Determination of Transport Kinetics of Chick MCT3 Monocarboxylate Transporter from Retinal Pigment Epithelium by Expression in Genetically Modified Yeast

Evelyn F. Grollman,[‡] Nancy J. Philp,[§] Peter McPhie,[||] Rita D. Ward,[⊥] and Brian Sauer*[,#]

Laboratory of Cell Biochemistry and Biology, Laboratory of Biochemical Pharmacology, NIDDK, and Laboratory of Neurobiology, NINDS, National Institutes of Health, Bethesda, Maryland 20892, Department of Pathology, Anatomy, & Cell Biology, Jefferson Medical College, 1020 Locust Street, Philadelphia, Pennsylvania 19107, and Developmental Biology Program, Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma 73104

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ABSTRACT: Monocarboxylate transporters (MCTs) comprise a group of highly homologous proteins that reside in the plasma membrane of almost all cells and which mediate the 1:1 electroneutral transport of a proton and a lactate ion. The isoform MCT3 is restricted to the basal membrane of the retinal pigment epithelium where it regulates lactate levels in the neural retina. Kinetic analysis of this transporter poses formidable difficulties due to the presence of multiple lactate transporters and their complex interaction with MCTs in adjacent cells. To circumvent these problems, we expressed both the MCT3 gene and a green fluorescent protein-tagged MCT3 construct in *Saccharomyces cerevisiae*. Since L-lactate metabolism in yeast depends on the *CYB2* gene, we disrupted *CYB2* to study the MCT3 transporter activity free from the complications of metabolism. Under these conditions L-lactate uptake varied inversely with pH, greater uptake being associated with lower pH. Whereas the V_{max} was invariant, the K_m increased severalfold as the pH rose from 6 to 8. In addition, MCT3 was highly resistant to a number of "classical" inhibitors of lactate transport. Last, studies with diethyl pyrocarbonate and *p*-chloromercuribenzenesulfonate set limitations on the locus of potential residues involved in the critical site of lactate translocation.

The monocarboxylate transporter MCT3[1] is a member of a large family of homologous proteins found in the plasma membrane of most cells (*1*−*3*). MCT3 is a moderately large (M_r = 55 000), nonglycosylated integral membrane protein with 12 predicted membrane spanning domains (*2*). Both the coding region and the internal organization of the gene are conserved between chicken and human (*4*, *5*). Members of this transporter family mediate the transport of pyruvate and short-chain fatty acids and function, for example, in the 1:1 electroneutral translocation of a proton and lactate ion (reviewed in *6*).

In vertebrate tissues, MCT3 is located in the basal membrane of the retinal pigment epithelium (RPE) which forms the outer blood retinal barrier and governs the flow of metabolites between the neuroretina and choroidal blood supply. Glucose, entering the basal membrane of the RPE, exits from the apical membrane into the subretinal space where it becomes available for use by the neuroretinal and adjoining Müller glial cells (*7*). The latter cells have only a limited number of mitochondria, and thus spill their excess lactate into the subretinal space where it serves to meet, in part, the high-energy demands of the photoreceptor cells (*7*). This symbiotic relationship for the production and utilization of an energy source by contiguous cells is known as the lactate shuttle and is a prominent feature of neural tissue (*8*). Transepithelial lactate transport across the retinal pigment membrane is mediated by two different isoforms: MCT1, located in the apical membrane, and MCT3, located in the basal membrane (*3*). The latter transporter effectively regulates the pH and osmolarity of the subretinal space by virtue of the cohort fluid accompanying the lactate efflux to the choroid (*9*). Why two different lactate transporters are expressed in the RPE is unclear. Several possibilities exist: for example, the two transporters could have different kinetic properties that are critically important for proper homeostasis, or the two transporters may need to be differentially regulated in order to respond efficiently to changes in metabolic demands.

As is obvious from the above brief survey, kinetic studies of the monocarboxylate transporters in vertebrate tissues are difficult to interpret due to the inherently complex shuttling within the several cell structures and also because of the lack of specific inhibitors capable of blocking unique steps (*6*). A further complication to analysis is that a single cell type commonly exhibits more than one MCT isoform and that alternate types of anion transporters are present which participate in the transport of L-lactate (*6*). To circumvent all of these problems, we have expressed MCT3 in *Saccharomyces cerevisiae* and have thus been able unambiguously to determine kinetic characteristics for this member of the monocarboylate transporter family.

* To whom correspondence should be addressed at the Developmental Biology Program, M.S. 49, Oklahoma Medical Research Foundation, 825 NE 13th St., Oklahoma City, OK 73104. Tel.: 405-271-8168. FAX: 405-271-7312. Email: sauerb@omrf.ouhsc.edu.

[‡] Laboratory of Cell Biochemistry and Biology, NIDDK.

[§] Jefferson Medical College.

[||] Laboratory of Biochemical Pharmacology, NIDDK.

[⊥] Laboratory of Neurobiology, NINDS.

[#] Oklahoma Medical Research Foundation.

[1] Abbreviations: CYB2, flavocytochrome b_2; EGFP, enhanced green fluorescent protein; MCT, monocarboxylate transporter; CHC, α-cyano-4-hydroxycinnamate; RPE, retinal pigment epithelium; pCMBS, 4-chloromercuribenzenesulfonic acid; DMSO, dimethyl sulfoxide; DEPC, diethyl pyrocarbonate; PCR, polymerase chain reaction.

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Table 1: Yeast Strains

strain	genotype	plasmid	source
MH272−3c	*MAT***a** *trp1-1 his3 leu2-3,112 ura3-52*	−	Davis et al. (*13*)
EG47	*MAT***a** *trp1-1 his3 leu2-3,112 ura3-52*	pYES2-MCT3-EGFP	this work
EG50	*MAT***a** *trp1-1 his3 leu2-3,112 ura3-52*	pYES2-MCT3	this work
EG53	*MAT***a** *trp1-1 his3 leu2-3,112 ura3-52 cyb2::TRP1*	−	this work
EG54	*MAT***a** *trp1-1 his3 leu2-3,112 ura3-52 cyb2::TRP1*	pYES2-MCT3-EGFP	this work
EG55	*MAT***a** *trp1-1 his3 leu2-3,112 ura3-52 cyb2::TRP1*	pYES2-MCT3	this work

In contrast to the multiple metabolic pathways for L-lactate found in mammalian cells, *S. cerevisiae* utilizes but a single one. Here, L-lactate is metabolized by the transfer of electrons within flavocytochrome b_2 [L-lactate cytochrome *c* oxidoreductase (EC 1.1.2.3)] (*10*). This enzymatic activity, encoded by the *CYB2* gene, is unique to yeast where it is induced by lactate and repressed by glucose. In mammalian cells, L-lactate is used by lactate dehydrogenase to regenerate NAD^+. In contrast, ethanol and glycerol serve this purpose in yeast. Second, and importantly, the mitochondrial monocarboxylate (pyruvate) carrier in yeast does not translocate L-lactate as it does in mammalian mitochondria (*11*), and therefore there are no complications from mitochondrial lactate transport when measuring the activity of the plasma membrane transporter. Last, there is but one known endogenous monocarboxylate transporter present in *S. cerevisiae* responsible for the uptake of lactic acid (*12*). By careful selection of both an appropriate yeast strain and suitable culture conditions, we can ensure the complete absence of endogenous lactate transport activity. By also genetically blocking L-lactate metabolism in yeast, we have obtained an unambiguous kinetic analysis of MCT3 activity, without interference from other lactate transporters. Our results show that kinetically MCT3 is highly similar to MCT1, but, unlike MCT1, it exhibits a profound resistance to classical inhibitors of lactate transport.

EXPERIMENTAL PROCEDURES

Construction of Recombinant Plasmids. Complementary synthetic oligonucleotides (Midland Certified Reagent Co.) were used to convert the multiple cloning site (MCS) of pYES2 (Invitrogen) between the *Hin*dIII and *Xba*I sites to 5′ AAG CTT CCT AGG AGA TCT TAA GTC GAC GAA TTC TCT AGA 3′ (unique *Avr*II, *Sal*I, and *Xba*I sites underlined). The gene for an enhanced green fluorescent protein (EGFP) was amplified (94 °C, 30 s; 63 °C, 30 s; 72 °C, 2 min; 19 cycles) from pEGFP-N1 (Clontech) using the primers 5′ CTT TAT GTC GAC **ATG** GTT AGC AAG GGC GAG GAG CTG 3′ and 5′ AAT GTT CTA GAG CTT TAC TTG TAC AGC TCG 3′ (unique *Sal*I and *Xba*I sites underlined, initiating ATG in boldface) and then inserted into the modified pYES2 vector at the *Sal*I and *Xba*I sites to give pYES2-EGFP. To make pYES2-MCT3-EGFP, the chick MCT3 coding sequence +447 to +2072 (Accession No. U15685) was PCR-amplified (94 °C, 30 s; 65 °C, 30 s; 72 °C, 2 min; 25 cycles) from the cDNA on plasmid pClneo-MCT3 (*2*) using the primers 5′ GTC ATC CTA GGT AGA CG**A TG**G GGA GAG CTG 3′ (*Avr*II site underlined, initiating ATG in boldface) and 5′ TGG TTC AGT CGA CGC AAA ACT GTC CCT CTC CAC 3′ (*Sal*I site underlined). The *Avr*II−*Sal*I-digested product was ligated into the corresponding sites of pYES2-EGFP, fusing the MCT3 reading frame to that of EGFP, to generate pYES2-MCT3-EGFP. The MCT3-containing *Hin*dIII−*Sal*I fragment from pYES2-MCT3-EGFP was inserted into the *Hin*dIII and *Xho*I sites of pYES2 to give pYES2-MCT3. In all cases, DNA amplification was with the high-fidelity *Pfu* DNA polymerase (Stratagene).

Yeast Strains and Growth Conditions. Strains of the yeast *Saccharomyces cerevisiae* (Table 1) used in this work were propagated at 30 °C in YPD (1% yeast extract, 2% bactopeptone, 2% dextrose) or synthetic S medium (*14*) supplemented with the appropriate nutritional requirements, containing either 2% glucose (SD) or 2% galactose (SG). For galactose induction studies, cell were grown to mid-log phase [$(0.5-2) \times 10^7$ cells/mL] in SD medium, washed twice with water, and suspended at the same density in SG media and grown for an additional 17−20 h before harvesting.

DNA transformation of yeast was with the Frozen-EZ Yeast Transformation Kit (Zymo Research, Orange, CA), following the manufacturer's protocol. A *cyb2* disruption mutant of MH272-3c was constructed by one-step gene disruption (*15*) using the *Eco*RI−*Hpa*I-linearized *cyb2::TRP1* plasmid pESD22 (*16*). Plasmid pESD22 contains a 0.85 kb *Cla*I fragment of the *S. cerevisiae TRP1* gene [(*17*); positions −102 to +710 of the protein coding region] inserted at the *Cla*I site (position +450) of *CYB2* (*10*). PCR analysis with appropriate sets of flanking diagnostic primers confirmed proper homologous targeting with resulting disruption of *CYB2* in candidate Trp^+ MH272-3c transformants.

Assay of Lactate Transport. Cells grown in defined medium were harvested at the mid-log phase after overnight growth with galactose. Cells were washed twice and suspended in distilled water so that the final concentration of the cell suspension was 60−120 mg (dry weight) per milliliter. To initiate the uptake experiments, 50 μL of cell suspension was added to an equal volume of 0.1 M HEPES/TRIS buffer, pH 6.8, in a 12 × 75 mm glass tube and stirred with a mini-magnetic stirring bar at room temperature. After 2 min, uptake measurements were initiated by adding 100 μL of 2 mM L-lactate (or the indicated concentration) with 0.1 μCi of L-[U-^{14}C]lactic acid, sodium salt. At 3 min (or the indicated time), uptake was terminated by diluting samples with 3 mL of ice-cold water followed by immediate vacuum filtration using a stainless-steel screen filter holder and Whatman GF/C glass microfiber filter (Whatman International Ltd., Maidstone, England). The filter was washed with 3 mL of ice-cold water. Dilution, filtration, and washing were complete within 10 s. Additional washes were without effect on the measured uptake. The filter was transferred to a vial and counted with 5 mL of CytoScint (ICN Biomedical Research Products, Costa Mesa, CA). Nonspecific [^{14}C]lactic acid binding to cells or filter was corrected by measuring uptake in the presence of 100 μM carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP). This value (less than 0.01% of the added counts)

was subtracted from the values shown. The uptake measurements presented reflect the mean of a minimum of three independent experiments ± SE.

Lactate Metabolism in Yeast. After incubating cells for 2 and 5 min as described above, an aliquot of the incubation mixture (75 μL) was added to 1 mL of ice-cold water and centrifuged immediately. The pellet was suspended in 5 μL of ammonium hydroxide and separated using HPTLC Silica Gel 60 precoated plates (EM Separations, Gibbstown, NJ) and ethanol−chloroform−ammonium hydroxide−water (70:40:20:2) as solvent. In this solvent system, lactic acid has an R_f of 35 and pyruvic acid an R_f of 3 (*18*). Radioactive spots were located by autoradiography using X-OMAT AR film (Eastman Kodak Co., Rochester, NY).

pH Dependence of Lactate Transport. L-Lactate uptake was measured at 3 min using incubation media buffered over the range of pH 5.8−8.1 with 50 mM (final concentration) MES/TRIS or HEPES/TRIS. $[\text{L-Lactate}]_{out}$ was between 1 and 6 mM. K_m and V_{max} were calculated assuming that transport obeyed Michaelis−Menten kinetics ($E + S \leftrightarrow ES \rightarrow E + P$). The pH dependence of transport was fitted assuming dependence on a single group, whose pK_a can be derived from the following equation: $y = [V_{max}/K_m\ ]_0 \cdot 10^{(pK_a - pH)}/[1 + 10^{(pK_a - pH)}]$.

Treatment with Diethyl Pyrocarbonate (DEPC) and Hydroxylamine. Cells (200 μL) were incubated with the indicated concentration of DEPC (diluted in cold ethanol) with 50 mM phosphate buffer, pH 6.0, in a total volume of 1 mL (*19*). After 8 min incubation at room temperature with stirring, cells were washed twice with 5 mL of 50 mM phosphate buffer, pH 6.8. The cell pellet was suspended in 200 μL, and 50 μL aliquots were assayed as detailed above. In studies of reactivation with hydroxylamine, cells treated with 300 μM DEPC for 3−8 min were washed twice with 50 mM phosphate buffer, pH 7.4, and incubated in 1 mL of 50 mM phosphate buffer, pH 7.4, containing 50 mM freshly prepared hydroxylamine in water, a concentration that has no effect on uptake in untreated cells. After 15 min, cells were washed with 50 mM phosphate buffer, pH 6.8, and assayed as described above.

Subcellular Localization of MCT3. Cells containing the MCT3−EGFP fusion−protein construct (5×10^6 cells/mL) were immobilized by adding cells to glass slides coated with poly-L-lysine (Sigma) for 15−30 min. Cells were viewed and images captured using a Zeiss Axiovert 100 TV microscope equipped with a Hamamatsu Color Chilled 3CCD camera. Figures were assembled using Adobe Photoshop 5.0.

Materials. L-[U-^{14}C]Lactic acid, sodium salt (154 Ci/mmol), was from Amersham Pharmacia Biotech, Inc. (Piscataway, NJ). FCCP [carbonyl cyanide *p*-(trifluoromethoxy)-phenylhydrazone], sodium salts of L-lactate and D-lactate, propionate, DEPC (diethyl pyrocarbonate), α-cyano-4-hydroxycinnamate, and dimethyl sulfoxide were from Sigma; pCMBS (4-chloromercuribenzenesulfonic acid, monosodium salt) and phloretin were from Fluka. All other chemicals were the highest grade available.

RESULTS

MCT3 Expression in Yeast. The chick MCT3 cDNA was placed under the control of the yeast *GAL1* promoter to permit convenient expression in yeast by switching the carbon source from glucose to galactose. Initial constructs included the upstream untranslated region of MCT3 but produced no measurable transport activity. A second construct was made in which the upstream untranslated region of MCT3 was removed and the enhanced green fluorescent protein EGFP was fused to the *N*-terminus of chick MCT3 (EG54). With this construct, protein expression could be monitor directly. Induction of gene expression with galactose resulted in the production of fluorescent yeast cells (Figure 1A,B). The fluorescence was found to be localized to the plasma membrane of the cells when yeast were optically sectioned using a laser-scanning microscope (not shown). Noninduced EG54 cells showed no fluorescence (Figure 1C,D). These results showed both that the MCT3 fusion protein could be expressed in yeast and targeted to the plasma membrane and that the MCT3 coding region itself was not directly responsible for the poor expression from the initial constructs. An MCT3 expression construct was therefore designed without the upstream leader, and the resulting expression construct was used for subsequent functional expression experiments.

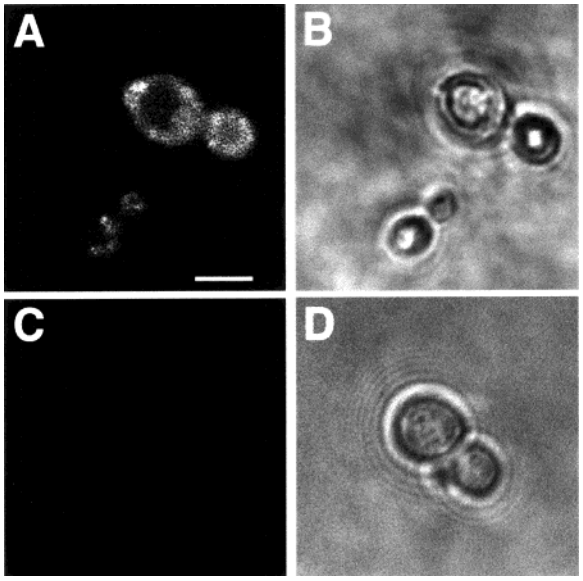

FIGURE 1: Expression of EFGP−MCT3 fusion protein. Cells were transformed with plasmid containing an EGFP−MCT3 fusion protein construct (EG54). Cells were grown overnight with galactose (induced) or with glucose (noninduced), and visualized as detailed under Experimental Procedures. Panel A shows the fluorescence signal in budding yeast and (C) the absence of signal in noninduced cells. Panels B and D show the cells visualized by phase contrast. Bar = 5 μm.

Several considerations guided our effort to develop an unambiguous system for characterizing lactate transport kinetics of MCT3 by expression of this transporter in *S. cerevisiae*. Features of the yeast system are shown diagrammatically in Figure 2. Lactate enters the cell at the plasma membrane transported by the transfected protein MCT3. JEN1 connotes an endogenous yeast lactate carrier that is unrelated to the MCT family (*20*). The JEN1 carrier is not expressed in yeast grown on glucose, but is induced when grown on lactate and is often induced when grown on other carbon sources. Screening of several laboratory strains of *S. cerevisiae* identified a yeast strain (MH272-3c) that exhibits no endogenous lactate transport activity under galactose

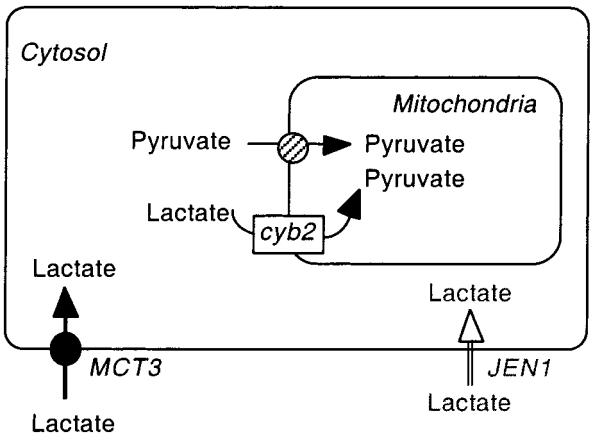

FIGURE 2: Diagram of lactate transport and metabolic pathways in *Saccharomyces cerevisiae*. Lactate is transported across the plasma membrane of *S. cerevisiae* by the heterologous expression of MCT3. The endogenous lactate transporter [proposed to be encoded by *JEN1* (*20*) and indicated by an open arrow] is not induced under the conditions used in the present report. The mitochondria pyruvate carrier indicated does not transport lactate (*11*). L-Lactate is metabolized by L-lactate cytochrome *c* oxidoreductase in the mitochondria (*10*). When *CYB2*, the gene that encodes this enzyme, is disrupted, L-lactate is no longer metabolized.

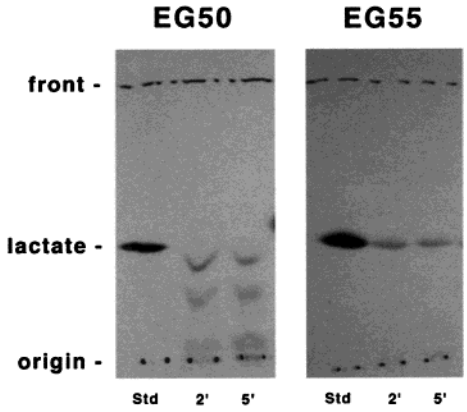

FIGURE 3: L-Lactate metabolism in yeast. L-Lactate metabolism in EG50 and EG55 was measured as described under Experimental Procedures. In brief, cells were incubated at room temperature for 2 and 5 min with L-[U-^{14}C]lactate and 1 mM lactate (final concentration) at pH 6.8 in a total volume of 200 μL. Uptake was terminated by adding 75 μL of cell suspension to 1 mL of ice-cold water and immediately centrifuged. The cell pellet was suspended in ammonium hydroxide and separated by thin-layer chromatography with the solvent system of ethanol−chloroform−ammonium hydroxide−water (70:40:20:2). Radioactive spots were located by autoradiography.

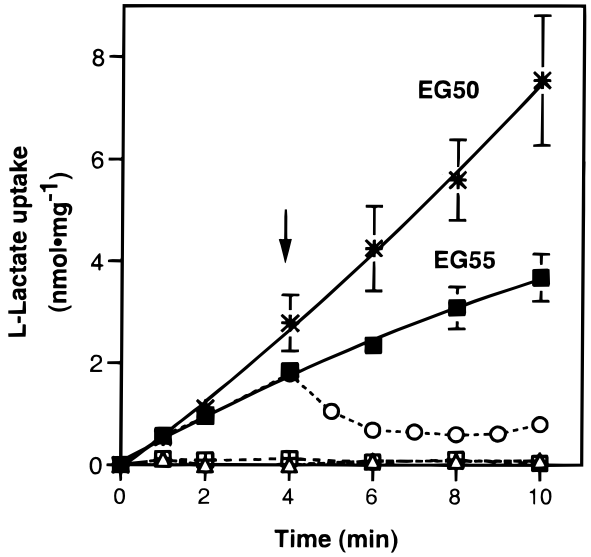

FIGURE 4: L-Lactate transport in *S. cerevisiae* as a function of time. Cells were grown overnight with galactose (induced) or with glucose (noninduced), and L-lactate uptake was measured at the indicated times as described under Experimental Procedures. The final concentration of lactate was 1 mM and the pH 6.8. Incubation was at room temperature. The cells used were EG50 induced (stars), EG55 induced (closed squares), EG55 noninduced (open squares), and wild-type EG53 induced (open triangles). At the time indicated by the arrow, D-lactate was added to EG55 at a final concentration of 200 mM (open circles). The results shown are the mean of 3 independent experiments ± SE. Uptake in the presence of 100 μM FCCP was subtracted from the values shown.

induction conditions, although this strain is still proficient for lactate transport when grown on lactate (data not shown). Use of this strain allowed us to eliminate endogenous lactate transport activity in the experiments in this study.

Lactate Metabolism in MCT3 Expressing Yeast. A second important concern in kinetic studies is the metabolism of added substrates. In particular, L-lactate is readily metabolized, and there presently exist no suitable inhibitors to block this activity (*6*). In *S. cerevisiae*, the *CYB2* gene encodes a mitochondrial enzyme required for the oxidation−reduction of L-lactate (*10*). By disrupting this gene, it is possible to measure lactate transport independent of L-lactate metabolism. The diagram in Figure 2 also depicts the mitochondrial monocarboxylate carrier in yeast that transports pyruvate but does not transport lactate (*11*). We therefore ablated the *CYB2* gene in MH272-3c by one-step gene disruption and thus eliminated L-lactate metabolism. This was demonstrated experimentally by directly measuring L-lactate metabolism in cells with a functional *CYB2* gene (EG50) and those with the disrupted gene (EG55). Cells were incubated with [^{14}C]-L-lactate 2 and 5 min and the solubilized cell pellets chromatographed (Figure 3). The single radioactive product in EG55 co-chromatographed with authentic [^{14}C]-L-lactate, while EG50 had at least four ^{14}C radioactive products evident as early as 2 min (Figure 3). This confirms that disruption of *CYB2* eliminated L-lactate metabolism and allowed us to specifically focus our study on L-lactate transport by the exogenous MCT3 transporter.

L-Lactate Uptake in Yeast Expressing MCT3. L-Lactate uptake at early time points in induced EG55 and EG50 cells was linear. Between 10 and 15 min, L-lactate uptake in EG55 leveled off but continued to be linear in EG50 for more than 20 min. In previous work, propionate had been used as a probe in studies of L-lactate transport by the endogenous lactate−proton symporter (*12*) and was not metabolized by yeast. To demonstrate that accumulation of [^{14}C]-L-lactate at the steady state was reversible, we therefore chose both propionate and D-lactate to challenge accumulation of L-lactate. Addition of a 200-fold excess of D-lactate or propionate at the time indicated by the arrow (Figure 4), reduced L-lactate accumulation in EG55 by more than 80%, but had no effect on EG50 (not shown). The inhibitory effects of D-lactate and propionate were similar. There was no measurable L-lactate uptake in cells grown with glucose, nor in cells transfected with the *GAL1* plasmid lacking the MCT3 insert (MH272-3c and EG53, Table 1) measured under the conditions used to induce plasmid expression, i.e., grown

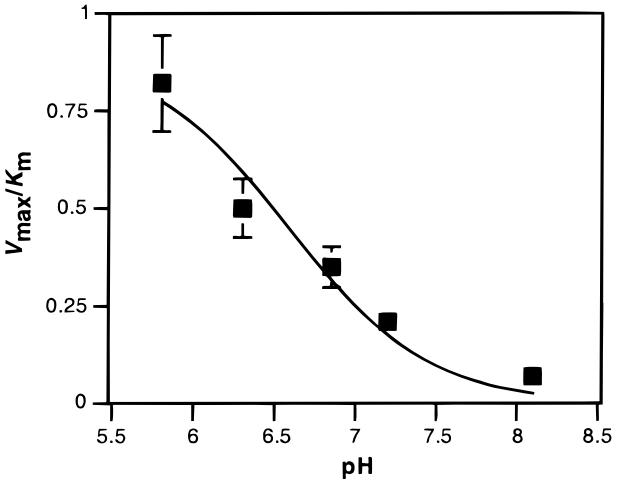

FIGURE 5: pH dependence of L-lactate transport. L-Lactate uptake was measured at 3 min using incubation media buffered over the range of pH 5.8 to pH 8.1, and the concentration of L-lactate was 1−6 mM. Measurement of L-lactate uptake and the calculation of V_{max}, K_m, and pK_a are described under Experimental Procedures. The V_{max}/K_m is shown as a function of pH. Values represent the mean of 3 independent experiments ± SE.

20 h with galactose (Figure 4).

Kinetics of L-*Lactate Transport in MCT3 Expressing Yeast.* The initial rate of L-lactate uptake was measured in EG55 cells at concentrations of L-lactate from 1 to 6 mM at pH 5.6−8.0. The uptake was pH-dependent, with greater uptake of L-lactate occurring at lower pH. The data were analyzed by fitting to the Michaelis−Menten equation, which indicated that V_{max} was independent of pH (1.44 ± 0.09 nmol mg^{-1} min^{-1}, mean ± SE), while the K_m increased with pH, from 1.9 mM at pH 5.8 to 8.3 mM at pH 7.2. At high pH, the concentrations of L-lactate fell below the derived K_m values, resulting in sizable errors. Higher concentrations of substrate approached that of the buffer, resulting in unacceptably large changes in the pH of the solutions and precluding more accurate determination of K_m values. Accurate values of V_{max}/K_m (the rate constant for reaction of free receptor with L-lactate) were determined from Woolf plots of v vs v/[S], and their variation with pH is shown in Figure 5. The best-fit curve through the points follows the protonation of a single group (presumably on the receptor) with a pK_a value of 6.6. Table 2 shows the kinetic constants of L-lactate transport determined for MCT3 expressed in yeast compared to those reported for MCT1 expressed in *Xenopus laevis* oocytes (*21*−*23*). The pK_a of MCT1 is also 6.6, and the K_m increases with increasing pH in MCT1 as it does with MCT3.

Effect of Inhibitors of Lactate Transporters. Although there was little difference between the kinetic constants of L-lactate transport determined for MCT1 and MCT3, several inhibitors are reported to discriminate among monocarboxylate transporters. To examine this issue more directly, L-lactate uptake was measured in yeast expressing MCT3 at 3 min, pH 6.8, and room temperature in the presence or absence of the indicated candidate inhibitors (Table 2). Cells routinely were preincubated with inhibitors prior to initiation of the assay. CHC, the most specific lactate transporter inhibitor, at concentrations as high as 5 mM, had no effect on L-lactate uptake by MCT3, in either EG50 (Cyb2$^+$) or EG55 (Cyb2$^-$). Two other inhibitors, phloretin and pCMBS, at the indicated concentration, also had no effect on L-lactate uptake, shown here as percent of uptake measured in the absence of added inhibitor (control). Uptake in cells preincubated with inhibitors for as long as 15 min did not differ from uptake in control cells that were not exposed to inhibitors. As discussed below, all three of these inhibitors have been reported to be good inhibitors of MCT1 expressed in oocytes (*21*, *23*).

Table 2: Kinetic Constants and Inhibitor Sensitivity of MCT3 Compared to MCT1[a]

property	MCT3	MCT1
pK_a	6.6	6.6[b]
K_m (pH 6.0)	2.0 mM	1.5 mM[c]
K_m (pH 7.0)	5.8 mM	5.6 mM[b]
inhibition by	(% control)	(% control)
CHC, 5 mM	101 ± 4	9[b]
CHC, 0.5 mM	96 ± 7	50[d]
phloretin, 0.1 mM	98 ± 5	14 ± 4[d]
pCMBS, 0.1 mM	99 ± 10	9 ± 2[d]

[a] pK_a and K_m for MCT3 were determined as described in Figure 5. For inhibition studies, L-lactate uptake was measured at 3 min pH 6.8 at room temperature as described under Experimental Procedures. Uptake was determined at a final L-lactate concentration of 1 mM in the presence or absence of inhibitors at the final concentration indicated. Inhibitors, added to the incubation medium for 2 min prior to initiation of the uptake assay, were in DMSO or ethanol at a final concentration of less than 0.5%, a concentration that had no effect on uptake. Stock solutions of pCMBS were made fresh by dissolving 10 mg in 1 mL of 0.04 N NaOH and adding 1 mL of 125 mM $NaPO_4$. The control uptake used for each inhibitor varied from 1.1 to 1.3 nmol/mg of protein at 3 min. Results are presented as percent of control, and the values shown are the mean ± SD of three independent experiments. [b] Taken from ref *21*. [c] Taken from ref *22*. [d] Taken from ref *23*.

Inhibition of L-*Lactate Uptake with DEPC.* A pK_a of 6.6 suggested a histidyl residue in MCT3 is involved in H$^+$/L-lactate symport in MCT-expressing yeast. Because DEPC is used to specifically modify histidyl residues (*24*, *25*), we determined the sensitivity of L-lactate transport to this agent. The effect of increasing concentrations of DEPC in reducing L-lactate uptake in EG55 cells is shown in Figure 6. At 8 min incubation in 400 μM DEPC, L-lactate uptake was reduced by 50%. Inhibition of L-lactate uptake by DEPC was not reversed by 15 min incubation with 50 mM hydroxylamine (not shown). Failure of hydroxylamine to reactivate may be ascribed to the modification by DEPC of residues other than histidyl, such as lysyl or arginyl residues (*24*).

DISCUSSION

Determination of the kinetic properties of the monocarboxylate transporters in mammalian tissue presents a number of inherent difficulties. This prompted us to develop a heterologous expression approach in the yeast *S. cerevisiae* to characterize the vertebrate MCT3 transporter. L-Lactate uptake in the yeast strain used in this report is devoid of L-lactate transport activity under a variety of growth conditions, so that all L-lactate uptake can be made completely dependent on the presence of a plasmid encoding MCT3. In particular, we chose a yeast strain that was unable to transport L-lactate when glucose was replaced by galactose. This allowed us to use the *GAL1* promoter to control heterologous expression of MCT3 without interference from endogenous L-lactate transport. To avoid complications from the metabolism of L-lactate after its entry into the cell, we availed ourselves of the observation that in *S. cerevisiae* L-lactate metabolism requires the transcriptional induction of *CYB2*,

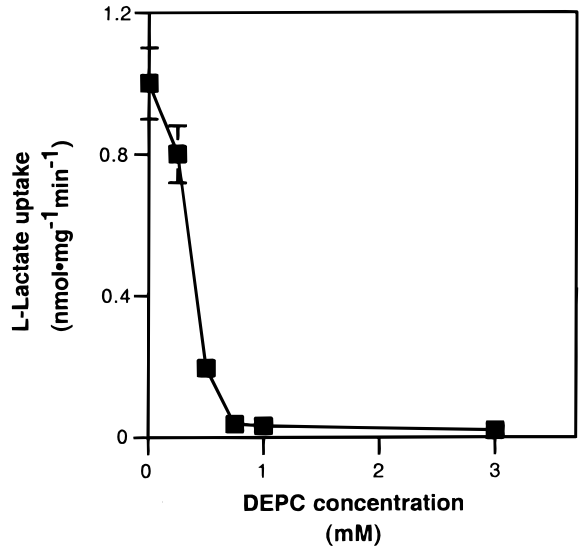

FIGURE 6: Effect of diethyl pyrocarbonate (DEPC) on L-lactate uptake. Treatment of cells with DEPC and measurement of L-lactate uptake are detailed under Experimental Procedures. In brief, induced cells (EG55) were incubated at room temperature in 50 mM phosphate buffer, pH 6.0, with the indicated final concentration of DEPC. The reaction was stopped at 8 min by washing cells twice with 50 mM phosphate buffer, pH 6.8, and L-lactate uptake was measured in the cell pellet. Uptake in the presence of 100 μM FCCP was subtracted from the values shown and was the same in both treated and untreated cells. The values shown are the mean of 3 independent experiment ± SE.

the gene encoding flavocytochrome b_2 (*10*). Disruption of this gene in yeast thus permitted the unambiguous kinetic characterization of L-lactate transport by MCT3. Confirmation that *CYB2* disruption had eliminated L-lactate metabolism in yeast was obtained by showing that L-lactate was the only radioactive compound present in yeast exposed to [^{14}C]-L-lactate.

We measured L-lactate accumulation under a variety of conditions, varying the concentration from 1 to 6 mM and the pH values between 6 and 8, conditions under which the V_{max} remained invariant. Values for the K_m, however, proved to be a function of pH, being 1.9 mM at pH 5.8 and 5.8 mM at pH 7.2. The kinetic properties we obtained for MCT3 closely mimic those reported for MCT1 as expressed in *Xenopus laevis* oocytes (*21*, *22*). Such dependence of uptake on pH is consistent with the proposal that transport involves an initial binding of a proton to the transporter followed by the lactate anion (*26*, *27*). The translocation and release of both species from the membrane are freely reversible (*27*). We note that the pK_a value for MCT3 derived from the best fit in Figure 5 may reflect that of the protonated group in the transporter. However, the hazards of over-interpretation of such curves are well-known (*28*). In accord with the suggested role of MCT3 as the isoform responsible for the efflux of L-lactate from the retinal pigment epithelium into the choroid, and that it is active at pH 7.2 (*29*), it seems unlikely that a pH gradient drives the loss of L-lactate. Rather, it appears that as the pH decreases at the site of L-lactate entry, transport may be stimulated via a lowered K_m in a fashion analogous to that reported for lactate−proton binding in skeletal muscle (*27, 30*).

Attempts to discriminate among the several monocarboxylate transporters are largely dependent upon the judicious use of allegedly specific inhibitors. Variability in sensitivity, however, has been reported depending on the particular cell or membrane system examined (*27, 31*). One such compound, α-cyano-4-hydroxycinnamate (CHC), is a relatively specific inhibitor of H^+-coupled transporters whereby the H^+ bonding of the lactate α-hydroxyl group to the transporter is believed to be disrupted by the analogous structure on CHC. For example, MCT1-mediated L-lactate transport is inhibited by CHC. In a like manner, L-lactate transport has been measured in cultured kidney cells that express MCT4, and that transport is also sensitive to inhibition by CHC (*32*). In contrast to studies using cultured cells, L-lactate transport measured in muscle membrane vesicles, which are enriched in MCT4, is insensitive to inhibition by CHC (>4 mM). In the work we describe here, we find that MCT3 expressed in yeast was insensitive to inhibition by CHC (>5 mM) even though its kinetic properties resembled those of MCT1. In addition, MCT3 was not inhibited by phloretin, another commonly used inhibitor of lactate transport. In a manner reminiscent of the CHC work, phloretin inhibits MCT1-mediated lactate transport when the assays are performed on intact cells, but lactate transport is unchanged in the presence of the inhibitor when muscle vesicles are used (*29*). These results underscore an important fact: studies using vesicles, unlike studies with intact cells, are not complicated by lactate metabolism nor by participation of mitochondrial transport.

In cells with functioning mitochondria, the inhibition of uptake into the mitochondria by CHC increases the cytosolic L-lactate concentration and thereby reduces further influx (*30*). This may be of particular importance when evaluating the inhibitor effect of CHC as mitochondrial lactate/pyruvate import is very sensitive (1 μM) to inhibition with CHC. Such mitochondrial inhibition can have a profound effect on lactic acid production and intracellular acidification (*29*). Moreover, MCT1 has been identified in muscle mitochondria (*33*). Studies of muscle vesicles may therefore better reflect plasma membrane transport properties of this tissue. By eliminating both mitochondrial import and lactate metabolism, the work here in yeast with MCT3 may more closely parallel the previously reported work with other lactate transporters in muscle vesicles.

Residues in the 8−11 membrane-spanning domains of MCTs are thought to be important for transporter activity (*22*), and are conserved in both MCT1 and MCT3, and also in MCT4. The overall sequence similarity between MCT3 and MCT4 isoforms most likely reflects their 'recent' common ancestor, and thus need not indicate conservation of lactate transporter kinetic parameters. A low affinity of MCT4 for L-lactate (K_m = 13−40 mM) is reported in studies of giant sarcolemmal vesicles from glycolytic muscle fibers (*30*). As noted above, this preparation also shows a low affinity for CHC. But MCT transport kinetics have also been reported to be similar to those of MCT1, for example, in studies of kidney epithelial cell lines expressing large amounts of MCT4 (*32*). Kinetic analysis can be complicated when cells exhibit more than one MCT isoform and when alternate types of anion transporters are present which participate in the transport of L-lactate. These complications could account for the K_m value reported for the kidney cell lines. Differences in affinity may also reflect differences in the type of assay and conditions used for assay. As demonstrated using model systems, there is a marked effect

of pH to influence the measured K_m (this report and ref *22*). MCT4 has not been analyzed in detail (*29*). We anticipate that further studies of MCT3 and MCT4, preferably using the same model system, will provide a true comparison of the lactate affinity of MCT3 and MCT4.

A second inhibitor, pCMBS, has been used to distinguish between isoforms MCT1 and MCT2, the former being sensitive and the latter insensitive to inhibition (*21*). This organomercurial thio reagent reacts with exposed cysteines, and at 100 μM was without effect on MCT3 expressed here in yeast. This result implicates a cysteine residue found on the trans membrane region 10 of MCT1, but missing on MCT2 and MCT3, as a likely candidate target for the site of inhibition. We note that helices 8 and 10, but not 9, have been reported to contain residues necessary for substrate recognition (*22*).

A third inhibitor in current use is diethyl pyrocarbonate (DEPC). This compound has been used to ask whether a histidyl residue might be involved in L-lactate translocation and thereby account for the apparent pH dependence of the dissociation constant of MCT3 (estimated $pK_a = 6.6$). In pursuit of this question, we showed that treatment with DEPC led to a concentration- and time-dependent inactivation of L-lactate transport by MCT3 that was not reversed by subsequent exposure to hydroxylamine. Since hydroxylamine removes the carbethoxy groups from modified histidyl and tyrosyl residues, its failure to reverse the action of DEPC suggests that one or more alternate amino acid residues, such as lysine or arginine, might be involved in the translocation complex of binding (*24*).

In summary, we have obtained high-quality kinetic data on the vertebrate MCT3 monocarboxylate transporter isoform that helps both to distinguish MCT3 from other members of this large transporter family and also to shed light on its biological role in the retinal pigment epithelium. This characterization of MCT3 relies on use of a heterologous expression approach in a genetically engineered yeast strain that (1) permits production of MCT3 under *GAL1* control without interference from endogenous L-lactate transport activities and (2) eliminates the confounding effects of subsequent L-lactate metabolism in the cell on transport kinetics. This genetic approach also promises to allow the powerful application of yeast genetics and molecular mutagenesis strategies for more detailed structure−function analysis of MCT3. Moreover, because the approach is generally applicable to all of the monocarboxylate transporters, it will undoubtedly facilitate study of the other members of this important transporter family, and thereby help clarify the various biological roles of these proteins in higher organisms.

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