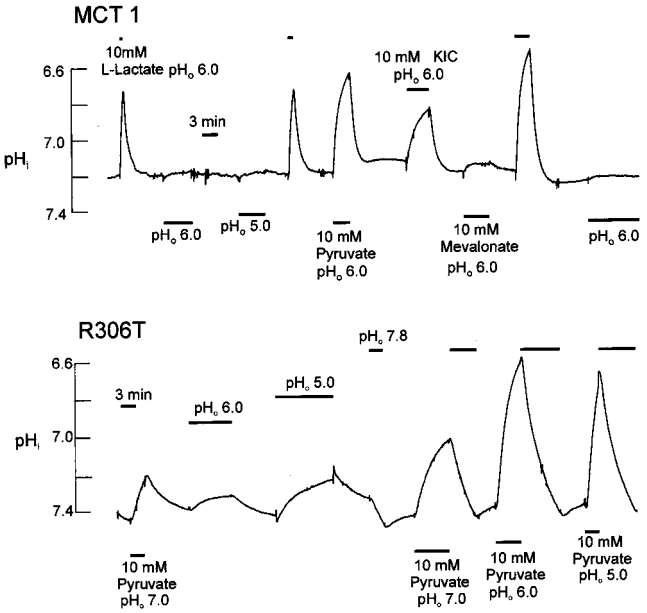

FIGURE 3: Changes in cytosolic pH elicited by superfusion of MCT1 and mutant R306T-expressing oocytes with substrate-containing and substrate-free solutions at different pH values. Each oocyte was injected with 12.5 ng of MCT1 or R306T cRNA followed by an expression period of 4−7 days. The cytosolic pH was recorded with a double-barrelled pH-sensitive microelectrode in MCT1 (upper trace)- or R306T-expressing oocytes (lower trace). The oocyte was superfused with pyruvate-containing solutions (pH 5.0, 6.0, and 7.0) and pyruvate-free solutions (pH 5.0, 6.0, and 7.8). Superfusion times are denoted with bars. During intervals which are not denoted with bars, oocytes were superfused with substrate-free buffer (pH 7.0).

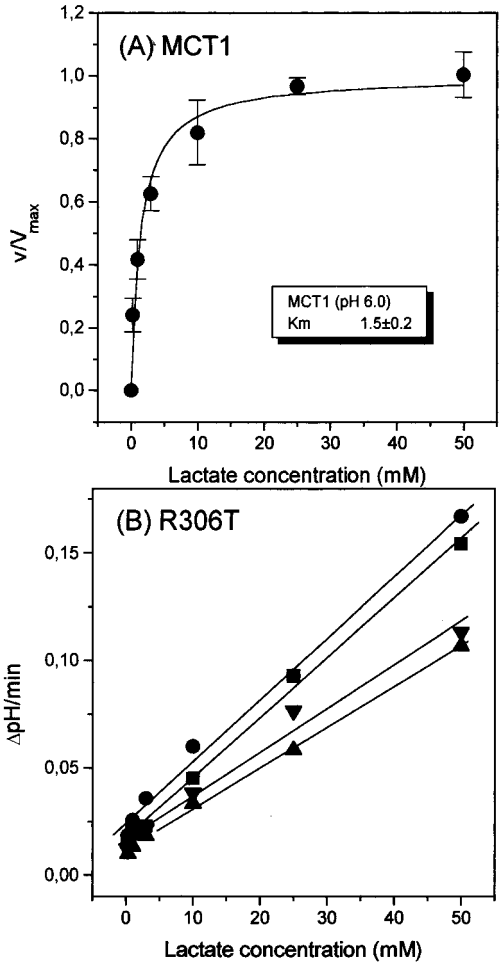

FIGURE 4: Analysis of kinetic parameters of the mutant R306T. The lactate uptake activity was analyzed at different substrate concentrations in MCT1 (mean of five oocytes; A)- and R306T-expressing oocytes (results from four oocytes; B). Initial slopes of cytosolic pH changes at different substrate concentrations were used to calculate the concentration dependence. In panel A, the differences in activity of single oocytes were normalized by dividing the actual transport velocity by the maximum attainable velocity.

histidine and cysteine side chains would fit to the pH dependence of lactate transport, these results exclude participation of the two residues in proton translocation as well as in substrate recognition by the transporter.

In contrast to the inconspicuous behavior of mutations in helix 9, mutation of R306 into threonine in helix 8 resulted in a complete loss of transport activity at low lactate concentrations (0.1 mM). However, by using pH-sensitive microelectrodes, H^+−monocarboxylate$^-$ cotransport could still be detected at high pyruvate concentrations as changes in cytosolic pH (Figure 3, lower trace). To improve the signal, the extracellular pH was decreased to pH 6.0 and 5.0, which resulted in an increased driving force (Figure 3, lower trace). Surprisingly, it was found that the cytosolic pH of oocytes expressing the R306T mutant was slowly reduced during superfusion with substrate-free oocyte ringer at acidic pH values (pH 6.0 and 5.0, Figure 3, lower trace, one of six similar experiments shown). This observation was in agreement with the more alkaline resting pH of oocytes expressing this mutant (pH 7.47 ± 0.12; $n = 14$) in comparison with that of wild-type MCT1-expressing oocytes (pH 7.32 ± 0.08; $n = 22$). The oocytes were routinely stored in oocyte ringer titrated to pH 7.8. During storage, the intracellular pH obviously adapted to the alkaline extracellular pH value. The R306T mutant, therefore, seemed to allow a limited movement of H^+ through the transporter in the absence of any substrate. Superfusion of native or MCT1-expressing oocytes with substrate-free oocyte ringer (pH 6.0) did not result in any changes in the intracellular pH (Figure 3, upper trace, one of five similar experiments shown). Even a decrease in the extracellular pH to 5.0 did not cause substantial changes in the intracellular pH in the absence of substrate. In the presence of substrate, transport of H^+ by the R306T mutant was strongly stimulated, showing that translocation of monocarboxylates was still largely accompanied by a movement of H^+.

The R306T mutant was characterized not only by a partial uncoupling of substrate and H^+ fluxes but also by strong changes in substrate binding. To evaluate the K_m value of the R306T mutant, the extracellular pH was set to 6.0. In the wild-type transporter, this drop in pH resulted in a decrease in the K_m value to 1.5 ± 0.2 mM ($n = 5$), which is in agreement with the ordered binding mechanism of MCT1 and the pH dependence of monocarboxylate transport. In contrast to the increased affinity of the wild-type transporter, increasing concentrations of lactate did not lead to saturation of the transporter in the R306T mutant (Figure 4A,B). Oocytes expressing the R306T mutant exhibited a nonsaturable dependence of the uptake rate on lactate concentration (Figure 4B; $n = 14$). This suggested that the binding of lactate to the transporter was strongly impaired in the mutated transporter. The substrate specificity of R306T was almost unaffected (Figure 5, lower trace, one of five similar

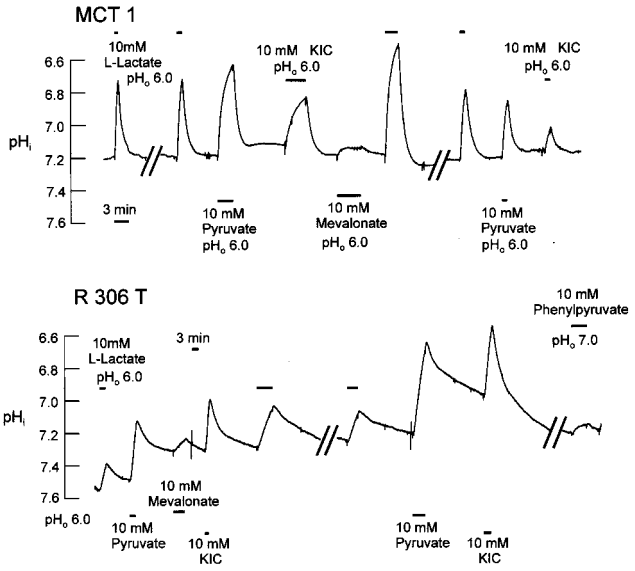

FIGURE 5: Changes in cytosolic pH elicited by superfusion of MCT1- and R306T-expressing oocytes with different substrates. Each oocyte was injected with 12.5 ng of MCT1 or R306T cRNA followed by an expression period of 4−7 days. The cytosolic pH was recorded with a double-barrelled pH-sensitive microelectrode in MCT1 (upper trace)- or R306T-expressing oocytes (lower trace). The oocyte was superfused with substrate-containing solutions (pH 6.0) and substrate-free solutions (pH 7.0). Superfusion times are denoted with bars.

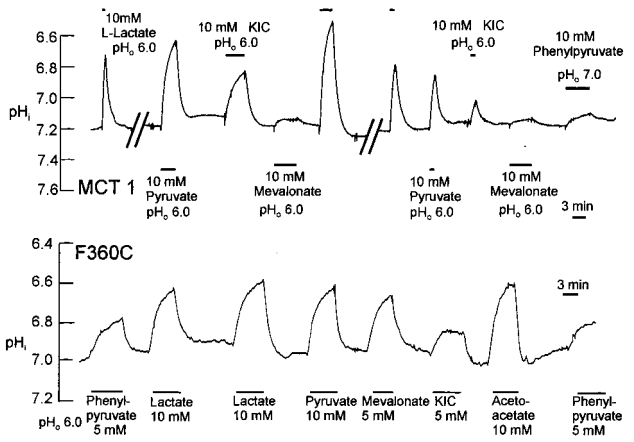

FIGURE 6: Changes in cytosolic pH elicited by superfusion of MCT1- and F360C-expressing oocytes with different substrates. Each oocyte was injected with 12.5 ng of MCT1 or F360C cRNA followed by an expression period of 4−7 days. The cytosolic pH was recorded with a double-barrelled pH-sensitive microelectrode in MCT1 (upper trace)- or F360C-expressing oocytes (lower trace). The oocyte was superfused with substrate-containing solutions (pH 6.0) and substrate-free solutions (pH 7.0). Superfusion times are denoted with bars.

experiments shown). Oocytes expressing the R306T mutant transported not only lactate but also pyruvate and α-ketoisocaproate. Subtle changes in the substrate specifcity were nevertheless visible. When exposed to equal concentrations (10 mM), the wild-type transporter exhibited the following order in the transport rate: lactate > pyruvate > α-ketoisocaproate. In the R306T mutant, however, the order was reversed: α-ketoisocaproate > pyruvate > lactate (Figure 5).

Aspartate 302 is located in close proximity to arginine 306 on the same face of helix 8 and therefore might as well be involved in substrate binding or could form a charge pair with R306. Changing D302 into asparagine resulted in a complete inactivation of the transporter. No uptake of labeled lactate could be detected in this mutant. In addition, no movement of protons could be detected by intracellular pH electrodes during superfusion with high concentrations of lactate (10 mM) or pyruvate (10 mM) (data not shown).

Even a conservative replacement of asparagine 302 by glutamate did not result in a functional transporter. A low transport activity was occasionally detected at a lactate concentration of 100 μM (Table 1). In an attempt to characterize the low activity of the D302E mutant, 50 nL of 100 mM lactate with a specific activity of 2200 dpm/nmol was injected into expressing oocytes. The efflux of labeled lactate was evaluated by fitting the data to a first-order rate equation. Rapid efflux of labeled lactate was detected in oocytes expressing the wild-type transporter ($k = 0.116 \pm 0.004$ min^{-1}), whereas both ooyctes expressing D302 ($k = 0.02 \pm 0.02$ min^{-1}) and native oocytes ($k = 0.03 \pm 0.01$ min^{-1}) exhibited similarly low levels of lactate efflux ($n = 7$).

The question of whether the exchange reaction was still conducted by the mutant transporter was tested further. For this, oocytes were injected with high concentrations of unlabeled lactate (final cytosolic concentration of ~20 mM), and subsequently, the level of uptake of labeled lactate (0.1 mM) was determined. Again, no difference was found between native and D302E-expressing oocytes (data not shown). The negligible activity of the D302 mutants excluded further analysis of this mutant.

Mutation of arginine 306 resulted in dramatic changes in the K_m value, whereas substrate specificity seemed to be almost unaffected. Contrasting observations were realized when F360 was converted into cysteine. This mutant has been described as a spontaneously occurring mutant of MCT1 allowing the transport of mevalonate (*2*, *14*). Although mevalonate [$HOCH_2CH_2C(CH_3)OHCH_2COOH$] is a monocarboxylate, MCT1 does not accept the simultaneous presence of a methyl and a hydroxyl group at the β-carbon atom and probably also does not accept a hydroxyl group at the δ-carbon atom (Figure 6, upper trace). Generally, the transport activity of the F360C mutant was much lower than the transport activity of the wild type (Table 1). Transport properties of F360C were therefore also analyzed at an extracellular pH of 6.0. The K_m value for lactate (at pH 6.0), in contrast to the situation with R306T, remained close to the value of the wild type in the F360C mutant (0.6 ± 0.6 mM; $n = 4$). Although the mutant exhibited a normal substrate affinity, mutation of F360 to cysteine resulted in a gain in the level of substrate recognition. In addition to the monocarboxylates recognized by MCT1, it could be confirmed that mevalonate is an additional substrate of F360C (Figure 6, one of five similar experiments shown).

As shown in Table 1, the mutation of E391 to glutamine resulted in a 30% decrease in the transport activity. Analysis of the kinetic parameters of lactate transport revealed that the pH dependence and K_m value of this mutant were close to those of the wild type for the substrate lactate as well as pyruvate (Table 1). The V_{max} value for lactate transport at pH 7.0, in contrast, dropped from 0.69 ± 0.03 nmol/2.5 min (wild type; $n = 7$) to 0.55 ± 0.03 nmol/2.5 min (E391Q; $n = 7$). The loss of transport activity in this mutant most likely resulted from a reduced expression level.

DISCUSSION

In this study, monocarboxylate transport was followed by using labeled lactate and pH-sensitive microelectrodes. Both methods complement each other. At low concentrations, labeled substrates offer the highest sensitivity, which is at least 10-fold higher than the sensitivity achieved with electrophysiological techniques. pH-sensitive microelectrodes in contrast allow us to follow transport at high lactate concentrations in severely impaired mutants, in which transport activity is hardly detected by using labeled lactate.

The only mutant which could not be analyzed by either technique was the D302N or D302E mutant. The D302N mutant did not exhibit any transport activity when expressed in oocytes. The low level of surface expression of the mutant points to a possible misfolding of the transporter. This idea is corroborated by the result that even the conservative replacement D302E did not result in the rescue of the transport defect, although normal surface expression was detected. If the p*K* value of the γ-carboxyl group would be critical for functioning of the transporter, replacement of aspartate by glutamate should still result in a reasonable transport activity. The low transport activity of the D302E mutant might result from an involvement of the carboxyl group in inter- or intrahelix hydrogen bonding in which the distance is crucial. For the lactose permease, it has been proposed that the release of hydrogen bonds might be the key event in the coupling of lactose transport to H^+ movement (*22*). Although the phenotype of D302 mutants could not be analyzed, the location of this residue in helix 8 in close proximity to R306 suggests that it is important for the transport mechanism.

In contrast to the limited information gained from the D302 mutant, analysis of R306T strongly suggests that this residue is involved in the binding of lactate. The affinity of a certain substrate for MCT1 is mostly governed by the oxidation status of the α- and β-carbon atoms. 2-Oxoacids invariably have K_m values of about 1 mM for MCT1, whereas 2-hydroxyacids have K_m values of about 5 mM (*10*). The unsubstituted short fatty acids have K_m values similar to those of the 2-hydroxyacids. 2-Chloroacids in contrast have affinities more similar to those of the 2-oxoacids (*1*). The oxidation status of the β-carbon atom has less pronounced effects on the K_m values; 3-oxoacids and 3-hydroxyacids have lower affinities than their counterparts with substitution in the α-position (*8*). These comparisons suggest that the K_m value is determined by the functional groups of the β- or γ-carbon atom. However, the p*K* value of the carboxyl group also varies with the extent of oxidation of the β- or γ-carbon atom. The resulting changes in the proportion between dissociated and undissociated monocarboxylates could also affect the apparent K_m value of the transporter. The distal side chain, in contrast, seems to be of little importance (see below). The linear correlation between substrate concentration and transport rate indicates that the R306T mutant is no longer able to bind the substrate. In analogy to the substrate binding site of the lactate dehydrogenase (*15*), it is tempting to speculate that the carboxyl group is recognized by arginine 306. Similar conclusions have been drawn by Price et al. (*5*) from sequence comparisons between members of the MCT family. The substrate specificity of the R306T mutant seemed to be similar to that of the wild type. However, the question of whether substrates which do not have a carboxyl group but otherwise are similar to monocarboxylates such as acetone, ethandiol, or glycerol are translocated by the R306T mutant was not investigated.

In addition, this mutant also exhibited a partial uncoupling of H^+ translocation from substrate translocation. Although uncoupled H^+ transport is expected to be electrogenic, analysis of H^+-induced currents appeared to be inconclusive. First, even coupled lactate transport has a small electrogenic component (*10*), and second, oocytes strongly depolarized at an external pH 6.0, which hampered analysis of inward currents at this pH value. The increased extent of proton translocation which could be detected in the presence of substrate might result from cotransport of H^+ with substrate or from influx of protonated lactic acid into the oocytes. Since native oocytes do not exhibit any appreciable permeability for protonated lactic acid, even at pH 6.0 (*10*), this influx must be mediated by the mutant transporter.

Residue F360 located in helix 10 could constrict the substrate binding site of MCT1. Comparison of K_m values of different substrates reveals that the highest affinity is reached when the carboxyl group and the functional group at C-2 are in one plain as in the case of the 2-ketoacids. A tetrahedral configuration at C-2 is accepted with two hydrogen atoms or with a hydrogen atom and a hydroxyl group as exemplified by short fatty acids and 2-hydroxyacids, respectively. A methyl group at this position increases the K_m value for the hydroxyacids to values above 100 mM (*8*). This discrimination, although less pronounced, also applies to the C-3 position. Mutation of F360 to cysteine might expand the binding site, allowing mevalonate to be transported. The small decrease in the K_m value for lactate in this mutant possibly indicates that the expansion also facilitates binding of the hydroxyacid lactate. The F360C mutation could also influence the hydrophobicity of the substrate binding site. Analysis of monocarboxylate transport in erythrocytes has led to the proposal that the transporter should contain a hydrophobic pocket (*6*). The arrangement of three phenylalanine residues on the same face of helix 10 is a possible candidate for a hydrophobic site in the transporter. The F360C mutation could result in alterations of this hydrophobic pocket, which could explain the reduced V_{max} values for all substrates. Mutation of two residues in helix 9 did not result in any changes of the transport activity. Taken together with the low degree of amino acid conservation in this helix (*5*), participation of this helix in substrate binding is less likely.

An alignment of all mammalian members of the MCT family shows that R306 is conserved in all members of the family besides MCT5 (MCT4 of ref *5*). Therefore, one could speculate that MCT5 might not be a monocarboxylate transporter. The phenylalanine residues in helix 10 are not well conserved in the MCT family. MCT1 and MCT2, which have almost identical substrate specificities (*12*), both contain F360 in all the species that were investigated. MCT4 in contrast has a tyrosine in this position. The substrate specificity of this transporter might therefore be different from that of MCT1 and MCT2.

The analysis of the mutants in helices 8−11 suggests that substrate recognition in MCT1 mostly occurs in the carboxy-terminal half of the transporter. This proposal is in agreement with a general bias in the location of the substrate binding

site in transport proteins toward the carboxy terminus (*23*−*25*). With regard to helices 8−10, it is tempting to suggest that helices 8 and 10 are lining the translocation pore of MCT1, because both helices contained residues which are critical for substrate recognition. Structural data from channels and other transporters indicate that only a limited number of helices actually line the translocation pore. The results of this work suggest that R306 and F360 are in close contact with the substrate. This, however, would define only a part of the translocation pathway. Further work will be devoted to the question of whether the C-terminal half of the transporter is sufficient to form the translocation pore.

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