Analysis of the Binding Moiety Mediating the Interaction between Monocarboxylate Transporters and Carbonic Anhydrase II*

Received for publication, November 7, 2014, and in revised form, December 31, 2014. Published, JBC Papers in Press, January 5, 2015, DOI 10.1074/jbc.M114.624577

Sina Ibne Noor†, Steffen Dietz‡, Hella Heidtmann§*, Christopher D. Boone‡, Robert McKenna‡, Joachim W. Deitmer§, and Holger M. Becker††

From the †Department of Biology, Division of Zoology/Membrane Transport and the §Department of Biology, Division of General Zoology, University of Kaiserslautern, D-67653 Kaiserslautern, Germany and the ‡Department of Biochemistry and Molecular Biology, University of Florida, Gainesville, Florida 32610

Proton-coupled monocarboxylate transporters (MCTs) mediate the exchange of high energy metabolites like lactate between different cells and tissues. We have reported previously that carbonic anhydrase II augments transport activity of MCT1 and MCT4 by a noncatalytic mechanism, while leaving transport activity of MCT2 unaltered. In the present study, we combined electrophysiological measurements in Xenopus oocytes and pulldown experiments to analyze the direct interaction between carbonic anhydrase II (CAII) and MCT1, MCT2, and MCT4, respectively. Transport activity of MCT2-WT, which lacks a putative CAII-binding site, is not augmented by CAII. However, introduction of a CAII-binding site into the C terminus of MCT2 resulted in CAII-mediated facilitation of MCT2 transport activity. Interestingly, introduction of three glutamic acid residues alone was not sufficient to establish a direct interaction between MCT2 and CAII, but the cluster had to be arranged in a fashion that allowed access to the binding moiety in CAII. We further demonstrate that functional interaction between MCT4 and CAII requires direct binding of the enzyme to the acidic cluster 431EEE in the C terminus of MCT4 in a similar fashion as previously shown for binding of CAII to the cluster 489EEE in the C terminus of MCT1. In CAII, binding to MCT1 and MCT4 is mediated by a histidine residue at position 64. Taken together, our results suggest that facilitation of MCT transport activity by CAII requires direct binding between histidine 64 in CAII and a cluster of glutamic acid residues in the C terminus of the transporter that has to be positioned in surroundings that allow access to CAII.

The SLC16 gene family of monocarboxylate transporters (MCTs)2 comprises 14 isoforms, the first four of which carry high energy metabolites, such as lactate, pyruvate, and ketone bodies together with H⁺ in a 1:1 stoichiometry (1–5). MCT1 is found in nearly all tissues, where it operates either as a lactate importer or exporter (3–5). MCT1 has an intermediate Km value of 3–5 mM for l-lactate (1, 2). MCT2, which is primarily found in liver, kidney, testis, and brain (6–8), has the highest affinity for l-lactate among all MCTs with a Km value of ~0.7 mM (9). In liver and kidney, MCT2 facilitates the uptake of lactate, which is used for glyconeogenesis in these tissues (3). In the brain, MCT2 is expressed in neurons, where it facilitates the import of lactate, which is exported from astrocytes and vascular endothelial cells via MCT1 and MCT4 (10–13). Expression of MCT3 is restricted to retinal pigment epithelium and choroid plexus epithelia, where it primarily serves as a lactate exporter (14–16). MCT3 transports l-lactate with a Km of ~6 mM (17). MCT4 is a low affinity, high capacity carrier with a Km value of 20–35 mM for l-lactate (18). It primarily acts as a lactate exporter in glycolytic cells and tissues like astrocytes, skeletal muscle, and (hypoxic) tumor cells (3, 5, 19, 20). All MCTs have a 12-transmembrane helix structure, with both the C and N termini located intracellularly (3, 5). Trafficking, but also regulation of transport activity of MCT1–4, is mediated by ancillary proteins. MCT1 and MCT4 are associated with basigin (CD147), whereas surface expression and transport activity of MCT2 is facilitated by embigin (GP70) (21, 22).

Mammalian carbonic anhydrases (CA), included in the α-class of CAs, of which 16 isoforms are identified, catalyze the reversible hydration of CO₂ to HCO₃⁻ and H⁺ (23, 24). Cytosolic CAII has been found to bind to and facilitate transport function of many acid/base transporting membrane proteins, including the Cl⁻/HCO₃⁻ exchangers AE1 and AE2 (25–29), the Na⁺/HCO₃⁻ cotransporters NBCe1 and NBCn1 (30–36) and the Na⁺/H⁺ exchanger NHE1 (37, 38), a phenomenon

*This work was supported by Stiftung Rheinland-Pfalz für Innovation Grant 961-386261/957 (to H. M. B.) and by the Landesschwerpunkt Membrantransport (to H. M. B. and J. W. D.).
†To whom correspondence should be addressed: Dept. of Biology, Div. of Zoology/Membrane Transport, University of Kaiserslautern, P.O. Box 3049, D-67653 Kaiserslautern, Germany. E-mail: h.becker@biologie.uni-kl.de.

This is an Open Access article under the CC BY license.

© 2015 by The American Society for Biochemistry and Molecular Biology, Inc. Published in the U.S.A.
coined “transport metabolon.” In all cases, CAII was found to bind to an acidic cluster within the C-terminal tail of the transport protein. In AE1, 886LDADD was identified as CAII binding site (27, 28), whereas binding of CAII to AE2 requires the corresponding cluster 1216LDANE (27). In NBCe1, the acidic clusters 958LDV and 986DNDD are suggested to bind CAII (33, 34), and in NBCn1, binding to CAII is mediated by Asp1135 and Asp1136 (35). Binding of CAII to NHE1 involves the penultimate group of 13 amino acids of the transporter C terminus (790RIQRCL), to be crucial for the functional interaction with CAII (49).

In the present study, we could demonstrate that introduction of a CAII binding site into the C terminus of MCT2 induced binding between MCT1-CT and CAII. This suggests that cytosolic CAII can bind to the C terminus of MCT1, which presumably positions the enzyme close enough to the pore of the transporter for efficient $H^+$ shuttling.

In the present study, we could demonstrate that introduction of a CAII binding site into the C terminus of MCT2 induced CAII-mediated facilitation of MCT2 transport activity. However, binding and functional interaction between MCT2 and CAII could only be achieved when the two glutamic acid residues mediating binding to CAII were located in a moiety that allowed their access to the enzyme. This demonstrates that the sole presence of an acidic amino acid cluster within the C terminus of a transport protein is not sufficient to mediate binding of CAII to this transporter but that the amino acid environment needs to allow free access of the binding site to CAII. We further show that binding between CAII and MCT4 is mediated by the amino acids His$^{64}$ in CAII and Glu$^{489}$/Glu$^{491}$ in the C terminus of MCT4, whereas binding between CAII and MCT1 involves CAII-His$^{64}$ and MCT1-Glu$^{489}$/Glu$^{491}$.

**EXPERIMENTAL PROCEDURES**

**Site-directed Mutation of MCT2 and MCT4—**Site-directed mutation of MCT2 and MCT4 was carried out by PCR using a mix of $Taq$ and $Pfu$ polymerases (Fermentas high fidelity PCR enzyme mix; Thermo Fisher) and modified primers, which contained the desired mutation. Primers used for creation of the different mutants are shown in Table 1. Rat MCT2 and MCT4, cloned in the oocyte expression vector pGEM-He-Juel (9, 18), were used as template. PCR was cleaned up using the Gene Jet plasmid miniprep kit (Thermo Fisher), and the template was digested with DpnI (Fermentas FastDigest DpnI; Thermo Fisher) before transformation into Escherichia coli DH5α cells.

**Pulldown of CAII with GST Fusion Proteins—**C termini of rat MCT1-WT, rat MCT2-WT, rat MCT4-WT, and mutants of the C termini were cloned into the expression vector pGEX-2T (GE Healthcare Europe GmbH) and transformed into E. coli BL21 cells. Protein expression was induced by addition of 0.8 mM isopropyl-$\beta$-D-thiogalactopyranoside. 3 h after induction, the cells were harvested and resuspended in PBS and lysed with lysis buffer (PBS, 2 mM MgCl$_2$, 1% Triton X-100) in the presence of protease inhibitors (protease inhibitor mixture tablets; Roche). Bacterial lysates were centrifuged for 15 min at 4 °C at 12,000 $\times$ g, and the supernatant containing the GST fusion protein (bait protein) was collected for further use. CAII-WT and CAII-H64A, respectively, were expressed in Xenopus oocytes, as described in the next section. For each experiment, 25 oocytes were lysed in lysis buffer in the presence of protease inhibitors (Roche). Oocyte lysates were centrifuged for 15 min at 4 °C at 12,000 $\times$ g, and the supernatant (prey protein) was collected for further use.

The pulldown experiment was carried out using the Pierce GST protein interaction pulldown kit (Thermo Fisher). Briefly, for immobilization of GST fusion protein, 400 μg of bacterial lysate was added to a column containing 50 μl of 50% slurry of glutathione agarose and incubated for 2 h at 4 °C with end over
Binding between MCTs and CAII

end mixing. After incubation, the excess bait protein was removed by centrifugation (4 °C, 700 x g), and the beads were washed five times with wash buffer (1 PBS: 1 lysis buffer). 400 μl of oocyte lysate containing CAII-WT or CAII-H64A was added to the column and incubated for 2 h at 4 °C with end over end mixing. After incubation, the excess prey protein was removed by centrifugation (4 °C, 700 x g), and the beads were washed five times with wash buffer. Protein was eluted from the beads with 250 μl of elution buffer (10 mM glutathione in PBS, pH 8.0).

To determine the relative amount of GST and CAII, an equal volume of the samples was analyzed by Western blotting. GST was detected using a primary anti-GST antibody (dilution 1:400, anti-GST tag mouse monoclonal IgG, no. 05-782; Millipore) and a goat anti-mouse IgG horseradish peroxidase-conjugated secondary antibody (dilution 1:12000; Santa Cruz). CAII was detected using a primary anti-CAII antibody (human erythrocytes) (dilution 1:400, rabbit anti-carbonic anhydrase II polyclonal antibody, AB1828; Millipore) and a goat anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (dilution 1:12000; Santa Cruz). Membranes were analyzed after incubation with Luminata classic Western HRP substrate (Millipore) with an Odyssey Fc dual mode imaging system (Li-Cor Biosciences). Quantification of the band intensity was carried out with the software ImageJ. To overcome variations in the signal intensity between different blots, the signal intensity of each band for CAII was normalized to the signal intensity of the band from the pulldown of CAII with the GST fusion protein of the full-length C terminus. To account for variations in the amount of GST fusion protein, each normalized signal for CAII was divided by the corresponding, normalized signal for GST.

Heterologous Protein Expression in Xenopus Oocytes—Plasmid DNA of rat MCT1-WT, rat MCT2-WT, rat MCT4-WT, rat GP70, human CAII-WT, and mutants of the proteins, respectively, cloned into the oocyte expression vector pGEM-He-Juel, which contains the 5’ and the 3’ untranscribed regions of the Xenopus β-globin flanking the multiple cloning site, was linearized with Sall (Fermentas FastDigest Sall; Thermo Fisher) and transcribed in vitro with T7 RNA polymerase (Ambion mMessage mMachine; Life Technologies) as described earlier (50). Xenopus laevis females were purchased from Xenopus Express (Vernassal, France). Segments of ovarian lobules were surgically removed under sterile conditions from frogs anesthetized with 1 g/liter of 3-amino-benzoic acid ethylester (MS-222; Sigma-Aldrich) and rendered hypothermic. The procedure was approved by the Landesuntersuchungsamt Rheinland-Pfalz, Koblenz (23 177-07/A07-2-003 66). Oocytes were singularized by collagenase (collagenase A; Roche) treatment and stored overnight in oocyte saline (82.5 mM NaCl, 2.5 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 1 mM Na2HPO4, 5 mM HEPES). In lactate-containing saline, NaCl was replaced by an equivalent amount of sodium l-lactate. Application of lactate was always carried out in HEPES-buffered solution at pH 7.0, in the nominal absence of CO2/HCO3-, containing ~0.008 mM of CO2 from air and hence a HCO3− concentration of less than 0.2 mM. To check for CAII catalytic activity, a short CO2 pulse was applied. For this solution, NaCl was replaced by 10 mM NaHCO3, and the solution was aerated with 5% CO2/95% O2.

For measurement of intracellular H+ concentration and membrane potential, single-barreled microelectrodes were used; the manufacture and application have been described in detail previously (51, 52). Briefly, a borosilicate glass capillary of 1.5 mm in diameter was pulled to a micropipette and was silanized with a drop of 5% tri-N-butylchlorosilane in 99.9% pure carbon tetrachloride, backfilled into the tip. The micropipette was baked for 45 min at 450 °C on a hot plate. H+-sensitive mixture (no. 95291; Fluka) was backfilled into the silanized tip and filled up with 0.1 sodium citrate, pH 6.0. To increase the opening of the electrode tip, it was beveled with a jet stream of aluminum powder suspended in H2O. The reference electrode was filled with 3 mM KCl. Calibration of the electrodes was carried out in oocyte salines with a pH of 7.0 and 6.4. As described previously (2), optimal pH changes were detected when the electrode was located near the inner surface of the plasma membrane. During all measurements, the oocytes were clamped to a holding potential of −40 mV using an additional microelectrode, filled with 3 mM KCl, and connected to an Axoclamp 2B amplifier (Axon Instruments). All experiments were carried out at room temperature (22–25 °C). The measurements were stored digitally using custom made PC software based on the program LabView (National Instruments). The rate of change of the measured [H+]i was analyzed by determining the slope of a linear regression fit using the spreadsheet program OriginPro 8.6 (OriginLab Corporation). Conversion and analysis of the data has been described in detail previously (52).

Calculation and Statistics—Statistical values are presented as means ± S.E. of the mean. For calculation of significance in differences, Student’s t test or, if possible, a paired t test was used. In the figures shown, a significance level of p ≤ 0.05 is marked with *, p ≤ 0.01 is marked with **, and p ≤ 0.001 is marked with ***.
acidic and hydrophobic faces of CAII. The C terminus of MCT4 is based on the model of the MCT1 C terminus, with minor rearrangements to facilitate proper CAII-His64 interactions. CAII is based on the crystal structure of human CAII (Protein Data Bank code 2ILI (53)). Models were created utilizing SWISS Model. Docking of the C terminus of MCT1/MCT4 to CAII was performed using the interactive software COOT (54) and then energy minimizing using the program CNS (55).

Graphical representations of the structures were created with MacPyMOL (DeLano Scientific LLC).

RESULTS

Introduction of a CAII-binding Site Allows CAII-mediated Facilitation of MCT2 Transport Activity—We have previously shown that CAII augments transport activity of MCT1, while leaving transport activity of MCT2 unaltered (42, 43, 45, 46, 49).
Binding between MCTs and CAII

Although the C terminus of MCT1 contains a CAII binding site (485EEE), MCT2 lacks such a cluster (Fig. 1A). To test the hypothesis that the failure of CAII to enhance MCT2 transport activity is due to the lack of an appropriate CAII-binding site in the MCT2-C terminus, we introduced a putative CAII-binding site into the C terminus of MCT2. Therefore, we replaced lysine 485 and serine 487 by glutamate (MCT2-K485E/S487E), which would create a putative CAII-binding site (485EEE) at a similar position as found in MCT1 (489EEE). MCT2-WT and MCT2-K485E/S487E, respectively, were coexpressed together with their chaperone embigin (GP70) in Xenopus oocytes, either with or without 50 ng CAII protein injected. MCT2 transport activity was determined by measuring the rate of rise in intracellular H\(^{+}\) concentration (\(\Delta[H^+]/\Delta t\)) during application of 0.3, 1, and 3 mM lactate, respectively, in HEPES-buffered, nominal CO\(_2\)/HCO\(_3\)\(-\)-free solution (Fig. 1B). Injection of CAII did not result in an increase in MCT2 transport function, neither in MCT2-WT nor in MCT2-K485E/S487E, carrying the putative CAII binding site (Fig. 1B). However, in MCT2-K485E/S487E the putative binding site 485EEE is surrounded by polar or bulky amino acids (see inset in Fig. 1B) that are not found at this position in MCT1. To determine whether these amino acids might obscure the CAII-binding site, we exchanged the seven most distal amino acids in the MCT2 C terminus to the corresponding amino acids of the MCT1 C terminus (R483P/K484A/K485E/E486E/S487E/S488S/I489P, MCT2-PAEEESP) and coexpressed the mutant with embigin in Xenopus oocytes. Indeed, injection of CAII increased transport activity of MCT2-PAEEESP by a factor of 2, indicating a functional interaction between CAII and the MCT2 mutant (Fig. 1B). Catalytic activity of CAII was controlled in each oocyte by measuring \(\Delta[H^+]/\Delta t\) during application of 5% CO\(_2\)/HCO\(_3\)-free solution (Fig. 1B). Injection of CAII caused a threefold increase in transport activity of MCT2-WT by a factor of 2, as determined by \(\Delta[H^+]/\Delta t\) during application of lactate (Fig. 1C). Truncation of the MCT4 C terminus at position 430 (MCT4-S430X; removal of all three potential binding sites) resulted in a complete loss of the CAII-mediated augmentation in MCT4 transport activity (Fig. 2C). In contrast, truncation of the MCT4 C terminus at position 446 (MCT4-S446X; removal of 448EVE and 456EPE) had no effect on the CAII-induced augmentation in MCT4 transport activity (Fig. 2C). Transport activity of MCT4 itself in the absence of CAII was not altered by truncation of the C terminus (Fig. 2C, gray bars). These data indicate that functional interaction between MCT4 and CAII is mediated by an amino acid cluster located between Ser\(^{430}\) and Ser\(^{446}\) in the MCT4 C terminus, most likely the glutamic acid cluster 431EEE.

To further analyze which glutamic acid residues within this cluster are required for functional interaction between MCT4 and CAII, we replaced the three glutamic acid residues Glu\(^{431}\)– Glu\(^{433}\) by glutamine in any possible combination and expressed the mutants in Xenopus oocytes with and without CAII (Fig. 2, D and E). Single replacement of any glutamic acid residue by glutamine (MCT4-E431Q, MCT4-E432Q, and MCT4-E433Q, respectively) had no effect on the CAII-induced augmentation in MCT4 transport activity (Fig. 2C). Transport activity of MCT4 itself in the absence of CAII was not altered by truncation of the C terminus (Fig. 2C, gray bars). These data indicate that functional interaction between MCT4 and CAII is mediated by an amino acid cluster located between Ser\(^{430}\) and Ser\(^{446}\) in the MCT4 C terminus, most likely the glutamic acid cluster 431EEE.
at least one of the two glutamic acid residues Glu$^{431}$ and Glu$^{433}$ in the MCT4 C terminus is required for the functional interaction between MCT4 and CAII. Catalytic activity of CAII was controlled in each oocyte by measuring $\Delta$[H$^+$/H$^-$]$_t$ during application of 5% CO$_2$, 10 mM HCO$_3^-$ at the end of the experiment. Fig. 2B shows example traces of MCT4-WT (black trace, third pulse) as example for a non-CAII-injected oocyte, and traces of MCT4-WT + CAII (gray trace, third pulse), MCT4-S446X + CAII (green trace, third pulse), and MCT4-S430X + CAII (blue trace, third pulse) as examples for CAII-injected oocytes. In non-CAII-injected oocytes $\Delta$[H$^+$/H$^-$]$_t$ varied between 27.0 ± 1.0 nM/min and 30.8 ± 1.4 nM/min, whereas $\Delta$[H$^+$/H$^-$]$_t$ in CAII-injected cells varied between 163.4 ± 5.4 nM/min and 199.9 ± 12.4 nM/min (data not shown). No statistical differences were found between the batches, indicating that all batches of oocytes were injected with approximately the same amount of catalytically active CAII protein.

To test whether CAII binds to the C terminus of MCT4, we pulled down CAII from lysates of CAII-expressing Xenopus oocytes with GST fusion proteins of the MCT4 C terminus, bound to agarose beads. The amount of captured CAII was quantified by Western blot analysis (Fig. 3A, upper row). To overcome variations in the signal intensity between different blots, signal intensity of each band for CAII was normalized to the signal intensity of the band from the pulldown of CAII with GST-MCT4-WT. To account for variations in the amount of GST fusion protein, each normalized signal for CAII was divided by the corresponding, normalized signal for GST. Pull-down of CAII with a GST fusion protein of the full-length C terminus of MCT4 (GST-MCT4-WT) resulted in a robust signal for CAII on the Western blot, whereas pulldown of CAII with GST alone resulted in almost no signal for CAII (3.0 ± 1.6% of the signal achieved with GST-MCT4-WT), indicating direct binding of CAII to the C terminus of MCT4, but not to...
GST (Fig. 3B). Truncation of the C terminus at position 430 (GST-MCT4-S430X) resulted in vast reduction of the signal to 4.8 ± 1.5% (as compared with GST-MCT4-WT), whereas truncation at position 446 (GST-MCT4-S446X) had no significant effect on the amount of captured CAII (Fig. 3B). Single replacement of any glutamic acid residue by glutamine (GST-MCT4-E431Q, GST-MCT4-E432Q, and GST-MCT4-E433Q, respectively), as well as replacing either one of the flanking glutamic acid residues and the glutamic acid residue in the middle of the cluster by glutamine (GST-MCT4-E431Q/E432Q and GST-MCT4-E432Q/E443Q, respectively) also showed no significant effect on the amount of precipitated CAII (Fig. 3B). In contrast to that, replacement of the two glutamic acid residues at the rim of the cluster by glutamine (GST-MCT4-E431Q/E433Q), as well as simultaneous replacement of all three glutamic acid residues by glutamine (GST-MCT4-E431Q/E432Q/E433Q), led to a significant reduction in the amount of captured CAII (Fig. 3B). These data indicate that CAII binds to the C terminus of MCT4 and that at least one of the two glutamic acid residues Glu431 and Glu433 within the MCT4 C terminus is required for the binding.

Assuming that a CAII-binding site in MCT4 should be conserved along different mammalian species, we aligned the protein sequence of the MCT4 C terminus of the five mammalian species analyzed so far (Fig. 3C). Indeed the binding site was found to be conserved along rat (431EEE), human (425EEE), horse (420EEE), and bovine (431EE-), leaving only one glutamic acid residue (Glu431) that would mediate binding of CAII.

MCT1 and MCT4 Bind to CAII Histidine 64—Previous studies have shown that CAII-His64 is crucial for CAII-mediated increase in transport activity of MCT1 and MCT4, because exchange of His64 by alanine resulted in the loss of CAII-medi-
ated facilitation of transport activity (48). To test whether binding between MCT1/4 and CAII is mediated by His64 in CAII, we pulled down CAII-WT or CAII-H64A with GST-MCT1 and GST-MCT4, respectively (Fig. 4A). Although pulldown of CAII-WT with GST-MCT1 and GST-MCT4 resulted in a robust signal for CAII, virtually no signal could be detected when CAII-H64A was pulled down with GST-MCT1 or GST-MCT4 (Fig. 4B). To make sure that the antibody used for detection of CAII in the pulldown experiment is able to recognize CAII-H64A, we directly blotted lysates of Xenopus oocytes expressing CAII-WT and CAII-H64A, respectively, and stained for CAII using the same antibody as used for CAII detection after the pulldown (Fig. 4C). The plot showed robust signals both for CAII-WT and CAII-H64A, indicating that CAII-H64A can be recognized by the antibody. These results indicate that binding of MCT1 and MCT4 to CAII is likely mediated by CAII-His64.

DISCUSSION

The present study shows that introduction of a CAII-binding site into the C terminus of MCT2 induces CAII-mediated augmentation in MCT2 activity. However, binding and functional interaction could only be achieved when the ambient amino acids were arranged in a fashion that allowed access of the introduced binding site to CAII.

We have previously shown that CAII facilitates transport activity of MCT1, but not MCT2, when the proteins are heterologously expressed in Xenopus oocytes (42, 43, 45–49). Facilitation of MCT1 transport function has been found to be independent of the catalytic activity of the enzyme (42, 43) but required both the intramolecular H+ shuttle of CAII (CAII-His64) (48) and the CAII-binding site489EEE in the C terminus of MCT1 (49). This led to the conclusion that CAII, directly bound to the transporter, can act as a H+ -collecting/distributing antenna, which shuttles protons between transporter and surrounding protonatable residues to support proton/lactate cotransport (48). The requirement for H+ -collecting antennae has been proposed for H+ -cotransporters such as MCTs, whose substrate is present at very low concentrations (56). By modeling intracellular diffusion of ions following Brownian movement, the authors found that transporters that utilize substrates like Ca2+ or H+, which are available in the cell only at very low concentrations, show experimental rates of transport that are considerably faster than the rates at which the aqueous phase may possibly feed their binding sites. From this paradoxical finding the authors concluded that Ca2+ and H+ transporters do not extract their substrates directly from the bulk cytosol but from an intermediate “harvesting” compartment located between the aqueous phase and the transport site (56), which in the case of MCTs might be CAII. An efficient proton transfer requires close proximity between transporter and enzyme. Therefore, the failure of CAII to increase MCT2 transport activity was attributed to the lack of an appropriate CAII binding site in the C terminus of MCT2. Indeed, the present study shows no binding between CAII and the C terminus of MCT2-WT. To investigate whether introduction of a CAII-binding site into the C terminus of MCT2 might induce facilitation of MCT2 transport activity by CAII, we first introduced a cluster of three glutamic acid residues (485EEE) into the C terminus of MCT2. However, although this potential binding cluster was located at the analogous position of the CAII-binding cluster in MCT1 (489EEE), neither binding of MCT2 to CAII nor CAII-induced facilitation of MCT2 transport activity was observed. Only when the ambient amino acid residues were mutated to the corresponding residues in MCT1 (R483P, D484A, and I489P) was CAII able to bind and facilitate transport activity of MCT2. We speculate that the newly introduced CAII-binding site489EEE was either obscured by ambient, bulky amino acids or that binding was suppressed because of electrostatic problems, because in the mutant four instead of three acidic amino acid residues are present in a row (DEEE in MCT2-K485E/S487E, instead of AEEE in MCT1-WT). Only when these constraints are removed can the MCT C terminus bind to CAII-His64. This leads to the conclusion that the mere presence of an appropriate acidic amino acid cluster within the C terminus of a transport protein is not sufficient to mediate binding of CAII to this transporter, but that amino acids surrounding this acidic cluster must allow free access of the binding site to CAII and hence functional interaction between these proteins.

We further could show that direct binding between MCT4 and CAII-mediated by Glu431 and Glu433 in the C terminus of MCT4 and CAII-His64 is essential for functional interaction between the two proteins. This arrangement is similar to the
binding between MCT1-Glu489/Glu491 and CAII-His64, which allows CAII-mediated facilitation of MCT1 transport activity. Therefore, it appears likely that CAII has to be recruited to the transporter by direct binding to the C-terminal tail of MCT1/4 to be close enough to the transporter pore to establish a proton shuttle between transporter and surrounding protonable residues. A structural model of the binding of CAII to the C terminus of MCT1 and MCT4, respectively, is shown in Fig. 5. The eight most distal amino acids of the MCT1 C terminus (AEEFPEAEESPV) fit well into the surface groove of CAII (Fig. 5A). The side chains of Glu489 and Glu491 of the MCT1 C terminus, which have both been shown to be crucial for binding and functional interaction between MCT1 and CAII (49), point toward the CAII interface within hydrogen bond distance from CAII-His64 in the "out" configuration. In contrast, Glu490 of MCT1-CT points outward to the bulk solvent and would therefore not be involved in the interaction with CAII (Fig. 5A). In contrast to MCT1, the C terminus of MCT4 does not fully cover the surface groove of CAII, but only the three glutamic acid residues AEEE, which form the CAII binding site, are in close contact with the enzyme (Fig. 5B). The side chains of Glu431 and Glu433 of the MCT4 C terminus, which have both been shown to be crucial for binding and functional interaction between MCT4 and CAII, point toward the CAII interface within hydrogen bond distance from CAII-His64 in the "out" configuration. In contrast, Glu432 of MCT4-CT points outward to the bulk solvent and would therefore not be involved in the interaction with CAII (Fig. 5B).

Although MCT1 and MCT4 each carry a CAII-binding cluster of three glutamic acid residues, in which the two flanking residues are directly involved in binding, several differences between the CAII-binding sites in MCT1 and MCT4 could be observed. Although in MCT1 both flanking glutamic acid residues (Glu489 and Glu491) are required for binding and functional interaction with CAII (49), only one of the two flanking glutamic acid residues (Glu431 or Glu433) in the binding cluster of MCT4 is required for physical and functional interaction with CAII (this study). This difference may indicate a stronger binding of CAII to MCT4 than to MCT1. However, without further analysis of the binding kinetics, this assumption remains speculative.

REFERENCES

1. Bröer, S., Rahman, B., Pellegrin, G., Pellerin, L., Martin, J. L., Verleysdonk, S., Hamprecht, B., and Magistretti, P. J. (1997) Comparison of lactate transport in astroglial cells and monocarboxylate transporter 1 (MCT 1) expressing Xenopus laevis oocytes: expression of two different monocarboxylate transporters in astroglial cells and neurons. *J. Biol. Chem.* **272**, 30096–30102

2. Bröer, S., Schneider, H.-P., Bröer, A., Rahman, B., Hamprecht, B., and Deitmer, J. W. (1998) Characterization of the monocarboxylate transporter 1 expressed in Xenopus laevis oocytes by changes in cytosolic pH. *Biochem. J.* **333**, 167–174

3. Halestrap, A. P., and Price, N. T. (1999) The proton-linked monocarboxylate transporter (MCT) family: structure, function and regulation. *Biochem. J.* **343**, 281–299

4. Halestrap, A. P., and Meredith, D. (2004) The SLC16 gene family: from monocarboxylate transporters (MCTs) to aromatic amino acid transporters and beyond. *Pflugers Arch.* **447**, 619–628

5. Halestrap, A. P. (2013) The SLC16 gene family: structure, role and regulation in health and disease. *Mol. Aspects Med.* **34**, 337–349

6. Garcia, C. K., Brown, M. S., Pathak, R. K., and Goldstein, J. L. (1995) cDNA cloning of MCT2, a second monocarboxylate transporter expressed in different cells than MCT1. *J. Biol. Chem.* **270**, 1843–1849

7. Jackson, V. N., and Halestrap, A. P. (1996) The kinetics, substrate, and inhibitor specificity of the monocarboxylate (lactate) transporter of rat liver cells determined using the fluorescent intracellular pH indicator, 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein. *J. Biol. Chem.* **271**, 861–868

8. Jackson, V. N., Price, N. T., Carpenter, L., and Halestrap, A. P. (1997) Cloning of the monocarboxylate transporter isoform MCT2 from rat testis provides evidence that expression in tissues is species-specific and may involve post-transcriptional regulation. *Biochem. J.* **324**, 447–453

9. Bröer, S., Bröer, A., Schneider, H.-P., Stogen, C., Halestrap, A. P., and Deitmer, J. W. (1999) Characterization of the high-affinity monocarboxylate transporter MCT2 in Xenopus laevis oocytes. *Biochem. J.* **341**, 529–535

10. Pellerin, L., Pellegrin, G., Bittar, P. G., Charnay, Y., Bouras, C., Martin, J. L., Stella, N., and Magistretti, P. J. (1998) Evidence supporting the existence of an activity-dependent astrocyte-neuron lactate shuttle. *Dev. Neurosci.* **20**, 291–299

11. Pellerin, L., Pellegrin, G., Martin, J. L., and Magistretti, P. J. (1998) Expression of monocarboxylate transporter mRNAs in mouse brain: support for a distinct role of lactate as an energy substrate for the neonatal vs. adult brain. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 3990–3995
12. Berghersen, L., Waerhaug, O., Helm, I., Thomas, M., Laake, P., Davies, A. J., Wilson, M. C., Halestrap, A. P., and Ottersen, O. P. (2001) A novel post-synaptic density protein: the monocarboxylate transporter MCT3 is co-localized with $\delta$-glutamate receptors in postsynaptic densities of parallel fiber-Purkinje cell synapses. *Exp. Brain Res.* **136**, 523–534

13. Pierre, K., and Pellerin, L. (2005) Monocarboxylate transporters in the central nervous system: distribution, regulation and function. *J. Neurochem.* **94**, 1–14

14. Yoon, H., Fanelli, A., Grollman, E. F., and Philp, N. J. (1997) Identification of a unique monocarboxylate transporter (MCT3) in retinal pigment epithelium. *Biochem. Biophys. Res. Commun.* **234**, 90–94

15. Philp, N. J., Yoon, H., and Grollman, E. F. (1998) Monocarboxylate transporter MCT1 is located in the apical membrane and MCT3 in the basal membrane of rat RPE. *Am. J. Physiol.* **274**, R1824–R1828

16. Philp, N. J., Yoon, H., and Lombardi, L. (2001) Mouse MCT3 gene is expressed preferentially in retinal pigment and choroid plexus epithelia. *Am. J. Physiol. Cell Physiol.* **280**, C1319–C1326

17. Grollman, E. F., Philp, N. J., McPhie, P., Ward, R. D., and Sauer, B. (2000) Determination of transport kinetics of chick MCT3 monocarboxylate transporter from retinal pigment epithelium by expression in genetically modified yeast. *Biochemistry* **39**, 9351–9357

18. Dimmer, K. S., Friedrich, B., Lang, F., Deitmer, J. W., and Bröer, S. (2000) Low-affinity monocarboxylate transporter MCT4 is adapted to the export of lactate in highly glycolytic cells. *Biochem. J.* **350**, 219–227

19. Pinheiro, C., Reis, R. M., Ricardo, S., Longatto-Filho, A., Schmitt, F., and Baltazar, F. (2010) Expression of monocarboxylate transporters 1, 2, and 4 in human tumours and their association with CD147 and CD44. *J. Biomed. Biotechnol.* **2010**, 427694

20. Pinheiro, C., Longatto-Filho, A., Azevedo-Silva, J., Casal, M., Schmitt, F. C., and Baltazar, F. (2012) Role of monocarboxylate transporters in human cancers: state of the art. *J. Bioenerg. Biomembr.* **44**, 127–139

21. Wilson, M. C., Meredith, D., Fox, J. E., Manoharan, C., Davies, A. J., and Halestrap, A. P. (2005) Basigin (CD147) is the target for organomercurial inhibition of monocarboxylate transporter isoforms 1 and 4: the ancillary protein for the insensitive MCT2 is EMBIGGIN (gp70). *Biochemistry* **44**, 523–534

22. Philp, N. J., Yoon, H., and Grollman, E. F. (1998) Carbonic anhydrase II binds to and enhances activity of the Na+/HCO$_3^-$ exchanger. *J. Biol. Chem.* **272**, 27213–27221

23. Sterling, D., Reithmeier, R. A., and Casej, J. R. (2001) Intramolecular proton shuttle supports not only catalytic but also noncatalytic function of carbonic anhydrase II. *Biochemistry* **39**, 13344–13349

24. Li, X., Liu, Y., Alvarez, B. V., Casey, J. R., and Fliegel, L. (2006) A novel carbonic anhydrase II binding site regulates NHE1 activity. *Biochemistry* **45**, 2414–2424

25. Johnson, D. E., and Casey, J. R. (2009) Bicarbonate Transport Metabolons. In *Drug Design of Zinc-Enzyme Inhibitors: Functional, Structural, and Disease Applications* (Supuran, C. T., and Winum, J.-Y., eds), John Wiley & Sons, Inc., Hoboken, NJ

26. Deitmer, J. W., and Becker, H. M. (2013) Transport metabolons with carbonic anhydrases. *Front. Physiol.* **4**, 291

27. Becker, H. M., Klier, M., and Deitmer, J. W. (2014) Carbonic anhydrases and their interplay with acid/base-coupled membrane transporters. In *Sub-cellular Biochemistry* (Frost, S. C., and McKenna, R., eds) pp. 105–34, Springer, Dordrecht, The Netherlands

28. Becker, H. M., Hirt, D., Fecher-Trost, C., Sülttemeyer, D., and Deitmer, J. W. (2005) Transport activity of MCT1 expressed in Xenopus oocytes is increased by interaction with carbonic anhydrase. *J. Biol. Chem.* **280**, 39882–39889

29. Becker, H. M., and Deitmer, J. W. (2008) Nonenzymatic proton handling by carbonic anhydrase II during $H^+$-lactate cotransport via monocarboxylate transporter 1. *J. Biol. Chem.* **283**, 21655–21667

30. Becker, H. M., Klier, M., and Deitmer, J. W. (2010) Nonenzymatic augmentation of lactate transport via monocarboxylate transporter isoform 4 by carbonic anhydrase II. *J. Membr. Biol.* **234**, 125–135

31. Klier, M., Schüler, C., Halestrap, A. P., Sylw, W. S., Deitmer, J. W., and Becker, H. M. (2011) Transport activity of the high-affinity monocarboxylate transporter MCT2 is enhanced by extracellular carbonic anhydrase IV but not by intracellular carbonic anhydrase II. *J. Biol. Chem.* **286**, 27781–27791

32. Klier, M., Andres, F. T., Deitmer, J. W., and Becker, H. M. (2014) Intracellular and extracellular carbonic anhydrases cooperate non-enzymatically to enhance activity of monocarboxylate transporters. *J. Biol. Chem.* **289**, 2765–2775

33. Almquist, J., Lang, P., Prätz-Wolters, D., Deitmer, J. W., Jrstrand, M., and Becker, H. M. (2010) A kinetic model of the monocarboxylate transporter MCT1 and its interaction with carbonic anhydrase II. *J. Comput. Sci. Syst. Biol.* **03**, 107–116

34. Becker, H. M., Klier, M., Schüler, C., McKenna, R., and Deitmer, J. W. (2011) Intramolecular proton shuttle supports not only catalytic but also noncatalytic function of carbonic anhydrase II. *Proc. Natl. Acad. Sci. USA* **108**, 3071–3076

35. Stridh, M. H., Alt, M. D., Wittmann, S., Heidtmann, H., Aggarwal, M., Riederer, B., Seidler, U., Wennemuth, G., McKenna, R., Deitmer, J. W., and Becker, H. M. (2012) Lactate flux in astrocytes is enhanced by a non-catalytic action of carbonic anhydrase II. *J. Physiol.* **590**, 2333–2351

36. Becker, H. M., Bröer, S., and Deitmer, J. W. (2004) Facilitated lactate transport by MCT1 when coexpressed with the sodium bicarbonate cotransporter (NBC) in Xenopus oocytes. *Biophys. J.* **86**, 235–247

37. Deitmer, J. W. (1991) Electrogenic sodium-dependent bicarbonate secretion by glial cells of the leech central nervous system. *J. Gen. Physiol.* **98**, 637–655

38. Becker, H. M. (2014) Transport of lactate: Characterization of the transporters involved in transport at the plasma membrane by heterologous...
protein expression on Xenopus oocytes. Neuromethods 90, 25–43
53. Fisher, S. Z., Maupin, C. M., Budayova-Spano, M., Govindasamy, L., Tu, C., Agbandje-McKenna, M., Silverman, D. N., Voth, G. A., and McKenna, R. (2007) Atomic crystal and molecular dynamics simulation structures of human carbonic anhydrase II: insights into the proton transfer mechanism. Biochemistry 46, 2930–2937
54. Emsley, P., and Cowtan, K. (2004) Coot: model-building tools for molecular graphics. Acta Crystallogr. D Biol. Crystallogr. 60, 2126–2132
55. Brünger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang, J. S., Kuszewski, J., Nilges, M., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T., and Warren, G. L. (1998) Crystallography & NMR system: A new software suite for macromolecular structure determination. Acta Crystallogr. D. Biol Crystallogr. 54, 905–921
56. Martínez, C., Kalise, D., and Barros, L. F. (2010) General requirement for harvesting antennae at Ca^{2+} and H^{+} channels and transporters. Front. Neuroenergetics 2, 27