Nonenzymatic Proton Handling by Carbonic Anhydrase II during \(H^+\)-Lactate Cotransport via Monocarboxylate Transporter 1*

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Carbonic anhydrase (CA) is a ubiquitous enzyme catalyzing the equilibration of carbon dioxide, protons, and bicarbonate. For several acid/base-coupled membrane carriers it has been shown that the catalytic activity of CA supports transport activity, an interaction coined “transport metabolon.” We have reported that CA isoform II (CAII) enhances lactate transport activity of the monocarboxylate transporter isoform 1 (MCT1) expressed in Xenopus oocytes, which does not require CAII catalytic activity (Becker, H. M., Fecher-Trost, C., Hirnet, D., Sültemeyer, D., and Deitmer, J. W. (2005) J. Biol. Chem. 280, 39882–39889). Coexpression of MCT1 with either wild type CAII or the catalytically inactive mutant CAII-V143Y similarly enhanced MCT1 activity, although injection of CAI or coexpression of an N-terminal mutant of CAII had no effect on MCT1 transport activity, demonstrating a specific, nonenzymatic action of CAII on lactate transport via MCT1. If the \(H^+\) gradient was set to dominate the rate of lactate transport by applying low concentrations of lactate at a high \(H^+\) concentration, the effect of CAII was largest. We tested the hypothesis of whether CAII helps to shuttle \(H^+\) along the inner face of the cell membrane by measuring the pH change with fluorescent dye in different areas of interest during focal lactate application. Intracellular pH shifts decayed from the focus of lactate application to more distant sites much less when CAII had been injected. We present a hypothetical model in which the effective movement of \(H^+\) into the bulk cytosol is increased by CAII, thus slowing the dissipation of the \(H^+\) gradient across the cell membrane, which drives MCT1 activity.

Transport of acid/base equivalents across cell membranes plays a crucial role both for pH regulation and cellular import and export of metabolites. Many \(Na^+\)-dependent and \(Na^+\)-independent transport modes of metabolites (e.g. neurotransmitter substances) also transport \(H^+\), \(OH^-\), or \(HCO_3^-\) as co- or counter-substrate. The energetic compounds lactate and pyruvate are primarily transported by monocarboxylate transporters (MCT) in an electroneutral 1 proton-1 organic anion transport mode. Members of the MCT family, which belong to the human gene family SLC16 which comprise 14 isoforms, are expressed in most tissues, in particular those with a large energy consumption like muscle and brain (1, 2). In the brain MCT1 is located mainly in glial cells, where it facilitates the export of lactate, which is then taken up by neurons via the high affinity MCT2; thereby the two isoforms are believed to shuttle lactate from glial cells to neurons which seems to be pivotal for brain energy metabolism (3–6). In muscle, MCT1 is highly expressed in oxidative type I fibers, where it mediates import of lactate that is released by glycolytic muscle cells via MCT3 and MCT4 (7–9).

MCT1, when expressed in Xenopus oocytes, operates with a \(K_m\) value of 3.5 mM for lactate influx and of 6.4 mM for efflux of lactate. Both the rate and the amplitude of the lactate-induced acidification strongly depend on the extracellular \(H^+\) concentration (10, 11). Expressed in frog oocytes, MCT1 has been shown to cooperate with the sodium bicarbonate cotransporter NBCe1 (12, 13) and with carbonic anhydrase isoform II (CAII) (14). Remarkably, the support of MCT1 activity by CAII was independent of the catalytic activity of the enzyme, which catalyzes the equilibrium of \(CO_2\), \(H^+\), and \(HCO_3^-\) (14).

CAII binds to, and enhances the activity of various acid/base transporters like the chloride/bicarbonate exchanger AE1 (15, 16), the sodium bicarbonate cotransporter NBCe1 (17, 18), and the sodium/hydrogen exchanger NHE1 (19). In muscle cells, coexpression of CAII and acid/base-transporting proteins like NBC, NHE1, and also the MCTs have been found to be pivotal in acid/base homeostasis (20). In addition, it has been shown that extracellular carbonic anhydrase activity facilitates lactic acid transport in rat skeletal muscle fibers (21). In the brain CAII is highly expressed in astrocytes where it plays a supportive role in pH regulation (22) and supports the lactate shuttle from astrocytes to neurons (23).

In the present study we have focused on the mechanism by which MCT1 activity is enhanced by nonenzymatic proton handling of CAII. Our results suggest that CAII, presumed to accumulate exclusively at the inner face of the cell membrane, accelerates the disposal of protons away from the MCT1 domain at the inner face of the cell membrane into the bulk cytosol. This would help to prevent local proton microdomains

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2 The abbreviations used are: MCT, monocarboxylate transporter; AE1, anion exchanger isoform 1; AOI, area of interest; BCEO-AM, 2',7'bis(carboxyethyl)5(andro)carboxyfluorescein, acetoxymethyl ester; CA, carbonic anhydrase; NBCe1, electrogenic sodium bicarbonate cotransporter isoform 1; EZA, ethoxyzolamide; WT, wild type.
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developing at the pore of the transporter and, thus, maintain a proton gradient which allows a sustained lactate flux via MCT1.

EXPERIMENTAL PROCEDURES

Constructs, Oocytes, and Injection of cRNA and Carbonic Anhydrase—The human CAII cDNA (CAII-WT) as well as the N-terminal mutant CAII-HEX with mutations of the amino acids at position H3P, H4Q, L9A, H10K, H15K, and H17S were kindly provided by Dr. Reinhart Reithmeier, Toronto, CA (24, 25). The catalytically inactive mutant CAII-V143Y was a gift from Dr. Carol Fierke, Ann Arbor, MI (26, 27). The two constructs were subcloned into the oocyte expression vector pGEM-He-Juel, which contains the 5′ and the 3′ untranscribed regions of the Xenopus β-globulin flanking the multiple cloning site. Rat MCT1 cDNA (MCT1) cloned in oocyte expression vector pGEM-He-Juel was kindly provided by Dr. Stefan Bröer, Canberra, Australia (28). Plasmid DNA was transcribed in vitro with T7 RNA polymerase (mMessage mMACHINE, Ambion Inc.) as described earlier (13). Xenopus oocytes of the stages V and VI were injected with 5 ng of MCT1-cRNA dissolved in diethyl pyrocarbonate-H₂O. Control oocytes were injected with an equivalent volume of diethyl pyrocarbonate-H₂O. Measurements were carried out 3–6 days after injection of cRNA. CAII was either injected as protein or coexpressed with the MCT1. For injection of protein, 2, 10, 50, or 200 ng of CAII, isolated from bovine erythrocytes (C3934, Sigma-Aldrich, Taufkirchen, Germany), and CAI, isolated from human erythrocytes (C4396, Sigma-Aldrich), respectively, dissolved in 25 nl of diethyl pyrocarbonate-H₂O, were injected 20–24 h before electrophysiological measurement. Control oocytes were injected with 25 nl of diethyl pyrocarbonate-H₂O. For coexpression of CAII, 12 ng of CAII-cRNA were injected either alone or together with the MCT1-cRNA. The oocyte saline had the composition 82.5 mM NaCl, 2.5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 1 mM Na₂HPO₄, 5 mM HEPES, titrated with NaOH to the desired pH. In lactate-containing saline NaCl was replaced by an equivalent amount of Na-t-lactate. In the bicarbonate-containing saline, NaCl was replaced by an equivalent amount of NaHCO₃, and the solution was aerated with 5% CO₂. Application of lactate was carried out in HEPES-buffered solution at pH 7.0 in the nominal absence of CO₂/HCO₃⁻ containing around 0.008 mM of CO₂ from air and, hence, a HCO₃⁻ concentration of around 0.06 mM.

Intracellular pH Measurements—For measurement of intracellular pH and membrane potential, double-barreled microelectrodes were used; the manufacture and application have been described in detail previously (13, 29). Briefly, for double-barreled microelectrodes, two borosilicate glass capillaries of 1.0 and 1.5 mm in diameter were twisted together and pulled to a micropipette. The ion-selective barrel was silanized with 5% tri-N-butylchlorosilane in 99.9% pure carbon tetrachloride, backfilled into the tip. H⁺-sensitive mixture (Fluka 95291, Fluka, Buchs, Switzerland) was backfilled into the tip of the silanized ion-selective barrel and filled up with 0.1 mM sodium citrate, pH 6.0. The reference barrel was filled with 3 mM KCl. To increase the opening of the electrode-tip, it was beveled with a jet stream of aluminum powder suspended in H₂O. Calibration of the electrodes was carried out in oocyte saline by changing the pH by 0.6 units.

As described previously (11), 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 experiments were carried out at room temperature.

Calculation of [H⁺]ᵢ—The measurements of intracellular pH were stored digitally using homemade PC software based on the program LabView (National Instruments Germany GmbH, München, Germany) and were routinely converted into intracellular H⁺ concentration, [H⁺]ᵢ. This should provide changes in the [H⁺]ᵢ, which take into account the different pH base line, as e.g. measured in HEPES- and CO₂/HCO₃⁻-buffered saline (12). The rate of change of the measured [H⁺]ᵢ, was analyzed by determining the slope of a linear regression fit using the spread sheet program OriginPro 7 (OriginLab Corp., Northampton, MA).

Voltage Clamp Recording—A borosilicate glass capillary 1.5 mm in diameter was pulled to a micropipette and backfilled with 3 mM KCl. The resistance of the electrodes measured in oocyte saline was around 1 megaohms. For voltage clamp, the electrode was connected to the head-stage of an Axoclamp 2B amplifier (Axon Instruments). Oocytes were clamped to a holding potential of −40 mV.

Fluorescent Staining of MCT1 and CAII in Oocytes—Frog oocytes, either injected with cRNA for MCT1 and CAII, as well as native control oocytes were fixed in 4% paraformaldehyde in phosphate-buffered saline. Oocytes were treated with 100% methanol and permeabilized with 0.1% Triton X-100. Unspecific binding sites were blocked with 3% bovine serum albumin and 1% normal goat serum. The cells were incubated in phosphate-buffered saline containing the primary antibodies (chicken anti-MCT1 polyclonal antibody (1:300) and rabbit anti-carbonic anhydrase II polyclonal antibody (1:1000), Chemicon International, Inc.) overnight at 4 °C. Oocytes were then incubated with the secondary antibodies (Alexa Fluor 488 goat chicken IgG and Alexa Fluor 546 goat anti-rabbit IgG, Molecular Probes, Inc.). The stained oocytes were analyzed with a laser-scanning microscope (LSM 510, Carl Zeiss GmbH, Oberkochen, Germany) using whole oocytes through which cross-sectional optical planes were laid or the cell surface was viewed. Additionally, oocytes were embedded in 2% agarose and sectioned into 300-μm thick slices with a microtome (752M Vibroslice, Campden Instruments Ltd.).

Imaging of Cytosolic H⁺ Changes in Oocytes—MCT1-expressing oocytes either injected with 200 ng of CAII or H₂O were stained with 7 μM 2',7'bis(2carboxyethyl)5(and6) carbboxyfluorescein, acetoxyxymethyl ester (BCECF-AM) ester (B-1170, Molecular Probes, Leiden, The Netherlands) for 45 min at 18 °C in oocyte saline, pH 7.8. Oocytes were placed into a cavity 1 mm in diameter in a bath with the vegetative (white) hemisphere faced up. Cells were continuously perfused with oocyte saline, pH 7.0. A class micropipette with a tip diameter of 20 μm was filled with 100 mM lactate in oocyte saline, pH 6.0, and connected to a pressure applicator (PDES-02D pneumatic drug ejector, npi electronic GmbH). The tip was placed close to the oocyte membrane, and lactate was focally applied with a pressure of 0.2 bar for 20 s against the direction of perfusion to wash lactate away from the cell to exclude global lactate appli-
cation. Changes in the fluorescence intensity were measured using an imaging system (TILL Photonics, Munich-Martin- sried, Germany). BCECF-loaded oocytes were excited by monochromatic wavelengths of 440 and 490 nm. The fluorescence emissions were continuously recorded at 2 Hz. After calculation of the ratio, areas of interest (AOI) with a size of 40 μm × 40 μm were chosen with respect to the position of the application pipette. For each AOI the rate of rise in fluorescent intensity during application of lactate was calculated and plotted against the relative position of the AOI from the application pipette. The change in fluorescence ratio refers to changes in intracellular pH according to a modified Henderson-Hasselbalch equation, yielding a nonlinear correlation between changes in fluorescent intensity and intracellular pH over a wide pH range (30, 31). However, because the expected changes in pH, as calculated from measurements with ion-selective electrodes, range between pH 7.2 and 6.8, where a nearly linear relationship between the fluorescent signal and intracellular pH is found, changes in the fluorescent signal can directly account for changes in intracellular H⁺ concentration. To determine the rate of rise in fluorescent intensity, the slope of a linear regression fit was calculated for the time course during lactate application for every AOI.

**Determination of CAII Activity**—Activity of CAII was determined by monitoring the 18O depletion of doubly labeled 13C18O2 through several hydration and dehydration steps of CO2 and HCO3⁻ at 25 °C (32, 33). The reaction sequence of 18O loss from 13C18O18O (m/z = 49) over the intermediate product 13C18O16O (m/z = 47) and the end product 13C18O16O (m/z = 45) was monitored with a quadrupole mass spectrometer (MSD 5970; Hewlett Packard, Waldbronn, Germany). The relative 18O enrichment was calculated from the measured 45, 47, and 49 abundance as a function of time according to the following equation: log enrichment = log(49 × 100/(49 + 47 + 45)). For the calculation of the CAII activity of the sample, the rate of 18O degradation was obtained from the linear slope of the log enrichment over the time using the spreadsheet analyzing software OriginPro 7. The rate was compared with the corresponding rate of the noncatalyzed reaction. Enzyme activity in units was calculated from these two values as defined by Badger and Price (34). From this definition, 1 unit corresponds to 100% stimulation of the noncatalyzed 18O depletion of doubly labeled 13C18O2. For the experiments, the cuvette was filled with 8 ml of oocyte saline with a pH of 7.25 according to the mean intracellular pH of CAII-injected oocytes at 25 °C. To determine the catalytic activity, oocytes either expressing or injected with catalytic antibodies against human CAII and secondary antibodies against chicken and rabbit linked with a fluorescent dye. A and B show the signal for CAII and D show staining for MCT1, and E–H shows an overlay of the signals for MCT1 and CAII. G and H show native control oocytes stained for MCT1 and CAII as described for A–F. J shows staining for CAII in an oocyte expressing the catalytically inactive mutant CAII-V143Y. J shows staining for CAII in an oocyte expressing the N-terminal mutant CAII-HEX. K shows a cross-section through a bovine CAII-injected oocyte sectioned into 300-μm slices and stained for CAII. The right and left rows (A, C, E, G, I, and J) show optical cross-sections through the oocytes, and the middle row (B, D, F, H) shows a surface view on the oocytes membrane.

**RESULTS**

**CAII-mediated Support of MCT1 Activity**—Expression of the proteins used in this study was checked via fluorescence staining using antibodies against MCT1 and CAII. Optical cross-sections (Fig. 1, A, C, E, G, I, and J) and surface views (Fig. 1, B, D, F, and H) of oocytes were taken with confocal microscopy. Staining for MCT1 (Fig. 1, C and D), the wild type form of CAII (CAII-WT; Fig. 1, A and B), the catalytically inactive mutant CAII-V143Y (Fig. 1I), and the N-terminal mutant CAII-HEX (Fig. 1J) were observed at the plasma membrane, whereas no staining was evident in the cytoplasm. Specificity of the antibodies for MCT1 and CAII was checked by double labeling of a native control oocyte, which showed no visible staining (Fig. 1, G and H). To confirm that cytosolic CAII was only localized at the plasma membrane but not in the cytosol and that the fluorescence...
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![Image](https://example.com/image.png)

**FIGURE 2. Coexpression of MCT1 with CAII-WT and CAII-V143Y.** A, original recordings of the intracellular H⁺ concentration in oocytes expressing MCT1, MCT1 + CAII-WT, and MCT1 + CAII-V143Y during application of 10 mM lactate in HEPES-buffered solution in the nominal absence of CO₂/HCO₃⁻ at pH 7.0 in the absence (black traces) and presence of 10 μM EZA (gray traces). B, rate of change in the intracellular H⁺ concentration induced by application of 3 and 10 mM lactate in the absence and presence of 10 μM EZA. The asterisks on the bars for MCT1 + CAII-coexpressing oocytes refer to the corresponding values for MCT1-expressing cells. C, original recordings of the intracellular H⁺ concentration in oocytes expressing MCT1, MCT1 + CAII-WT, and MCT1 + CAII-V143Y during application of 5% CO₂, 10 mM HCO₃⁻ in the absence and presence of 10 μM EZA. D, rate of change in the intracellular H⁺ concentration during application of 5% CO₂, 10 mM HCO₃⁻, indicating CAII catalytic activity before application and in the presence of EZA. The asterisks on the bars for MCT1 + CAII-coexpressing oocytes for the control (−EZA) refer to the corresponding values of MCT1-expressing cells, and the asterisks on the bars for the values in EZA (+10 μM EZA) refer to the corresponding bars for the values of the control without EZA. ***, p ≤ 0.001; n.s., not significant.

was not quenched when optical slices were taken from intact oocytes, a CAII-injected oocyte was sectioned into 300-μm thick slices. The confocal image of the upper part of the slice shows that CAII is only found at the surface of the oocyte plasma membrane but not in the cytosol (Fig. 1A).

Injection of CAII into MCT1-expressing oocytes resulted in an increase in MCT1 transport activity during application of lactate (14). Blocking the catalytic center of the enzyme by application of ethoxzolamide (EZA, 10 μm) had no effect on the interaction between MCT1 and CAII. To exclude an involvement of the catalytic activity in the enhancement of MCT1 activity, we coexpressed CAII either with the wild type form of CAII (CAII-WT) or with the catalytically inactive mutant CAII-V143Y. Transport activity of the MCT1 was determined with pH-sensitive microelectrodes as the rate of rise of intracellular H⁺ concentration during application of 3 and 10 mM lactate in oocytes either expressing MCT1 alone and in oocytes coexpressing MCT1 with either CAII-WT or CAII-V143Y in voltage clamp at a holding potential of −40 mV (Fig. 2A). As previously shown, injection of CAII into MCT1-expressing oocytes leads to no changes in the oocyte buffer capacity (14). With no changes in the intracellular buffer capacity, the rate of changes in intracellular H⁺ concentration is directly linked to changes in acid/base flux (13). Because changes in H⁺ concentration, in contrast to changes in pH, also account for changes in the pH base line, we used the changes in intracellular pH instead of changes in acid/base flux for calculation of changes in the MCT1 transport activity.

Coexpression of MCT1 with CAII-WT and the catalytically inactive CAII-V143Y increased transport activity of MCT1 equally by a factor of about 2.5 at both lactate concentrations (p ≤ 0.001, Fig. 2B). To completely rule out any influence of CAII catalytic activity on the enhancement of MCT1 activity, we also applied lactate in the presence of 10 μM EZA. Application of EZA had no effect either for MCT1 or for MCT1 + CAII-WT- and MCT1 + CAII-V143Y-coexpressing oocytes on the rate of rise in intracellular H⁺ concentration during application of 3 and 10 mM lactate (Fig. 2B). The catalytic activity of CAII was checked by measuring the rate of rise of the intracellular acidification induced by application of 5% CO₂, 10 mM HCO₃⁻ (Fig. 2C). Coexpression of CAII-WT resulted in a 7-fold increase in the rate of rise as compared with oocytes coexpressing MCT1 and CAII-V143Y or expressing MCT1 alone (p ≤ 0.001, Fig. 2D). The increase in the rate of rise of acidification in oocytes coexpressing MCT1 and CAII-WT could be suppressed after inhibition of CAII activity by 10 μM EZA (p ≤ 0.001, Fig. 2D). In the presence of EZA, similar rates of acidification were obtained by CO₂/HCO₃⁻ application in all three types of oocytes. These results clearly indicate that CAII can increase transport activity of the MCT1 even without its catalytic activity.

To determine the dependence of the CAII-induced augmentation in MCT1 activity on the concentration of intracellular CAII, we monitored transport activity after the injection of different amounts of the enzyme into MCT1-expressing oocytes (Fig. 3A). Activity of MCT1 was measured by determining the rate of change in intracellular H⁺ concentration during application (black triangles, acidification) and removal (white triangles, alkalization) of 3 and 10 mM lactate in oocytes injected with 2, 10, 50, or 200 ng/oocyte of CAII or with no CAII injected (Fig. 3C). Amounts of CA were chosen from the lowest concentration where CA activity could be determined by pH-sensitive electrodes up to the maximal concentration that could be injected without causing severe damage to the oocytes. Both influx and efflux of protons during application and removal of lactate were accelerated with increasing intracellular concentration of CAII. Up to a concentration of 10 ng CAII/oocyte, only a small increase in MCT1 activity could be observed, whereas at higher CAII concentrations a steep increase in transport activity was measured. Even at a concentration of 200 ng of CAII/oocyte, the effect of CAII on MCT1 activity showed...
no signs of saturation. The results also indicate that CAII facilitates both influx and efflux of $\text{H}^+$ (and lactate) via the MCT1; influx and efflux occurred with similar rates and show the same dependence on the intracellular CAII concentration.

The rate of rise in intracellular $\text{H}^+$ concentration as induced by application of 5% $\text{CO}_2$, 10 mM HCO$_3^-$ showed a typical saturation curve (Fig. 3E). CAII increased the rate of the $\text{CO}_2$-induced acidification but also accelerated the alkalinization during removal of $\text{CO}_2$. For the rate of the $\text{CO}_2$-induced acidification, an EC$_{50}$ of 6.7 ± 1.0 ng CAII/oocyte and a $V_{\text{max}}$ of 519 ± 20 nM H$^+$/min was obtained. For the rate of alkalinization during removal of $\text{CO}_2$, an EC$_{50}$ of 4.8 ± 3.5 ng of CAII/oocyte and a $V_{\text{max}}$ of 354 ± 30 nM H$^+$/min were determined. Interestingly, the efflux of $\text{CO}_2$, as indicated by the rate of change in H$^+$ was larger during addition, as compared with during removal of CO$_2$/HCO$_3^-$. This might be attributable to different kinetics of CAII activity and CO$_2$ efflux, caused by a limited supply of CO$_2$ in the oocyte cytosol as compared with the large reservoir of CO$_2$ in the bulk solution.

To check for the specificity of CA isoform II for the interaction with MCT1, we also injected 2, 10, 50, or 200 ng of CA isoform I isolated from human erythrocytes into MCT1-expressing oocytes. Transport activity of MCT1 was again determined by monitoring the change in intracellular $\text{H}^+$ concentration during application of 3 and 10 mM lactate (Fig. 3B). Injection of CAI had no effect on the rate of rise in intracellular $\text{H}^+$ concentration during application of lactate or at the alkalinization during removal of the substrate (Fig. 3D). Catalytic activity of CAI, as determined by the rate of rise in intracellular $\text{H}^+$ concentration induced by application of 5% CO$_2$, 10 mM HCO$_3^-$, was lower than the activity of CAII but still induced a significant increase in the rate of CO$_2$-induced acidification and also showed a typical saturation curve (Fig. 3F). For the rate of the CO$_2$-induced acidification, an EC$_{50}$ of 11.0 ± 1.6 ng CAI/oocyte and a $V_{\text{max}}$ of 349 ± 14 nM H$^+$/min was obtained. For the rate of alkalinization during removal of CO$_2$/HCO$_3^-$, an EC$_{50}$ of 9.6 ± 1.9 ng CAI/oocyte and a $V_{\text{max}}$ of 217 ± 27 nM H$^+$/min was determined. These findings illustrate that enhancement of MCT1 activity is not per se due to an increased amount of any protein near the plasma membrane. Furthermore, the results suggest that enhancement of MCT1 activity requires a feature of CAII that appears to be absent in CAI.

CAI is a homologous isoform to CAII possessing 60% sequence identity. Nevertheless, it has been shown that CAI, in contrast to CAII, does not bind to AE1 (24). As a potential binding motif for CAII, a group of histidine residues within the N-terminal region of CAII, not found in CAI, was hypothesized. When six of these target CAII residues were mutated to the corresponding sequence in CAI, binding of CAII to AE1 was lost (24). To check if binding of CAII to MCT1 requires the same binding motif as for AE1, we expressed the same mutant.
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FIGURE 4. Coexpression of MCT1 with CAII-WT and CAII-HEX. A, original recordings of the intracellular H⁺ concentration in oocytes expressing MCT1, MCT1 + CAII-WT, and MCT1 + CAII-HEX during application of 10 mM lactate in HEPES-buffered solution in the nominal absence of CO₂/HCO₃⁻ at pH₇. B, rate of change in the intracellular H⁺ concentration induced by application of 3 and 10 mM lactate. The asterisks on the bars for MCT1- and MCT + CAII-HEX-coexpressing oocytes refer to the values for MCT1 + CAII-WT-expressing cells. n.s., not significant. C, rate of change in the intracellular H⁺ concentration during application of 5% CO₂, 10 mM HCO₃⁻. The asterisks on the bars for MCT1 + CAII-V143Y- and MCT + CAII-HEX-coexpressing oocytes refer to the values for MCT1-expressing cells. Meaning of the colors of the bars is the same as in B, **, p ≤ 0.01; ***, p ≤ 0.001.

FIGURE 5. Enzymatic activity of CAII injected or expressed in oocytes as determined by mass spectrometry. A, recording of the log enrichment of either 20 native oocytes (gray traces) or MCT1-expressing oocytes (black traces) injected with bovine CAII or coexpressing human CAII-WT or the N-terminal mutant CAII-HEX. The beginning of the traces shows the rate of degradation of the ¹⁸O-labeled substrate in the noncatalyzed reaction; the black arrow indicates the addition of oocytes with expressed CAII or with injected CAII protein. B, enzymatic activity of CAII in units/ml at extracellular pH of 7.25. One unit is defined as 100% stimulation of the noncatalyzed ¹³C¹⁸O₂. C, calibration curve for the determination of the active CAII concentration in the oocytes by measuring the activity of a defined amount of CAII (0.25, 0.5, 1, and 2 μg) and fitted by linear regression to calculate the amount of expressed or injected CAII in ng/oocyte, plotted in D.

(CAII-HEX), already used by Vince et al. (25), together with the MCT1. Transport rate of MCT1 was determined by measuring the rate of rise in intracellular H⁺ concentration during application of 3 and 10 mM lactate in HEPES-buffered solution, pH₇ 7.0. As a control, oocytes expressing MCT1 alone or coexpressing MCT1 with CAII-WT were used (Fig. 4A). Coexpression of MCT1 with CAII-HEX resulted in no change in the rate of rise during application of lactate, whereas coexpression with CAII-WT still induced a significant increase in MCT1 activity (Fig. 4B).

Expression of CAII-HEX and CA-WT led to the same increase in ΔH⁺/t during application of 5% CO₂, 10 mM HCO₃⁻, indicating the similar catalytic activity for both mutant and wild type CAII (Fig. 4C). Therefore, it could be speculated that the functional interaction between MCT1 and CAII is mediated by the same motif as found for the interaction of CAII with AE1.

To compare the amount of expressed CAII-WT and CAII-HEX with the amount of injected CAII, we determined the catalytic activity of oocytes coexpressing MCT1 and CAII-WT or CAII-HEX and native or MCT1-expressing oocytes injected with 50 ng of bovine CAII by mass spectrometry. Original recordings of the log enrichment of either 20 native oocytes injected with 50 ng of bovine CAII each or H₂O and of 20 MCT1-expressing oocytes injected with 50 ng of bovine CAII each or coexpressing CAII-WT or CAII-HEX are shown in Fig. 5A. The first part of the curve gives the noncatalyzed degradation of labeled CO₂. The black arrow indicates the addition of oocytes. The statistical analysis of the enzyme activity calculated from the difference of the noncatalyzed and the CAII-catalyzed change in the log enrichment shows CAII activity in all oocytes injected with or coexpressing CAII (Fig. 5B). Twenty native oocytes injected with 50 ng of bovine CAII each showed a catalytic activity of 27.4 ± 0.4 units/ml, whereas 20 MCT1-expressing oocytes injected with 50 ng of bovine CAII each had an activity of 27.9 ± 0.2 units/ml. Oocytes either coexpressing MCT1 + CAII-WT or MCT1 + CAII-HEX showed a catalytic activity of 30.3 ± 0.6 and
The Role of the Substrate Gradients for the CAII-mediated Support of MCT1 Activity—The dependence of the CAII-induced increase in MCT1 activity on substrate concentration and a potential effect of CAII on substrate affinity of MCT1 was studied at 5 different concentrations of lactate (0.1, 0.3, 1, 3, and 10 mM) at extracellular pH values of 6.0, 7.0, and 7.5. In MCT1-expressing oocytes injected either with CAII or H2O 1 day before the experiment, transport activity was determined by measuring the rate of rise of the lactate-induced increase in the H+ concentration at pHo 7.0 (Fig. 6A). Plotting of ΔH/t shows a dependence of MCT1 activity on the lactate concentration (Fig. 6, B and C). For MCT1-expressing oocytes a Vmax value of 58.1 nM H+/min and an EC50 of 4.0 mM lactate could be determined. Injection of 50 and 200 ng of CAII into MCT1-expressing oocytes resulted in a Vmax of 103.9 nM H+/min and 160.2 nM H+/min and an EC50 of 3.3 and 2.8 mM lactate, respectively. For all lactate concentrations used, both injection of 50 and 200 ng of CAII resulted in an increase in the rate of rise; however, this difference between oocytes with and without CAII decreased with increasing lactate concentration, as evident from the relative change in ΔH/t (Fig. 6D). Using an Eadie-Hofstee plot, a Km value of 3.3 mM lactate could be determined. This Km value corresponds to those reported previously for lactate in MCT1-expressing oocytes (3–5 mM (11)). Injection of CAII caused a loss of the linear dependence between substrate and substrate affinity. Instead, an increase in substrate affinity could be observed at decreasing substrate concentrations. For high lactate concentrations (3–30 mM) Km values close to the Km for MCT1-expressing oocytes could be observed (3.7 and 3.3 mM for 50 ng and 200 ng of CAII, respectively), whereas application of lower lactate concentrations resulted in a substantial increase in substrate affinity (Fig. 6E).

To check if the decrease in CAII-induced augmentation of MCT1 activity is due to changes in lactate concentration at the inner membrane, affecting H+ dissipation, or an interaction of lactate with CAII, affecting its ability to transport H+, we preincubated MCT1-expressing oocytes injected with either 50 ng active CAII in native and MCT1-expressing oocytes injected with bovine CAII resulted in an amount of 49.7 ± 0.2 and 50.8 ± 0.3 ng of CAII/oocyte, respectively, indicating that virtually all CAII injected into the oocytes displayed catalytic activity (see also Becker and Deitmer (17)). Oocytes coexpressing either MCT1 + CAII-WT or MCT1 + CAII-HEX expressed 54.3 ± 1.1 and 59.6 ± 0.4 ng of CAII/oocyte, respectively, a value that corresponds well with the amount of CAII injected into each oocyte (50 ng). In native control oocytes, an amount of 3.0 ± 0.8 ng of CAII/oocyte was determined.
**H+ Handling by Carbonic Anhydrase**

![Graphs and images](49x405)

**FIGURE 7.** Dependence of the rate of rise in H+ concentration on the proton gradient. A, original recordings of the intracellular H+ concentration in MCT1-expressing oocytes injected with 50 ng of CAII (upper trace) or H2O (lower trace), respectively, during application of 0.3 and 10 mM lactate in HEPES-buffered solution in the nominal absence of CO2/HCO3 at pH 7.5 and 6.0. For checking the CAII activity, 5% CO2, 32 mM HCO3, pH 7.5, was also applied. B, rate of rise of H+ concentration in MCT1-expressing oocytes injected with 50 ng of CAII or H2O, respectively, during application of 5% CO2, 32 mM HCO3, C and D, rate of rise in intracellular H+ concentration as induced by application of 3 and 10 mM lactate in MCT1-expressing oocytes injected with CAII or H2O in HEPES-buffered solution at an external pH of 7.5 and 6.0. The asterisks at the bars for 6.0 refer to the values of the bars for 7.5. **p < 0.01; ***p < 0.001; n.s., not significant.

CAII or water at the beginning of the experiment was 7.38 ± 0.03 (n = 8) and 7.33 ± 0.02 (n = 8), respectively, resulting in a small inwardly directed electrochemical proton gradient at pH 7.5 (at an holding potential of ~40 mV) and a steep inwardly directed proton gradient at pH 6.0. Thus, the H+ equilibrium potential changed from ~7 to ~10 mV at pH 7.5 to about ~80 mV at pH 6.0. As expected, transport activity of MCT1 not only depends on the lactate concentration but also greatly on the H+ gradient. Both with and without CAII injected, application of 0.3 and 10 mM lactate at an extracellular pH of 6.0 resulted in a much larger rate of intracellular H+ rise than at an extracellular pH of 7.5 (p ≤ 0.001; Fig. 7, C and D). With the larger H+ gradient at pH 6.0, injection of CAII increased the transport activity of MCT1 at both lactate concentrations by a factor of two (p ≤ 0.01; Fig. 7, C and D). At pH 7.5, however, injection of CAII made no difference for the activity of MCT1 during lactate application (Fig. 7, C and D). Thus, injection of CAII augments the increase of MCT1 activity at a high proton gradient but has no or little effect on the increase in MCT1 activity when there is no gradient for protons.

Proton Handling of Carbonic Anhydrase II—Our results suggest that CAII increases transport activity of the MCT1 via a mechanism that differs from the kinetics of the catalytic function of the enzyme. To study the fate of protons imported into the oocyte via the MCT1, we used the pH-sensitive dye CECF-AM to monitor allocation of H+ along the inner face of the oocyte membrane. We focally applied lactate to MCT1-expressing oocytes, which were injected either with 200 ng of CAII or H2O, and measured the amplitude and the rate of rise in intracellular H+ concentration, indicated by the absolute change and the rate of change in the fluorescence ratio (see “Experimental Procedures”) at defined distances away from the region of application as shown in Fig. 8F. Each AOI used to determine the H+ concentration had a size of 40 × 40 μm. Application of lactate was performed by pressure ejection of lactate from a pipette positioned close to the cell surface (<5 μm) against continuous bath perfusion counterflow to minimize lactate being superfused over the entire oocyte. The size of the region to which lactate was applied during the experiment was checked by application of a dye (Fast Green) added to the pipette solution. Ejection of lactate for 20 s from the tip of a pipette filled with 100 mM lactate at pH 6.0 resulted in an increase in the intracellular H+ concentration that propagated from the region of where lactate was applied to more distant areas. To evaluate the kinetics of proton movement, we determined the rate of rise in fluorescence intensity in different AOI by fluorescence microscopy, here shown for three AOI in two MCT1-expressing oocytes, one injected with 200 ng of CAII (Fig. 8A) and the other with H2O (Fig. 8B). The steepest increase in H+ concentration was observed in the area of application (AOI-0, plotted at 0 μm distance to the pipette in Fig. 8C), with a higher rate of rise observed in MCT1-expressing oocytes injected with CAII as compared with non-CAII-injected cells. With increasing distance from this area (AOI-0), the rate of acidification decreased both in MCT1-expressing oocytes either injected with CAII or injected with H2O, indicated by a spatial decay of the signal with increased distance from the area of application.
from the pipette (Fig. 8C). Nevertheless, the decay of the signal observed with increasing distance from the area of application occurred to a lesser extent in MCT1-expressing oocytes injected with CAII as compared with MCT1-expressing oocytes that were not injected with CAII (Fig. 8C). The rate of rise normalized to that recorded in the area of application (AOI-0) allows a direct comparison of the H⁺ rise normalized to that recorded in the area of application. Whereas in cells injected with H₂O instead of CAII the rate decreased to 50% at a distance of 108 μm away from the pipette, when CAII is present in the cell, leading to a reduction in the decay of the fluorescent signal at more distant regions from the pipette, when CAII is present in the cell. To check whether the decrease in the change in fluorescence, measured in AOI with a larger distance from the point of application, is attributable to a decrease in the rate of rise in H⁺ concentration and not to some artifact associated with positioning of the AOI itself, we also bath-applied 10 mM lactate, pH 7.0, and measured the rate of rise in fluorescent ratio in the same AOIs as used for the measurements during focal application of lactate (Fig. 8E). Bath application of lactate resulted in a similar rate of change in fluorescence in all AOI. This indicates that the decrease in the rate of H⁺ rise during focal application of lactate is not per se due to the positioning of the areas of interest at the cell membrane. Focal application of lactate at pH 6.0 to native oocytes resulted in a small, local acidification, presumably due to the large H⁺ gradient across the cell membrane during the time of application (open diamonds, Fig. 8C).

To evaluate the build up of protons in different areas within the oocyte, we determined the amplitude of the change in fluorescence ratio during focal application of lactate at the same areas of interest as used for the calculation of the rate of change. Because the fluorescent ratio does not reach saturation during the 20-s lactate pulse (Fig. 8A and B), the time points shortly before and at the end of the lactate pulse were chosen to determine the amplitude of the change in fluorescence ratio during focal application of lactate. The maximum change in fluorescence was observed in AOI with a larger distance to the pipette, when CAII is present in the cell, leading to a reduction in the decay of the fluorescent signal at more distant regions from the pipette, when CAII is present in the cell.
**H⁺ Handling by Carbonic Anhydrase**

![Graph](image)

**Figure 9. Build up of protons during focal lactate application.** A, amplitude of the fluorescence ratio in different areas in MCT1-expressing oocytes with (filled circle) and without (open circle) 200 ng of CAII injected, respectively, and native control oocytes during focal application of 100 mM lactate, pH 6.0, as described in Fig. 8. B, amplitude of fluorescence ratio during focal application of 100 mM lactate, pH 6.0, in MCT1-expressing oocytes with and without 200 ng of CAII, respectively, normalized to the rate of rise in the area of application (AOI-0). C, amplitude of the fluorescence ratio during bath application of 10 mM lactate, pH 7.0, in MCT1-expressing oocytes with and without 200 ng of CAII. For these measurements the same AOIs were used as in the experiments shown in A, D, schematic drawing of the proposed mechanism underlying the increase in MCT1 activity induced by CAII. The upper panel illustrates the case without CAII with H⁺ accumulating at the pore of the MCT1 (as indicated by the yellow shadow), thereby decreasing transport activity. In the lower panel CAII allocates H⁺ along the inner face of the membrane and thereby increases the area where protons can move into the bulk water phase; shown are the distances between CA molecules (green circles) for injection of 50 ng of CAII. By this mechanism CAII removes H⁺ from the pore of the MCT1, thereby enhancing transport activity.

During application of lactate. With increasing distance from AOI-0, the amplitude decreased both in MCT1-expressing oocytes injected with CAII and in cells injected with H₂O. However, the spatial decay of the signal observed with increasing distance from the area of application occurred to a lesser extent in MCT1-expressing oocytes injected with CAII as compared with MCT1-expressing oocytes, which were not injected with CAII. The amplitude in fluorescence ratio normalized to that recorded in the area of application (AOI-0) displays a logarithmic decrease of the signal in MCT1-expressing oocytes injected with H₂O, reaching a value of 50% at a distance of 92 µm (Fig. 9B). In MCT1-expressing oocytes injected with CAII, the decay of the fluorescent signal showed nearly linear kinetics, with a reduction to 68% in the most distant area 400 µm away from the application pipette. These results support the assumption of an augmentation in proton movement, mediated by CAII, that leads to an increased build-up of H⁺ at more distant regions while keeping the H⁺ concentration stable in the area of application. Bath application of lactate resulted in a similar change in fluorescence in all AOIs (Fig. 9C), indicating that the decrease in the amplitude during focal application of lactate is not per se due to the positioning of the areas of interest at the cell membrane. A hypothetical model (Fig. 9D) has been developed for enhanced transport activity to the same extent as wild type CAII. In addition, application of EZA had no effect on the augmentation of MCT1 transport activity induced by coexpression with CAII-V143Y and CAII-WT, respectively. In another study coexpression of CAII-V143Y with the sodium bicarbonate cotransporter NBCe1 failed to increase NBCe1 activity, whereas coexpression of NBCe1 with wild type CAII increased transport activity (17). We, therefore, conclude that the increase in MCT1 transport activity does not depend on the catalytic activity of CAII. This suggests a mechanism for the interaction between MCT1 and CAII, which is different from “transport metabolons” formed between CAII and acid-base transporting proteins that require the catalytic activity of CAII (15, 17, 35, 36).

If catalytic activity of the enzyme is not required for the augmentation of MCT1 transport activity, CAII might alter the substrate affinity of the MCT1. Interestingly, a change in $K_m$ value due to injection of CAII could only be observed for low concentrations of lactate, whereas application of higher substrate concentrations resulted in a similar $K_m$ value as in MCT1-expressing oocytes without CAII. Moreover, increasing the extracellular lactate concentration resulted in a smaller augmentation of MCT1 activity induced by CAII. CAII only

**Discussion**

We have recently shown that injection of CAII into MCT1-expressing Xenopus oocytes induces an increase in transport activity of MCT1, an effect that can be observed in the nominal absence of CO₂/HCO₃⁻ and is independent of the catalytic activity of the enzyme (14). In the present study we have focused on the mechanism underlying the augmentation of MCT1 transport activity as induced by CAII. Our results suggest that H⁺ ions entering the cell via MCT1 accumulate in a microdomain at the inner face of the cell membrane, which leads to the dissipation of the H⁺ gradient and, hence, reduction of lactate transport. Injection or coexpression of CAII suppresses this H⁺ microdomain by promoting H⁺ removal from its site of entry by nonenzymatic operation. CA thereby helps to maintain the H⁺ gradient across the cell membrane and allows sustained MCT1 activity, resulting in an increased H⁺-lactate cotransport.

In the present study coexpression of MCT1 with CAII-V143Y...
increased transport activity of MCT1, when transport was supported by a H⁺ gradient. Without a H⁺ gradient, when lactate was the only driving force, little or no effect of CAII on MCT1 activity was observed. Furthermore, CAII increased the sensitivity of MCT1 activity to extracellular pH. This effect increased with decreasing lactate concentration. These findings indicate that cooperation of CAII with MCT1 is likely related to the H⁺ gradient across the cell membrane.

It could be speculated that injection or coexpression of CAII might affect the expression of MCT1, leading to an increase in the amount of MCT in the oocyte plasma membrane. Nevertheless, coexpression of MCT1 with the N-terminal mutant CAII-Hex, in which five histidine and one lysine residues were mutated to the analogous amino acids in CAI (25), as well as injection of CAI and injection of CAII together with a CAII antibody (14) did not enhance MCT1 activity. Because the CAII-WT and the mutant CAII-Hex differ only in six amino acids, it appears unlikely that this difference is responsible for a change of the oocytes expression system leading to an increased expression of MCT1 in the plasma membrane.

Diffusion of protons in the cytoplasm has been found to be slow; in rabbit cardiac myocytes, an apparent diffusion coefficient for H⁺ of 3.78 × 10⁻⁷ cm²/s was determined by imaging H⁺ diffusion with a pH-sensitive dye (37). This value is around 300-fold smaller than the H⁺ diffusion determined in a diluted, unbuffered solution, 1187 × 10⁻⁷ cm²/s (37, 38). Furthermore, it is suggested that intrinsic H⁺ mobility is dictated by the mobility of intracellular non-CO₂ buffers (37).

In frog oocytes import of H⁺ and lactate via the MCT1 would be expected to accumulate H⁺ at the mouth of the transporter. This would create a local H⁺ microdomain, reducing the effective H⁺ gradient and, hence, slow substrate flux via the MCT1. We hypothesize that removing H⁺ from the inner face of the membrane increases MCT1 activity by stabilizing the H⁺ gradient at the mouth of the transporter. The ability of intracellular CAII to facilitate cytoplasmic H⁺ mobility has been shown by imaging H⁺ movement with carboxy-seminaphthodafloflur (SNARF-1) in isolated mouse small intestinal enterocytes (39). In contrast to intracellular H⁺ movements, diffusion of lactate is considered to be fast. An apparent diffusion coefficient for lactate of 130 ± 20 μm²/s was determined in rat intracerebral 9L glioma cells by measuring the diffusion of [¹³C]-labeled metabolites in vivo with localized NMR spectroscopy (40). It can be expected that lactate, when transported into the oocyte via MCT1, would accumulate much less at the transporter microdomain and thereby will not reduce transport unless lactate accumulates globally in the cell interior. Metabolite microdomains produced by single sources like enzymes and transporters can be quantified using an analytical model of brownian diffusion (41). Aided by this mathematical tool, it is possible to predict that near its cytosolic mouth the influx activity of MCT1 generates a strong H⁺ microdomain but no significant lactate microdomain. The difference is explained by large differences in diffusion and concentration between the two molecules. These predictions of the modeling are consistent with our observation that CAII affects MCT1 activity preferentially when the carrier is driven by the H⁺ gradient. Furthermore, our results suggest that CAII not only acted as a “normal proton buffer” inside the cell but also increased movement of protons imported by the MCT1.

Interestingly, it seems that direct binding between MCT1 and CAII is necessary to establish the H⁺ shuttle between the proteins. In an earlier study (14) we have shown that injection of CAII into oocytes expressing a C-terminal-truncated mutant of MCT1 does not enhance transport activity, although binding of CAII to both the mutant and the wild type MCT1 could be shown by a pulldown assay. Combining these results with the findings that both CAI and the N-terminal-modified mutant CAII-Hex are not able to enhance transport activity of MCT1 indicates that a direct interaction between the C-terminal region of MCT1 and the N-terminal region of CAII is crucial to form a transport metabolon. A similar binding mechanism has already been shown for CAII and AE1 (25). An acidic motif (DADD890) was identified as the binding site for CAII at the AE1 (24), whereas the basic N-terminal motif with the high content of histidine residues within the CAII was found to be pivotal for binding to AE1 (25). It has been shown that di-, tri- and tetra-peptides incorporating the sequence DADD, serving as the CAII binding site at the AE1, strongly activate CAII (42). This activation was assigned to the binding of these acidic peptides to the histidine residues within the N terminus of CAII. Bound at the entrance of the enzyme active site, the peptides might facilitate the proton transfer reaction from the zinc-bound water molecule to the environment. By determining CAII catalytic activity via mass spectrometry, however, no difference in the activity of CAII either injected into native or MCT1-expressing oocytes was found.

CAII Increases Activity of MCT1 by Dissipation of Local H⁺ Microdomains—Injection of transporter cRNA into Xenopus oocytes has been found to yield around 10¹⁵ transporter molecules in the oocytes plasma membrane (43). Injection of 50 ng of CAII equals an amount of 10¹² molecules/oocyte, resulting in a ratio of 100 CAII molecules for each MCT1. With an average membrane surface of 2.5 × 10⁻⁹ μm² (44) and assuming that all injected CAII locates at the membrane, as indicated by fluorescence staining, showing CAII solely located at the oocyte plasma membrane but not in the cytoplasm (Fig. 1K), the density of CAII at the membrane would be around 4 × 10⁸ molecules/μm². Assuming an even distribution of CAII at the cytosolic face of the cell membrane, the average distance between two molecules would be around 1 nm (5 nm between the center of two molecules). This would indicate a nearly closed layer of CAII at the membrane that could allow shuttling of protons carried into the cell by the MCT1 along the inner membrane surface. Protonatable residues that are up to 1–1.5 nm apart from each other could form proton-attractive domains and could share the proton among them at a very fast rate, exceeding the upper limit of diffusion-controlled reactions (45). Protons carried into the cell via MCT1 might, hence, be removed from the transporter mouth by being shuttled along the membrane via neighboring carbonic anhydrases. We tested this hypothesis by fluorescence measurements of intracellular pH after focal lactate application. Protons transported into the cell at a point source of H⁺ were distributed faster along the inner face of the cell membrane when CAII was injected. Furthermore, an increased buildup of H⁺ could be observed in more
distant regions when CAII was injected, whereas at the “source” of H⁺, only small differences in absolute H⁺ concentration between CAII- and H₂O-injected oocytes were observed, indicating a more efficient distribution of H⁺ by CAII. The CAII-induced increase of MCT1 activity showed no signs of saturation even at a concentration of 200 ng of CAII/oocyte. This could mean that CAII could be arranged in more than one layer at the oocyte membrane, leading to a more effective distribution of H⁺. Carbonic anhydrase II is known to use an internal proton shuttle to speed up catalysis. Thereby, a proton derived from zinc-bound water is transferred to the side chain of histidine 64, utilizing a network of intervening hydrogen-bonded water molecules, before the proton is transferred to another buffer molecule (46–52).

It is proposed that a long range transfer of protons released by an integral membrane protein along the membrane surface to a H⁺ sink is faster than proton exchange with the bulk water phase (49). H⁺ exchange between the membrane surface and the bulk water phase proceeds as slow as 1 ms or less because of a kinetic barrier for electrically charged species. Movement of protons released by bacteriorhodopsin, e.g. along the purple membrane of *Halobacterium salinarium*, was measured using the pH-sensitive dyes fluorescein, linked to the membrane, and pyranine in the bulk water phase (50–52). The H⁺ that was released at the extracellular side was detected much earlier at the cytoplasmic cell surface than in the aqueous bulk phase, suggesting that it is retained at the surface and then migrates along the purple membrane back to the other side. The authors concluded that after H⁺ release from an integral membrane protein, a long range H⁺ transfer along the membrane surface is faster than proton exchange with the bulk water phase. H⁺ can effectively diffuse from a source to a sink along the membrane without loss of H⁺ into the aqueous bulk. This mechanism is attributed to surface-bound protonatable groups, which conduct protons rapidly along the surface away from a source but can also collect H⁺ from the bulk solution. Negatively charged residues of membrane proteins with overlapping Coulomb cages can form a “H⁺-collecting antenna” that collects protons from solution and “funnels” them to the entrance of a proton-transfer pathway of a membrane-anchored protein (53). If CAII acts as a buffer, stabilizing the H⁺ gradient by binding H⁺ entering the cell via MCT1, a decrease in the rate of rise in H⁺ concentration would be measured, as H⁺ entering the cell would be removed by CAII, hence becoming “invisible” to the ion-sensitive electrode. Because we have measured an increase in the rate of rise in intracellular H⁺ concentration, protons could not just be bound by CAII but have to be passed into the intracellular bulk where they are detected by the electrode at a higher rate, as measured in oocytes without CAII.

We hypothesize that protonation and deprotonation of CAII could facilitate the movement of H⁺ in a similar way along the plasma membrane after entry via MCT1. Because the membrane of MCT1-expressing oocytes lacks a sink for H⁺, CAII might facilitate the transfer of H⁺ from the electrically charged plasma membrane into the bulk water phase. By this mechanism, CAII could allocate H⁺ along the plasma membrane and thereby increase the area in which H⁺ can be released from the membrane into the bulk as shown in Fig. 9D. This would dissipate local proton microdomains at the pore of the transporter faster and would allow net flux via the MCT1 to increase. On the other hand, CAII could attract H⁺ and funnel them along the membrane to the MCT1, which would prevent depletion of protons at the side of the transporter and would facilitate efflux of H⁺ and lactate when the gradients are reversed.

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