# Studies of the membrane topology of the rat erythrocyte $H^+$/lactate cotransporter (MCT1)

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1. Hydrophobicity analysis of the monocarboxylate/proton cotransporter MCT1 (lactate transporter) suggests a structure with 12 transmembrane (TM) segments, presumed to be $\alpha$-helical. 2. A series of anti-peptide antibodies have been raised against regions of the MCT1 sequence, which each recognize a polypeptide of approx. 40 kDa in rat erythrocytes. The topology of rat MCT1 was investigated by studying the immunoreactive fragments derived from proteolytic digestion of the protein in intact rat erythrocytes and leaky membranes. 3. Reactivity with an anti-(C-terminus) antibody was prevented on treatment of leaky membranes, but not intact cells, with carboxypeptidase Y, indicating that the C-terminus of the protein is cytoplasmically disposed. 4. Treatment of intact cells in saline buffer with trypsin, chymotrypsin, bromelain and protease K (up to 1 mg/ml) resulted in no degradation of MCT1, indicating the absence of any large exposed extracellular loop. In a buffer of low ionic strength (containing sucrose), cleavage was observed with bromelain at an extracellular site, probably TM9/10. 5. Treatment of leaky membranes with low (less than 100 $\mu$g/ml) concentrations of several proteases resulted in fragmentation of MCT1, reflecting cleavage at the cytoplasmic face of the membrane. These treatments generated N-terminal fragments of apparent molecular mass approx. 17–19 kDa that were resistant to further degradation. The epitopes for the TM6/7 and C-terminal antibodies were either lost from the membrane or destroyed under most of these conditions, indicating that these regions of the protein are located in the cytoplasm. 6. More detailed structural prediction analysis of MCT-related sequences was made assuming the constraints placed upon the possible arrangements by the experimental data outlined above. This analysis provided additional strong evidence for the 12-TM-segment model, with cytoplasmic N- and C-terminal ends and a large internal loop between TM6 and TM7. The predicted helices were assigned moments of hydrophobicity and residue substitution; for a number of TM segments this permitted the prediction of the sides of the helix that faced membrane lipid and the interior of the protein.

## INTRODUCTION

Transport of lactate, pyruvate and the ketone bodies, acetoacetate and $\beta$-hydroxybutyrate, is of major physiological importance to almost all cells [1]. In mammalian cells this process is mediated by a family of $H^+$-monocarboxylate co-transporters (MCTs). Recently the first two members of this family, MCT1 and MCT2, have been cloned from hamster [2–4]. The sequences of MCT1 from rat, mouse and human have also been determined [5–7]. MCT1 and MCT2 are approximately 60 % identical, and there are cDNAs for other related proteins whose sequences have been determined recently. These include a human X-linked PEST [a sequence rich in proline, glutamate (aspartate), serine and threonine]-containing putative transporter [8], a chicken retinal epithelial membrane protein (REMP; [9]), and sequences from *Caenorhabditis elegans* (gene K05B2.5) and *Saccharomyces cerevisiae* (ORF YKL221w; [10]). It has not been determined whether all of these cDNA sequences encode proton-coupled transporters for monocarboxylates, or whether they have other substrates or ion-coupling. However, it is clear that these and other, as yet unknown, proteins form a new family of membrane transporters.

The most fully characterized mammalian MCT is that of the erythrocyte, which corresponds to the MCT1 isoform [11]. The kinetic properties of this transporter have been investigated in some detail [1,12,13], and it has been functionally reconstituted into liposomes [14] and labelled specifically with stilbene disulphonates [15,16]. MCT1 is expressed widely in mammalian tissues, and is likely to be the 'housekeeping' monocarboxylate transporter. Although there is a wealth of information about the functional properties of MCT1, we have little understanding of the structure and mechanism of this transporter or any other member of the family. Analysis of hydrophobicity by standard algorithms such as that of Kyte and Doolittle [17] reveals the presence of 12 hydrophobic segments of sufficient length to traverse the membrane as $\alpha$-helices. This leads to a topological model of the transporter with these 12 segments separated between TM segments TM6 and TM7 by a large hydrophilic loop, and an extended hydrophilic C-terminal tail. There is no evidence for a cleavable N-terminal signal sequence, and indeed this has been confirmed by our own N-terminal sequencing of rabbit MCT1, which demonstrated the sequence of the mature protein to be as predicted from the cDNA except for the loss of the initiator methionine [11]. Here we present the first study to determine the membrane topology of MCT1, capitalizing on the utility of the erythrocyte membrane as a well-characterized, simple and flexible system for vectorial proteolysis [18]. Analysis of the available sequences of MCT1 and MCT2 for hydrophobicity, as well as periodicity in hydrophobicity and residue conservation within hydrophobic segments, is used as a complementary approach. Our results provide substantial support for

Abbreviations used: CHO, Chinese hamster ovary; KLH, keyhole limpet haemocyanin; MCT, monocarboxylate transporter; PEST, sequence rich in proline, glutamate (aspartate), serine and threonine; sulfo-SMCC, sulphosuccinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate; TM, transmembrane; Tos-Phe-$CH_2Cl$, tosylphenylalanylchloromethane, 1-chloro-4-phenyl-3-L-toluene-*p*-sulphonamidobutan-2-one.

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the 12-helix model and locate the C-terminus and the loop between TM6 and TM7 in the cytoplasm.

## EXPERIMENTAL

### Materials

The sources of chemicals and biochemicals were as given previously [16], with the following additions. Keyhole limpet haemocyanin (KLH) was supplied by Calbiochem, Nottingham, U.K. Sulphosuccinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (Sulfo-SMCC), 'immunopure gentle Ag/Ab elution buffer' and Sulfolink coupling gel were obtained from Pierce (Chester, Cheshire, U.K.). Peptides were synthesized either by Dr. Graham Bloomberg (Molecular Recognition Centre, University of Bristol, Bristol, Avon, U.K.) or commercially (Zinsser Analytic, Maidenhead, Berks., U.K., or Severn Biotech Ltd., Kidderminster, Hereford and Worcester, U.K.). Carboxypeptidase Y and bromelain were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). Pepstatin was supplied by Cambridge Research Biochemicals (Cambridge, U.K.) and tosylphenylalanylchloromethane (Tos-Phe-$CH_2Cl$)-treated trypsin was from Worthington (Irvine, Scotland, U.K.). Chymotrypsin A4 was supplied by Boehringer.

### Erythrocytes and membranes

Rat erythrocytes were collected in 84 mM sodium citrate/1 mM EGTA (pH 7.4) and washed several times in this buffer. Lysis of erythrocytes and preparation of leaky 'ghost' membranes were performed as described previously [14,16]. When erythrocyte membranes were to be used for proteolytic digestions, they were prepared immediately before use to prevent resealing, and PMSF was omitted from the lysis buffer.

### Protease treatments

All treatments of intact erythrocytes were performed at a haematocrit of approx. 10%, and for leaky erythrocyte membranes the protein concentration was 1 mg/ml. These conditions resulted in a similar concentration of membranes from both preparations. Most incubations of proteases, with both intact erythrocytes and leaky membranes, were performed in PBS (135 mM NaCl/11 mM sodium phosphate) adjusted to pH 7.8. Incubations of intact cells were performed with the addition of 5 mM glucose to maintain cell integrity and usually included 1 mM dithiothreitol. Incubations were continued for 60 min and stopped by addition of an equal volume of the same buffer containing the protease inhibitors PMSF (1 mM) and Tos-Phe-$CH_2Cl$ (0.1 mM). The cells were washed twice more with this buffer before lysis as described above, with buffer containing 1 mM PMSF and 0.1 mM Tos-Phe-$CH_2Cl$. Erythrocyte membranes incubated with proteases were washed three times with lysis buffer containing PMSF and Tos-Phe-$CH_2Cl$. Samples prepared in this way were stored at $-20$ °C before electrophoretic analysis. For incubations with carboxypeptidase Y, the cells and membranes were washed and resuspended in a buffer containing 112.5 mM NaCl, 25 mM sodium phosphate, 50 mM EDTA and 2 $\mu$g/ml pepstatin, pH 5.8. Carboxypeptidase Y was added and incubations were continued at 37 °C overnight (typically 18 h). Incubations of intact erythrocytes were supplemented with 5 mM glucose to maintain cell integrity. The digestions were stopped by the addition of 1 mM PMSF. Cells were washed well with citrate buffer or PBS/1 mM PMSF before lysis in phosphate buffer/1 mM PMSF. Membrane incubations were usually washed with 5 mM sodium phosphate/1 mM PMSF (pH 8.0).

**Table 1 Anti-MCT1 antibodies**

The peptides were synthesized on the basis of the sequences of MCT1 from various mammalian species as indicated. A cysteine residue (underlined) was included as the N- or C-terminal amino acid to enable coupling of the peptide to KLH and to permit the production of affinity matrices. Reactivity of the antibodies was assessed in two ways: first, reactivity against the peptides was determined with an ELISA assay; secondly, the antibodies were used on immunoblots of rat erythrocyte membrane proteins.

| Peptide | Sequence | Reactivity with peptide | Immunoblot reactivity (rat MCT1) |
|---|---|---|---|
| Rabbit MCT1 (N-terminal; 2–16) | PPAVGGPVGYTPPDGGC | + | + |
| CHO MCT1 (TM6/7; 202–216) | CPGKIEKLKSKESLQE | + | ++ |
| Rat MCT1 (TM7/8; 278–293) | CSNYGKSKHFSSEKSAF | + | + |
| Rat MCT1 (TM11/12; 403–418) | CGRLNDMYGDYKYTYWA | + | + |
| CHO MCT1 (C-terminal; 478–494) | CPQQNSSGDPAEEESPV | + | +++ |

Incubations of intact cells with bromelain were performed in PBS (pH 7.8) or 20 mM Mops/300 mM sucrose (pH 7.8), and stopped, washed and lysed with buffers containing 0.1 mM Tos-Phe-$CH_2Cl$.

### Anti-peptide antibodies

A series of antibodies were raised against peptides, corresponding to hydrophilic loop regions of the MCT1 sequence (Table 1). Accordingly, the nomenclature used for these antibodies describes the location with respect to putative TM segments (e.g. the TM7/8 antibody was raised against a peptide within the loop between TM7 and TM8). The peptides were chosen from the sequence that we obtained for the N-terminus of rabbit MCT1 [11], the derived sequence of the Chinese hamster ovary (CHO) cell MCT-1 cDNA (TM6/7 and C-terminal peptides; [2,3]) and from the sequence derived from rat MCT1 cDNA, which we determined recently (TM7/8, TM11/12; [7]). The use of antibodies against peptides from MCT-1 of different species merely reflects the availability of peptide sequence data at the time at which the antibodies were raised. Each of these peptides had an additional N- or C-terminal cysteine residue that allowed cross-linking to KLH with the water-soluble heterobifunctional cross-linker Sulfo-SMCC. The ratio for the coupling reaction was 2 mg of KLH/mg of peptide. New Zealand White rabbits were injected with 200 $\mu$g of conjugate (with respect to KLH) at 14-day intervals. The first injection was with Freund's complete adjuvant, subsequent injections being with incomplete adjuvant. The serum was routinely collected 7–10 days after the fourth injection. The IgG fraction was prepared with octanoic acid [19], and affinity-purified. Affinity columns were prepared by coupling the individual peptides via their terminal cysteine residues to Sulfolink coupling gel essentially in accordance with the manufacturer's instructions. Elution of the peptide was achieved by use of Pierce 'Immunopure gentle Ag/Ab elution buffer'. The eluted antibodies were exchanged into 20 mM Mops/150 mM NaCl (pH 7.4) with PD10 gel-filtration columns (Pharmacia), and stored in aliquots at $-70$ °C.

### Analytical techniques

Protein concentrations were assayed by the method of Bradford [20]. SDS/PAGE was performed by the method of Laemmli [21] or, for fragments of low molecular mass, with the Tricine buffer system as described by Schagger and von Jaggow [22]. For immunoblots the separated proteins were transferred electro-

phoretically to poly(vinylidene difluoride) membranes (Immobilon P; Millipore) and developed with an enhanced chemiluminescence protocol as described previously [16]. To assess the specificity of protein bands detected, the peptides against which the antibodies were raised were used as specific blocking agents. The peptides (at 4 $\mu$g/ml) were preincubated with the diluted antibody and then incubated with the transferred proteins.

## RESULTS AND DISCUSSION

The proposed model representing the topology of MCT1 is presented in Figure 1. The model is based simply on the presence in the sequence of MCT1 (and MCT2) of 12 hydrophobic segments of sufficient length to span the lipid bilayer. According to this model the N-terminus, a large hydrophilic segment between TM6 and TM7 and the C-terminal domain are located in the cytoplasm. This arrangement, with the 12 TM segments separated by a large loop between TM6 and TM7, is a feature commonly found in other families of transporters such as many of those for sugars, multidrug resistance and cystic fibrosis transmembrane conductance regulator [23–25], that have little sequence similarity to the MCT family. According to this model there is no external consensus site for N-linked glycosylation; even where such a site exists (in the TM1/2 loop in CHO MCT1), it seems not to be utilized [6]. To test the topological model we have raised a series of anti-peptide antibodies.

### Characterization of anti-MCT1 antibodies

The anti-MCT1 antibodies (Table 1) all recognized the peptides against which they were raised in an ELISA assay. They also reacted with a broad band centred at 40 kDa on Western blots of rat erythrocyte membrane proteins, and the appropriate peptides blocked detection of this band (results not shown). Some of the antibodies recognized MCT1 from a number of species, whereas others were more species-specific. This is expected on the basis of sequence variation between MCT1 in different mammalian species. To identify any antibodies that reacted with an exofacial epitope, the affinity-purified preparations were tested for their ability to agglutinate rat erythrocytes. Although none of the antibodies was capable of agglutinating erythrocytes in PBS, this does not exlude the possibility that they recognized an extracellular epitope, because they might not bind to the intact protein in the membrane.

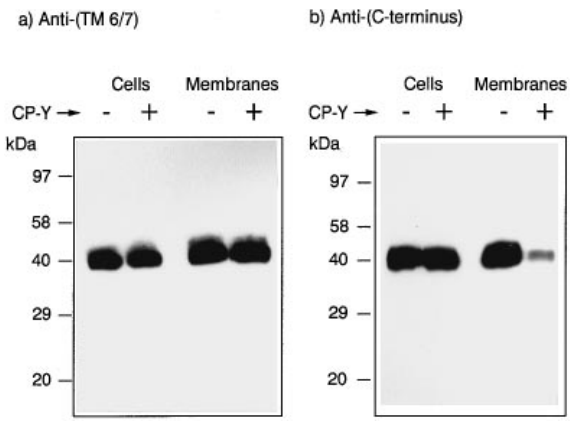


**Figure 2 Digestion of rat erythrocyte MCT1 with carboxypeptidase Y**

Incubations were performed as described in the Experimental section. 10 $\mu$g (erythrocyte incubations) or 5 $\mu$g (membrane incubations) of protein was loaded on each track, for membranes prepared from incubations of cells or leaky membranes with (+) or without (−) 40 units/ml carboxypeptidase Y. The Western blots were probed with either anti-TM6/7 antibody (**a**) or anti-C-terminus antibody (**b**).

### Digestion of rat MCT1 with carboxypeptidase Y

The location of the C-terminus of rat erythrocyte MCT1 was determined by performing digests of intact erythrocytes (extra-

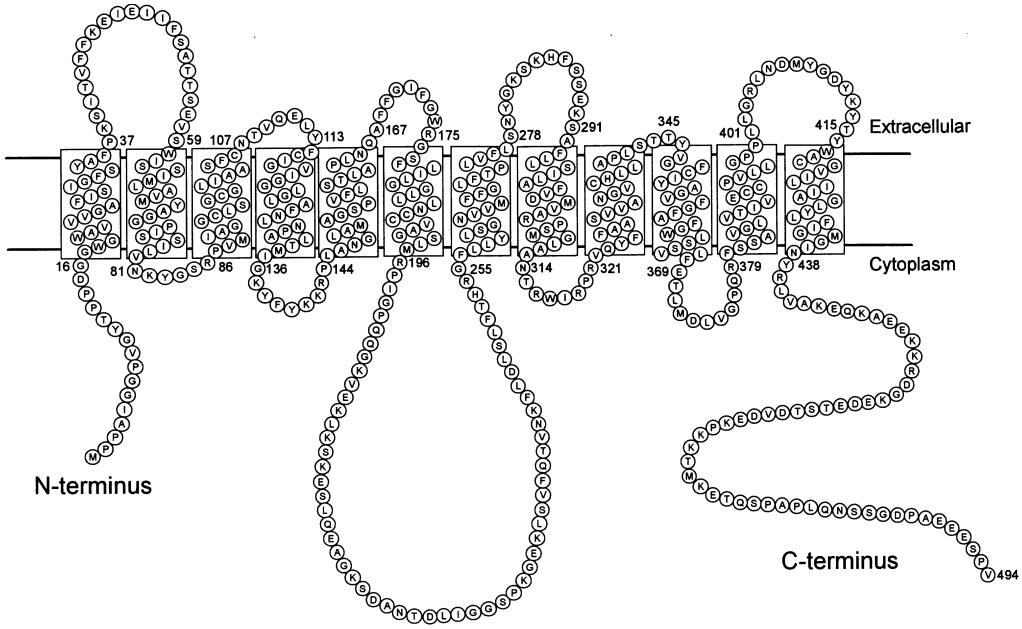


**Figure 1 Proposed topology of rat MCT1**

The protein sequence (derived from the cDNA sequence) is taken from Jackson et al. [7].

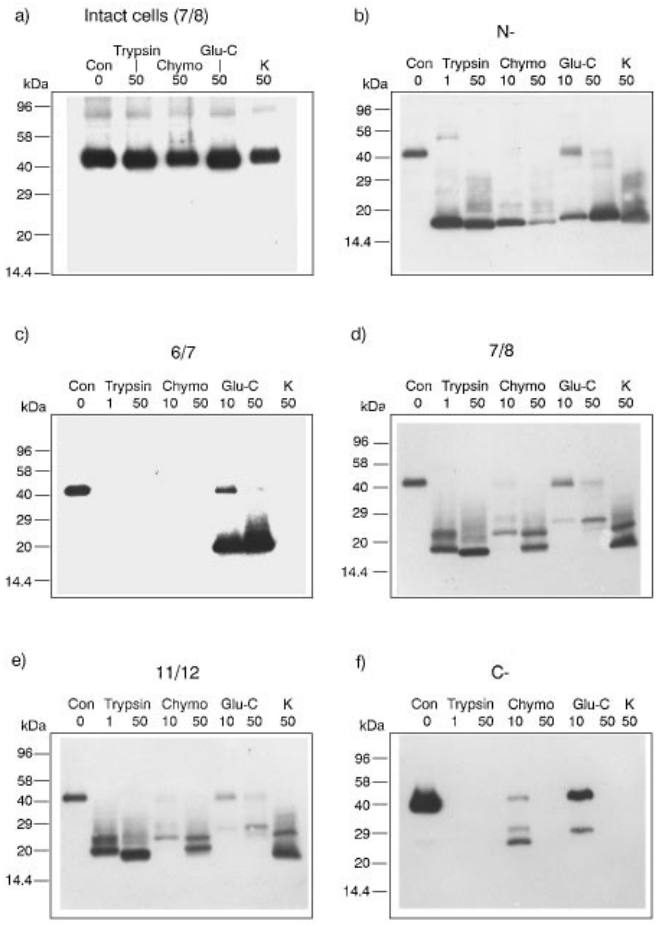


**Figure 3 Protease digestion of MCT1 in intact erythrocytes (a) and leaky membranes (b–f)**

Incubations were performed as described in the Experimental section. (**a**) Intact erythrocytes were incubated with the proteases trypsin, chymotrypsin, Glu-C and protease K at 50 $\mu$g/ml for 1 h at 37 °C. The immunoblot of the derived membrane preparation was probed with the TM7/8 antibody; similar results were seen with other antibodies. (**b–f**) Digestion pattern of MCT1 after incubations of leaky membranes with proteases as incubated. These Western blots were probed with anti-(N-terminus) (**b**), anti-TM6/7 (**c**), anti-TM7/8 (**d**), anti-TM11/12 (**e**) and anti-(C-terminus) (**f**) antibodies.

cellular face accessible) or leaky membranes (both membrane faces of the protein accessible) with carboxypeptidase Y, which cleaves amino acids sequentially from the C-terminus. Preliminary experiments (results not shown) demonstrated that long incubations (overnight) were required to remove most of the reactivity towards the anti-(C-terminal peptide) antibody on immunoblots. The results shown in Figure 2 demonstrate that carboxypeptidase Y removed the C-terminus of MCT1 (but not reactivity with the TM6/7 antibody) when incubated with membranes but not intact cells. These observations indicate clearly that the C-terminus of MCT1 is cytoplasmic.

### Digestion of rat MCT1 with endoproteinases

Further experiments were performed with a variety of proteases to digest intact cells and membranes and then detect immunoreactive fragments on immunoblots. For these experiments the proteins were separated with the gel system of Schagger and von Jaggow [22], which is effective at resolving small peptide fragments. The results of the proteolytic dissections are shown in Figure 3. It is clear from Figure 3(a) that treatment of intact erythrocytes with trypsin, chymotrypsin, endoproteinase Glu-C and protease K was unable to cause cleavage of MCT1. Similar observations were made with several anti-peptide antibodies and with higher concentrations of proteases, and indicate that there are few loops exposed on the extracellular face of the protein. In marked contrast, treatment of leaky membranes with the proteases resulted in considerable cleavage of the protein. Because the same proteases were without effect on intact cells, it is apparent that these peptide fragments are derived from cleavage of the cytoplasmic face of the transporter. Under several conditions no immunoreactive fragment was observed with the TM6/7 and C-terminal antibodies, indicating that these epitopes are lost from the protein and hence cytoplasmically disposed. This might be due either to destruction of the epitopes or to their presence in soluble fagments lost from the membrane during the washing procedure. Most of the treatments resulted in the appearance of N-terminal fragments of approx. 17–19 kDa. This would correspond to cleavage in the TM6/7 loop, which has many potential sites for the proteases used. No N-terminal fragments smaller than this were observed, indicating that this half of the transporter has no readily exposed cleavage sites, despite a number of residues as potential sites for cleavage in putative hydrophilic loops.

In most cases the C-terminal epitope was one of the first regions to be lost with endoproteinase digestion, but with some proteases (chymotrypsin, Glu-C) some minor C-terminus-containing fragments were observed. The size of these fragments would again correspond to cleavage within the TM6/7 loop. The results obtained with the TM7/8 and TM11/12 antibodies were the same for these proteases, indicating that there was no site of cleavage between these two epitopes. Taken together with the remaining data, the fragments detected with these antibodies represent gradual trimming from the C-terminus and the TM6/7 loop. One of the more interesting observations was of a fragment (approx. 19 kDa) detected with the TM6/7 antibody after digestion with Glu-C, which seems to correspond to an N-terminal peptide. The signal with the TM6/7 antibody was markedly enhanced in this fragment. Such an enhancement presumably reflects presentation of the peptide in a more favourable manner for binding of the antibody. This might be related to a conformational effect or it might more simply be that the site of cleavage exposes the peptide in a manner that closely matches that of the KLH-peptide immunogen. The high reactivity of the cleaved protein with the TM6/7 antibody indicates that the glutamate residues within the antigenic sequence are not major sites of cleavage by Glu-C. Although the precise identification of sites of cleavage (by N-terminal protein sequence analysis of fragments) would refine these studies, this would currently be extremely difficult for rat MCT1. Purification and N-terminal protein sequencing has been reported only for rabbit MCT1 [11], and our attempts to immunopurify rat MCT1 with the anti-peptide antibodies described here have not resulted in high yields in the immunoprecipitate.

### Exofacial digestion with bromelain

As has already been indicated, rat MCT1 is refractory to digestion at the external face of the membrane, presumably reflecting the presence of small extracellular loops and/or their lack of exposure owing to their close apposition to the membrane or other parts of the protein. Attempts to digest the transporter from the external face were made in buffer of low ionic strength which is known for band 3, the erythrocyte anion transporter, to increase the

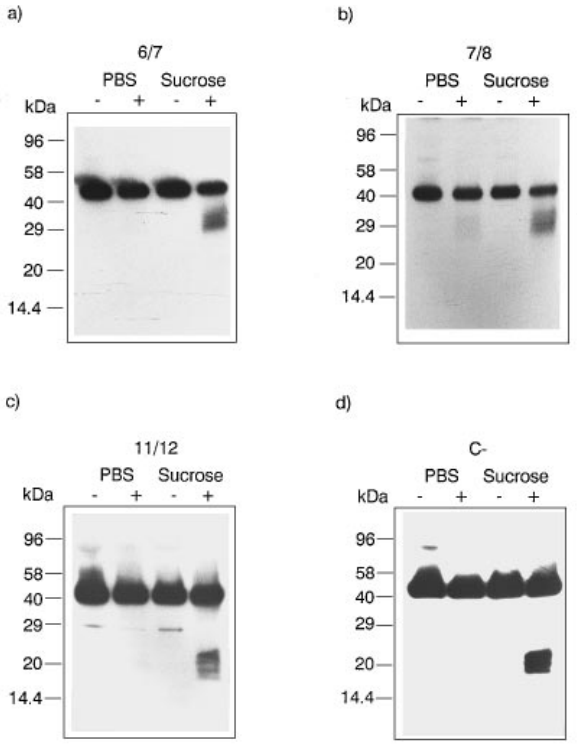


**Figure 4 Digestion of MCT1 in intact erythrocytes with bromelain**

Cells were incubated in saline or sucrose buffers as indicated, in the presence (+) or absence (−) of 1 mg/ml bromelain as described in the Experimental section. Western blots (20 $\mu$g of protein per track) of the membranes derived from these cells were probed with anti-TM6/7 (**a**), anti-TM7/8 (**b**), anti-TM11/12 (**c**) and anti-(C-terminus) (**d**) antibodies.

susceptibility to exofacial digestion by trypsin [26]. Presumably this reflects a conformational change and might be due to a loosening of loop structure. It is conceivable that there are extensive hydrophobic interactions within/between connecting loops that protect against proteolysis in ionic buffers and are released at low ionic strength. For most of the proteases tested (trypsin, chymotrypsin and Nagarse), sucrose buffer had little effect on the susceptibility to proteolysis, but with the non-specific protease bromelain there was partial digestion of MCT1. Two classes of product were observed; a fragment of approx. 29 kDa, reactive with antibodies to the N-terminus, TM6/7 and TM7/8, and a fragment of approx. 20 kDa, reactive with antibodies to TM11/12 and the C-terminus (Figure 4). These observations indicate that there is a single site of external cleavage between TM7 and TM12 (probably TM9/10, or possibly at the C-terminal end of TM7/8). Because the limited digestion of the transporter at this site occurs only in sucrose buffer, it probably reflects a hydrophobic association from which the cleaved region is released only at low ionic strength.

### Comparisons of the predicted topology with that predicted for other MCT-related sequences

The experimental results outlined here provide considerable support for many aspects of the topological model presented in Figure 1; in particular the TM6/7 loop and C-terminus must be disposed cytoplasmically. However, the loops between putative TM segments 1–6 and 7–12 cannot be defined unequivocally by this approach. It is worth while in this situation to analyse in some detail the sequence of MCT1 and related proteins to improve the predictions of putative TM segments. In addition to MCT-1 from rat, mouse, human and hamster [3,5–7] there are now several sequences related to MCT1 in the databases. These are MCT-2 from hamster [4], a cDNA isolated from chicken retinal epithelium (REMP; [9]), a human PEST-containing putative transporter (XPCT; [8]) and putative transporter sequences from *C. elegans* (gene K05B2.5) and *S. cerevisiae* (ORF YKL221w; [10]). Only for MCT-1 [3], MCT-2 [4] and REMP (Dr. N. Philp, personal communication) has MCT activity been demonstrated directly by expression. The pairwise identity of the sequences with rat MCT1 calculated with the GCG package [27] are as follows: hamster MCT2, 59.4%; REMP, 41.8%; XPCT, 24.4%; *C. elegans* gene K05B2.5, 26.2%; and *S. cerevisiae* ORF YKL221w, 23.4%. In all cases, by using the algorithm of Kyte and Doolittle [17] with an 11-residue window, hydropathy plots for the individual sequences suggested 12 hydrophobic segments, presumed to cross the membrane as $\alpha$-helices, although the C-terminal TM segments were defined less clearly than were the N-terminal segments. In addition, sequence conservation was greatest in these hydrophobic segments, consistent with the constraints required in the formation of the TM $\alpha$-helices. The largest variations, both in length and amino acid identity, were in the longer stretches of hydrophilic residues thought to comprise the N-terminus, the loop between TM6 and TM7, and the C-terminus.

The results obtained both with proteolytic cleavage of MCT1 in rat erythrocytes and by comparison of sequences strongly support the topological model proposed in Figure 1 in which both the C-terminus and TM6/7 loop are located in the cytoplasm. The location of the other loops must then follow from the sequence analysis, which predicts 12 TM spans, with six hydrophobic regions in the N-terminal half of MCT1. This defines the N-terminus as being in the cytoplasm. These conclusions are further supported by the absence of a cleavable signal sequence [11] and the presence, in XPCT, of an extension containing a PEST domain at the N-terminus. PEST domains are associated with proteins subject to rapid degradation [28]. If indeed this domain plays a role in regulation of protein turnover, it would be expected to reside in the cytoplasm.

Identification of glycosylation sites in membrane proteins is frequently able to provide useful topological information, because such sites are located in extracellular loops. Because CHO MCT1 has a consensus site for N-linked glycosylation at Asn-52 in the TM1/2 loop, we have compared the glycosylation of this protein with that of mouse MCT1, which like rat and human MCT1 lacks this site [6]. N-glycanase F had no effect on the mobility under SDS/PAGE of MCT1 from CHO cells and mouse Lettré cells; nor were the products of cell-free translation of these proteins changed on performing the translation reaction in the presence of microsomes. Thus we concluded that MCT1 is not subject to N-linked glycosylation, even when a putative extracellular site is present [6]; this might have been predicted because the TM1/2 loop is probably too small to be an efficient substrate for this process [29]. There is an additional consensus site for N-linked glycosylation in rat MCT1 but this is very near the C-terminus, known from the present work to reside in the cytoplasm. Accordingly it is not surprising that incubation with N-glycanase F was without effect on the mobility of MCT1 from rat erythrocytes (R. C. Poole, unpublished work).

The large hydrophilic segment between TM6 and TM7 is a common feature of 12-TM helix transporters including the mammalian glucose transporters [30,31]. Indeed the sensitivity of GLUT1 and MCT1 to proteases is very similar and in both cases attack is restricted to the C-terminal tail and the central loop

**Table 2 Analysis of periodicity of hydrophobicity and residue conservation in hydrophobic segments of MCT1 and MCT2**

Sequences of MCT1 from rat [7], Chinese hamster [2], mouse [6] and human [5], and MCT2 from Syrian hamster [4] were aligned and analysed for periodicity in hydrophobicity and residue substitution as described by Donnelly et al. [33,37]. Residue numbers are given for the sequence of rat MCT1. Quality of predictions is indicated with an arbitrary 4-point scale: 4, very good; 3, good; 2, marginal; 1, poor. Abbreviations used: TM no., helix number; helix, residues predicted to make up the helix; hydrophobicity, periodicity in hydrophobicity, calculated for the whole alignment by using the Eisenberg [34] matrix (which generally gave the most consistent results); conservation, periodicity in patterns of conserved residues [substitutions in the helix are indicated both in terms of the number of separate positions at which substitution occurs (no. posns.) and the total number of substitutions (no. subs.)]; P.R., region within helix giving best measure of periodicity; AP, predicted strength of periodicity (values of AP over approx. 3.0 are an unambiguous indication of helical periodicity); RPT, residues per turn giving the greatest degree of periodicity (should be as close to 3.6 as possible for an $\alpha$-helix); ang., angle of moment (N-terminal residue at 0, for helices alternately clockwise and anti-clockwise); QH, quality of predicted moment of hydrophobicity; QC, quality of predicted moment of conservation; diff., difference between the moments in degrees, on a scale of 0–180 [as the hydrophilic (inside) face is most likely to be conserved, for an 'ideal' prediction this should be approx. 180°].

| | | Hydrophobicity | | | | Conservation | | | | | | Quality of predictions | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| TM no. | Helix | P.R. | AP | RPT | Ang. (degrees) | No. posns. | No. subs. | P.R. | AP | RPT | Ang. (degrees) | QH | QC | Diff. (degrees) |
| 1 | 16–36 | 23–30 | 2.78 | 3.64 | 36 | 4 | 5 | 19–26 | 2.69 | 3.33 | 276 | 2 | 2 | 120 |
| 2 | 60–80 | 60–79 | 4.06 | 3.53 | 297 | 2 | 3 | 69–80 | 3.31 | 3.46 | 173 | 4 | 3 | 124 |
| 3 | 87–106 | 88–94 | 2.68 | 3.87 | 342 | 10 | 11 | 94–100 | 2.40 | 3.56 | 100 | 2 | 2 | 142 |
| 4 | 114–135 | 116–122 | 3.37 | 3.24 | 241 | 5 | 8 | 114–122 | 3.08 | 3.56 | 131 | 3 | 3 | 110 |
| 5 | 145–165 | 145–166 | 4.00 | 3.56 | 25 | 4 | 4 | 158–165 | 3.19 | 3.91 | 220 | 4 | 3 | 175 |
| 6 | 176–195 | 178–184 | 2.64 | 4.00 | 275 | 5 | 6 | 182–190 | 3.70 | 3.53 | 80 | 1 | 3 | 175 |
| 7 | 256–277 | 266–275 | 4.07 | 3.56 | 49 | 7 | 9 | 256–268 | 3.08 | 3.36 | 355 | 4 | 3 | 54 |
| 8 | 293–313 | 297–304 | 4.44 | 3.60 | 327 | 7 | 8 | 295–313 | 3.30 | 3.60 | 105 | 4 | 3 | 137 |
| 9 | 322–342 | 331–342 | 5.43 | 3.71 | 218 | 10 | 13 | 327–334 | 2.79 | 3.30 | 164 | 4 | 2 | 54 |
| 10 | 347–367 | 354–367 | 3.32 | 3.64 | 210 | 12 | 16 | 355–364 | 4.61 | 3.83 | 100 | 3 | 3 | 100 |
| 11 | 380–399 | 386–393 | 3.04 | 3.27 | 223 | 1 | 1 | 385–396 | 1.65 | 3.43 | 158 | 3 | 1 | 65 |
| 12 | 417–436 | 422–429 | 2.99 | 3.30 | 338 | 9 | 10 | 430–436 | 2.30 | 4.00 | 247 | 2 | 1 | 89 |

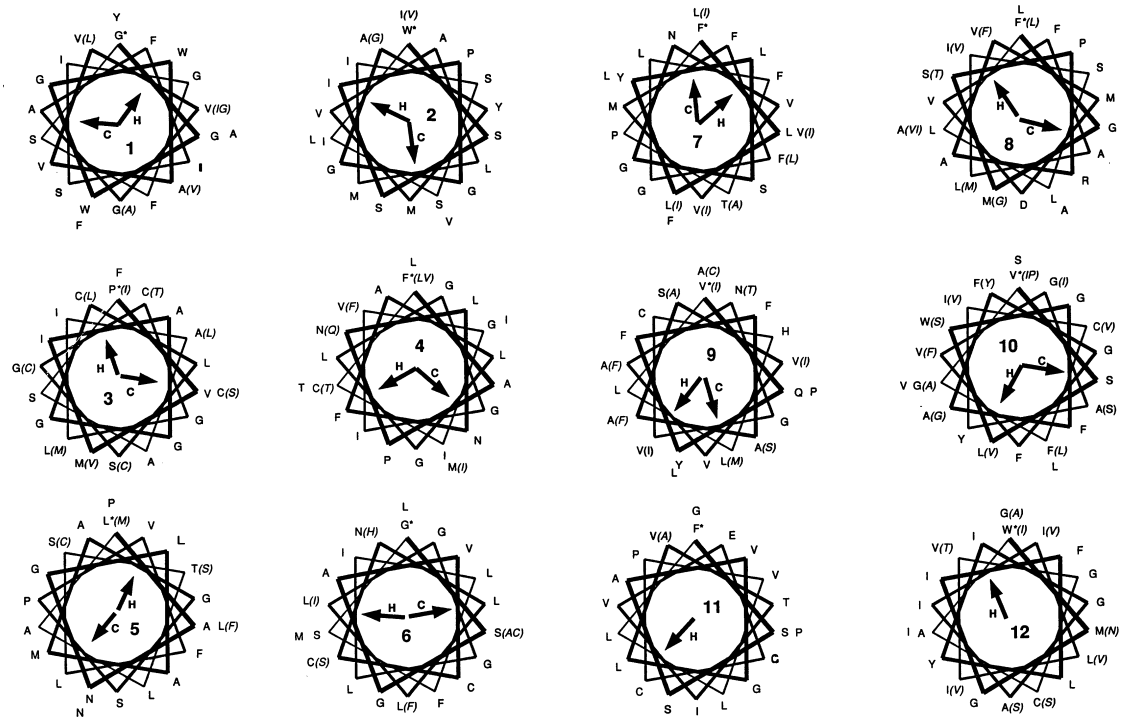

**Figure 5 Helical wheels for the 12 predicted TM helices of rat MCT1**

The sequence of rat MCT1 is given in normal type: at positions where substitutions occur in any of the MCT sequences, these are listed in brackets and italicized. Arrow H shows the position of the moment of hydrophobicity, and arrow C that of the moment of conservation. The N-terminal residue of each helix is at the top, marked with a star. In odd-numbered helices the N-terminus is at the near end of the helix, which twists in a clockwise direction; in even-numbered helices, the N-terminus is at the far end of the helix, which twists in an anti-clockwise direction. Moments of hydrophobicity are shown for all 12 helices. Moments of conservation are not shown if the prediction is 'poor', defined as either [(AP $<$ 3.0) and (RPT $-$ 3.6 $>$ 0.3] or (AP $<$ 2.0 or RPT $-$ 3.6) $>$ 0.4. AP and RPT are defined in Table 2.

which divides the protein into two TM domains [32]. In multiple alignments of MCT-related sequences there are 26 residues that are conserved in all sequences. Of these, more than half (15) are glycine residues, which presumably play an important structural role in the transporter [30]. They are likely to be involved in the formation of turns between TM segments, helix packing and flexibility for conformational changes.

### Periodicity in TM segments

In more detailed studies, those sequences that were known from expression studies to encode lactate/monocarboxylate transporters were taken and analysed further, for both amphiphilicity and patterns of residue substitution. The existence of a hydrophobic region that is also amphipathic is strong evidence for a TM segment because this is the pattern expected for a helix that has contacts with lipid (hydrophobic) on one face and protein/protein interactions and/or contact with an aqueous channel on the other face. For an $\alpha$-helix the amphiphilicity should be maximal with 3.6 residues per turn. This approach can be extended by also looking at the pattern of residue substitution among the different sequences, and determining a moment for these substitutions. It is generally accepted that the residues facing lipid will be both hydrophobic and poorly conserved. Thus it is possible to predict those faces of a putative TM segment that face into the protein/channel, as an aid in the design of further experiments in addition to structure predictions. It is also a first step in trying to understand how the individual TM segments pack together.

An alignment of complete sequences of all such lactate/monocarboxylate transporters – MCT1 from rat, mouse, hamster and human, and MCT2 from hamster – was analysed in more detail with the Fourier transform algorithm of Donnelly et al. [33]. There were no insertions or deletions in this group of sequences in any hydrophobic segment. With this approach, periodicity in both hydrophobicity and residue conservation is defined in terms of vector 'moments'; for an 'ideal' amphiphilic helical segment the direction of the moment of hydrophobicity is expected to be at approx. 180° to that of the moment of conservation (i.e. the hydrophilic face is expected to be the most conserved). Moments of hydrophobicity were calculated for the whole alignment by using three different hydrophobicity scales: Kyte and Doolittle [17], Eisenberg [34] and PRIFT [35]. In contrast with a similar analysis of the fucose transporter from *Escherichia coli*, FucP [36], the Eisenberg matrix gave slightly more consistent results than the others. Full results of this analysis are given in Table 2 and Figure 5, which shows helical wheels for the 12 putative TM segments giving moments of hydrophobicity and conservation where these can be defined. Strength of periodicity (AP) values of 3.0 and greater indicate the presence of significant helical periodicity, and values for residues per turn (RPT) of $3.6 \pm 10\%$ are consistent with an $\alpha$-helix. By these criteria TM segments TM2, TM4, TM5, TM7, TM8, TM9, TM10 and TM11 have well-defined moments of hydrophobicity, and segments TM2, TM4, TM5, TM6, TM7, TM8 and TM10 have well-defined moments of conservation. Of those helices where both moments were well predicted, the two moments were 100° or more apart for TM2, TM4, TM5, TM8 and TM10. These helices are all good candidates for TM segments adjacent to a lipid environment. The remaining TM segments show less clear periodicity and are perhaps more likely to lie towards the centre of a helical bundle. This information, particularly the indication for most helices of a hydrophobic face (which is likely to face lipid), allows the crude topological model to be refined.

### Conclusions

The results presented here provide the first investigation of the structure of a member of the MCT transporter family, and provide strong evidence for the model shown in Figure 1. This model will facilitate the design of experiments to investigate further the structure and function of MCT1. Identification of conserved and hydrophobic faces of TM segments will aid in deducing how helices pack together in the membrane and hence in the development of structural models. The identification of conserved residues and motifs will provide impetus for judicious use of site-directed mutagenesis to investigate structure and function. Finally, we have characterized a series of proteolytic fragments of MCT1. This will be invaluable in investigating the sites of labelling by specific ligands (e.g. stilbene disulphonates) because it should allow assignment of the labels to specific proteolytic fragments.

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