N-Terminal protein sequence analysis of the rabbit erythrocyte lactate transporter suggests identity with the cloned monocarboxylate transport protein MCT1

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An improved purification for the rabbit erythrocyte lactate transporter, using aminoethyl–Sephadex chromatography, is described. The process of purification of the 40–50 kDa transporter, labelled with 4,4′-diisothiocyanostilbene-2,2′-disulphonate (DIDS), was followed by Western blotting with anti-DIDS antibodies [Poole, R. C. and Halestrap, A. P. (1992) *Biochem. J.* **283**, 855–862]. Fractions highly-enriched in transporter were further purified by SDS/PAGE and the 40–50 kDa DIDS-labelled polypeptide was subjected to N-terminal protein sequencing. This analysis identified the first 16 amino acids of

the protein. With the exception of one conservative substitution, this protein sequence is identical to the N-terminal protein sequence predicted from a cDNA isolated from Chinese hamster ovary cells that encode a monocarboxylate transporter, MCT1 [Kim Garcia, C., Goldstein, J. L., Pathak, R. K., Anderson, R. G. W. and Brown, M. S. (1994) *Cell* **76**, 865–873]. This observation, along with similarities in functional properties, leads us to conclude that lactate transport in rabbit erythrocytes is mediated by the MCT1 monocarboxylate transporter isoform.

INTRODUCTION

The transport of lactate and other monocarboxylic acids such as pyruvate, β -hydroxybutyrate and acetoacetate across the plasma membrane is of major physiological importance to most mammalian cells. Indeed, almost all cells possess a H^+ -monocarboxylate cotransporter which is responsible for this process (for a recent review, see Poole and Halestrap, 1993). Monocarboxylate transport has been studied most extensively in erythrocytes, where it is stereoselective for L- over D-lactate, and sensitive to inhibition by derivatives of α -cyanocinnamate, organomercurial thiol reagents, and some stilbene disulphonates (Halestrap, 1976; Deuticke et al., 1978; Poole and Halestrap, 1991). Other cell types possess similar cyanocinnamate-sensitive proton-linked monocarboxylate transporters, and there is increasing evidence for a family of such carriers with different substrate and inhibitor selectivities (Poole and Halestrap, 1993).

Attempts to identify the protein responsible for transport of lactate in erythrocytes have used approaches based upon either chemical labelling or solubilization, fractionation and functional reconstitution (Jennings and Adams-Lackey, 1982; Poole and Halestrap, 1988, 1992). Jennings and Adams-Lackey (1982) were the first to demonstrate irreversible inhibition of lactate transport by 4,4′-diisothiocyanodihydrostilbene-2,2′-disulphonate (H_2 DIDS) and labelling of a protein of 40–50 kDa in rabbit erythrocytes by 3H_2 DIDS in parallel with inhibition of transport. However, the specificity of this labelling was difficult to assess (see Poole and Halestrap, 1992). More recently we demonstrated that both the stilbene disulphonate 4,4′-diisothiocyanostilbene-2,2′-disulphonate (DIDS) and its dihydro-derivative H_2 DIDS are affinity labels for the transporter (Poole and Halestrap, 1991). Subsequently we were able to demonstrate DIDS-labelling of proteins in the range 35–50 kDa in erythrocytes from different

mammalian species, in a manner than correlated with their capacity to transport lactate (Poole and Halestrap, 1992). Furthermore, inhibitors [e.g. α -cyano-4-hydroxycinnamate (CHC)] and substrates of the transporter protected against labelling of the rabbit and rat proteins by DIDS in precisely the manner expected. Only those stilbene disulphonates which were potent inhibitors of lactate transport were effective inhibitors of the labelling of the rabbit 40–50 kDa protein (Poole and Halestrap, 1992). Additionally, fractionation of monocarboxylate transport activity from rat erythrocytes, monitored after reconstitution into lipid vesicles, revealed copurification of activity with the DIDS-labelled protein (Poole and Halestrap, 1992).

Recently the cDNA encoding a mutant protein (MEV) which transports mevalonic acid (a 6-carbon monocarboxylate) has been cloned from a Chinese hamster ovary (CHO) cell variant (Kim et al., 1992). This protein has 494 amino acids and 12 putative transmembrane segments, consistent with a transport function, and migrates as a band of apparent molecular mass 43 kDa on SDS/PAGE analysis. It was found that normal CHO cells express an almost identical protein, but with the Phe replacing Cys at position 360 in the polypeptide. Thus, the mutation present in MEV results in a gain of mevalonate transport activity. Northern blots demonstrated that this or related transcripts are widely expressed in mammalian tissues, including heart, liver and spleen. The wild-type protein had no known function, but it was suggested that it might be responsible for transport of a compound similar to mevalonate (Kim et al., 1992). The transport of mevalonate by the CHO cell variant was also inhibited by DIDS, in the same concentration range that inhibits lactate transport (Faust and Krieger, 1987). In the present work we present an improved purification of the rabbit erythrocyte lactate transporter and N-terminal protein sequence of the DIDS-labelled carrier. These data show that lactate

Abbreviations used: DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulphonate; H_2 DIDS, 4,4′-diisothiocyanodihydrostilbene-2,2′-disulphonate; DBDS, 4,4′-dibenzamidostilbene-2,2′-disulphonate; DNDS, 4,4′-dinitrostilbene-2,2′-disulphonate; CHC, α -cyano-4-hydroxycinnamate; MEGA-10, decanoyl-N-methyl glucamide; $C_{12}E_8$, octaethyleneglycol monododecyl ether; PMSF, phenylmethylsulphonyl fluoride; CMC, critical micelle concentration; CHO, Chinese hamster ovary; ECL, enhanced chemiluminescence.

transport in erythrocytes is almost certainly mediated by the wild-type form of MEV. After completion of our studies, Kim Garcia et al. (1994) reported that expression of the wild-type form of MEV in a human breast carcinoma cell line does indeed lead to enhanced transport of lactate and other monocarboxylates, and named the carrier MCT1 (monocarboxylate transporter 1).

MATERIALS

All chemicals, biochemicals and radiochemicals were obtained from the sources given previously (Poole and Halestrap, 1991, 1992), with the following additions. The detergent octaethylene-glycol monododecylether ($C_{12}E_8$) was obtained from Fluka, Glossop, Derbyshire, U.K. and mevalonic lactone from Aldrich Chemical Co., Gillingham, Dorset, U.K. Solutions of sodium mevalonate (1 M) were prepared by alkaline hydrolysis of the lactone (Brown et al., 1978), as follows. Mevalonic lactone was dissolved in water and treated with a 0.2 molar excess of NaOH at 50 °C for 60 min, adjusted to pH 8.0 prior to storage at -20 °C. Purified *N*-glycanase F was obtained from Oxford Glycosystems. Aminoethyl-Sephadex was prepared from cyanogen bromide-activated Sepharose 4B (Sigma Chemical Co., Poole, Dorset, U.K.) as described by Casey et al. (1989).

METHODS

Purification of the DIDS-labelled lactate transporter from rabbit erythrocyte membranes

Washed rabbit erythrocytes, in 78 mM Na-citrate, 15 mM Hepes, 1 mM EGTA at pH 7.4 and 10% haematocrit were treated with 100 μ M DIDS at 37 °C for 60 min. This results in approx. 70% irreversible inhibition of lactate transport (Poole and Halestrap, 1991). The cells were washed free of reversibly bound inhibitor in citrate buffer +0.5% (w/v) BSA before preparation of membranes by hypotonic lysis and storage at -70 °C (Poole and Halestrap, 1992). All subsequent steps were performed at 4-7 °C in a buffer (pH 7.4) containing 20 mM Mops, 1 mM EGTA, 0.2 mM dithiothreitol and 1 μ g/ml of each of the proteinase inhibitors pepstatin, antipain and leupeptin. In most experiments the buffer also included 1 mM Na-pyruvate. Erythrocyte membranes were thawed and sedimented by centrifugation at 30000 *g* for 10 min at 4 °C, and stripped of peripheral membrane proteins by treatment with approx. 10 volumes of ice-cold reconstitution buffer containing 1 M KI and 0.5 mM phenyl-methylsulphonyl fluoride (PMSF). The stripped membranes were collected by centrifugation at 30000 *g* for 15 min at 4 °C, and washed once more in buffer. The membranes were then treated with approx. 5 volumes of buffer containing 0.05% (w/v) $C_{12}E_8$, a treatment which is known to extract sialoglycoproteins selectively (Yu et al., 1973). The membranes were sedimented and washed once more, before solubilization at a protein concentration of 2 mg/ml with 1% (w/v) $C_{12}E_8$, in the presence of 1 mM PMSF. After centrifugation at 30000 *g* for 30 min at 4 °C, the supernatant was applied to an aminoethyl-Sephadex column equilibrated with buffer containing 0.1% $C_{12}E_8$, attached to a Pharmacia f.p.l.c. system. After removal of non-bound protein, elution was performed by increasing the concentration of NaCl in the buffer. The DIDS-labelled 40-50 kDa protein was followed through purification by Western blotting of fractions with an anti-DIDS antibody, using the procedures described previously to reduce the background immunoreactivity (Poole and Halestrap, 1992). Immunoblots were developed using the enhanced

chemiluminescence system (ECL, Amersham International p.l.c., U.K.). The peak fractions containing the DIDS-labelled transporter were concentrated, usually by chromatography on a Mono-Q HR5/5 ion-exchange column equilibrated with buffer containing 0.2% $C_{12}E_8$. The protein was eluted with a steep 0-1.0 M NaCl gradient, and desalted by passage through a PD-10 gel filtration column (Pharmacia) equilibrated with 0.1% $C_{12}E_8$. If further concentration was required, this was performed by centrifugal filtration using a Centricon 10 (Amicon). The concentrated protein was stored at -20 °C until use.

Preparation of the lactate transporter for N-terminal microsequencing

A sample of the purified 40-50 kDa DIDS-labelled transporter (approx. 100 μ g) was treated with 60 Oxford Glycosystems units of *N*-glycanase F for approx. 3 h at 37 °C, and subjected to SDS/PAGE on a 10% (w/v) polyacrylamide gel. The protein was electroblotted onto a ProBlot membrane (Applied Biosystems) and stained with Serva Blue G. The broad 40-50 kDa band, which stained poorly with this dye, was excised as upper and lower halves and each was subjected to N-terminal microsequencing using an Applied Biosystems 477A pulsed liquid protein sequencer.

Measurement of monocarboxylate transport

Zero-*trans* uptake of 0.5 mM [U - 14 C]-L-lactate into erythrocytes at 7 °C was measured by a centrifuge-stop technique as described previously (Poole and Halestrap, 1991). Transport was also measured by following the proton fluxes which accompany the uptake of monocarboxylates on the transporter, using an external pH electrode and a microcomputer for data acquisition as described previously (Poole and Halestrap, 1991).

Analytical techniques

Protein was assayed by the dye-binding method of Bradford (1976), using BSA as standard. SDS/PAGE was performed by the method of Laemmli (1970), with silver staining by the method of Nielsen and Brown (1984).

RESULTS

The lactate transporter was purified from rabbit erythrocytes, since immunoblots with anti-DIDS antibodies reveal particularly high levels of the labelled protein in these cells (Poole and Halestrap, 1992). It is much easier to follow purification of the labelled protein than to follow reconstituted transport activity, as we found that this can be very labile during fractionation. A modified procedure for purification of the rabbit erythrocyte lactate transporter was developed for several reasons. Firstly, the Mono-Q ion-exchange fractionation that was successful for partial purification of the rat erythrocyte transporter failed to separate the rabbit DIDS-labelled protein from the major solubilized protein, band 3 (results not shown). Secondly, this column bound much of the protein so tightly that elution proved difficult. Finally, it is known that, at least for band 3, there is a tendency for aggregation to occur in detergents with a high critical micelle concentration (CMC), such as the decanoyl-*N*-methyl glucamide (MEGA-10) employed during this procedure (Casey and Reithmeier, 1993). We therefore used a weaker anion-exchange column (aminoethyl-Sephadex) and the detergent $C_{12}E_8$ which has a low CMC (approx. 0.06 mM). In Figure 1 we show the elution profile for such a chromatographic

Figure 1 Aminoethyl-Sephadex chromatography of rabbit erythrocyte membrane proteins

Rabbit erythrocyte membranes were pre-extracted and solubilized as described in Methods, loaded onto an aminoethyl-Sephadex column (18 ml bed volume) and recycled overnight. The non-bound protein was removed by washing until a constant A_{280} was achieved, before elution of protein by applying a gradient of increasing $[NaCl]$ in the buffer, as indicated. Fractions of 4 ml were collected, and elution of protein was monitored as A_{280} (a). In (b) and (c) SDS/PAGE separations of unfractionated solubilized protein (U) and fractions derived from the chromatography represented in panel (a) are shown. In (b) the gel was loaded with 2 μ g of unfractionated solubilized protein, and 5 μ l of the fractions as indicated, and was stained for protein (with silver). In (c) an immunoblot with anti-DIDS antibodies is shown for a gel loaded with 4 μ g of unfractionated solubilized protein and 12 μ l of the eluted fractions. The bands at 50–60 kDa present in all fractions are non-specific and not dependent upon DIDS-labelling (see Results section).

fractionation, along with SDS/PAGE separations stained for protein and an immunoblot probed with anti-DIDS antibodies. On immunoblots with anti-DIDS antibodies, band 3 and the lactate transporter are revealed as the major DIDS-labelled proteins (Poole and Halestrap, 1992). There was frequently non-specific cross-reactivity of these antibodies, which resulted in the appearance of bands at approx. 50–60 kDa, as in Figure 1c. Such bands were even found in tracks loaded with molecular mass standards, and thus appear to be independent of DIDS-labelling. We have demonstrated previously that DIDS labelling of the proteins of 35–50 kDa from rabbit and rabbit erythrocytes is sensitive to substrates and inhibitors of lactate transport, and that the DIDS-labelled protein of this molecular mass from rat erythrocytes co-fractionates with transport activity (Poole and Halestrap, 1992). The data of Figure 1b show clearly that a 40–50 kDa polypeptide was eluted before band 3. Densitometric scanning suggested that this polypeptide constituted approximately 50% of total protein in fraction 17 for the experiment shown in Figure 1. The migration and elution of this protein corresponded precisely with that observed for the DIDS-labelled lactate transporter (Figure 1c). Thus, this procedure results in effective purification of the transporter. Densitometric scanning of the ECL-developed Western blots (Figure 1c) demonstrated that there was an approx. 5-fold enrichment of the transporter by aminoethyl-Sephadex chromatography. An additional purification of at least 2-fold would be expected in the earlier stages, where peripheral proteins were removed from the membranes and $C_{12}E_8$ was used to extract sialoglycoproteins. When these considerations are taken into account, it can be estimated that the present procedure results in 10-fold purification of the protein from rabbit erythrocyte membranes, and that the transporter constitutes about 5% of membrane protein. Whilst this latter figure is likely to be a slight overestimate, it is in reasonable agreement with the value (2.25%) estimated by Jennings and Adams-Lackey (1982) on the basis of labelling with $[^3H]$ -H₂DIDS.

The DIDS-labelled protein migrates as a diffuse band on SDS/PAGE. This could be due to heterogeneous glycosylation, although treatment of the protein with *N*-glycanase F, under conditions we have found to deglycosylate other proteins, resulted in no change in electrophoretic mobility on immunoblots of SDS/PAGE separations (results not shown). This implies that the lactate transporter is either not glycosylated or that steric factors may have prevented its deglycosylation by the enzyme. Nevertheless, the 40–50 kDa protein, concentrated from the most purified fractions, was treated with *N*-glycanase F in order to remove any glycosylated minor contaminant that might underlie the 40–50 kDa band. The sample was further purified by SDS/PAGE to remove contaminating proteins of approx. 20 kDa and 30 kDa (Figure 1b), and transferred to ProBlot for sequencing. Since the transporter spans a large range of molecular mass and might contain minor contaminating species, the upper and lower halves of the 40–50 kDa band were each subjected to protein sequencing. The upper band yielded a single sequence. This was also the major sequence obtained from the lower band, which also contained lower levels of ill-defined sequence(s). The absence of appreciable contaminating sequence(s) confirms the high level of purity of the 40–50 kDa transporter after gel purification. Analysis of the data gave a very clear sequence for 16 rounds of the Edman degradation (Figure 2). The N-terminal amino acid sequence predicted from the cDNA encoding MCT1, cloned by Kim et al. (1992), is shown for comparison. These two sequences are identical, except for the loss of the initiator methionine in the lactate transporter protein sequence, and the conservative change of Ile to Val at position 4 in the lactate transporter sequence. It should be noted that the sequences are

PPAVGGPVGYTTPDGG . . Rabbit erythrocyte lactate transporter

MPPAIGGPVGYTTPDGG . . Hamster MCT1

Figure 2 Alignment of the N-terminal amino acid sequence obtained from purified rabbit erythrocyte lactate transporter with the deduced amino acid sequence for hamster MCT1

N-terminal microsequencing of the rabbit erythrocyte lactate transporter was performed as described in Methods. The N-terminal protein sequence for hamster MCT1 is derived from the cDNA sequence; Kim et al. (1992).

Figure 3 Mevalonate transport mediated by the rabbit erythrocyte lactate transporter

Monocarboxylate transport was measured by following the proton fluxes accompanying transport (extracellular alkalization) with constant stirring in a water-jacketed container at 37 °C. These measurements were performed with 4 ml of rabbit erythrocytes at 10% haematocrit in a lightly buffered citrate medium (84 mM Na-citrate/1 mM EGTA, pH 7.4) containing 5 µM DIDS and 100 µM acetazolamide to inhibit band-3-mediated pH changes. The inhibitors CHC (2 mM), DBDS (0.2 mM) and DNDS (0.2 mM) were added to the cell suspension for approx. 5 min before addition of 10 mM Na-mevalonate or L-lactate at the time indicated. The data shown are means of two traces in each case.

derived from different species; the lactate transporter protein sequence from rabbit and MCT1 from hamster (CHO cells).

MEV encodes a mutant protein which has gained the ability to transport mevalonate, due to a Phe to Cys substitution at position 360, whilst MCT1 transports mevalonate very poorly if at all (Kim et al., 1992; Kim Garcia et al., 1994). As shown below, mevalonate is related structurally to lactate, being an hydroxyl-substituted aliphatic monocarboxylate.

Work from this and other laboratories has demonstrated that the lactate transporter will accept a wide range of substituted aliphatic monocarboxylates up to C-5 or C-6, although the larger compounds and those with branched hydrocarbon chains are frequently less effective substrates (Deuticke, 1982; Poole et al.,

1990; Poole and Halestrap, 1993). The structure of mevalonate suggested that it may be a substrate for the lactate transporter, but that it is unlikely to be transported very efficiently. This was investigated in two ways: firstly by using mevalonate as a competitive inhibitor of L-lactate transport, and secondly by measuring inhibitor-sensitive proton fluxes induced by mevalonate. Mevalonate caused little inhibition of L-lactate transport at concentrations up to 30 mM, indicating a very low (if any) affinity for the transporter (data not shown). In order to measure transport of mevalonate itself, with high sensitivity, mevalonate-induced proton fluxes were measured at 37 °C, a temperature where lactate transport is too fast to resolve (see Figure 3). In these experiments uptake of mevalonate with a proton results in extracellular alkalization. This would also occur if mevalonate diffuses across the membrane as the free acid, a process which occurs to a greater or lesser extent with all monocarboxylates. However, transport on the lactate transporter, unlike diffusion, is sensitive to inhibition by derivatives of cyanocinnamate and to some stilbene disulphonates including 4,4'-dibenzamidostilbene-2,2'-disulphonate (DBDS). In contrast others such as 4,4'-dinitrostilbene-2,2'-disulphonate (DNDS) are much less effective (Poole and Halestrap, 1991). The data in Figure 3 demonstrate that addition of mevalonate induces proton fluxes, and that these were inhibited considerably by 2 mM CHC and to a similar extent by 0.2 mM DBDS, whilst the same concentration of DNDS gave very little inhibition. These observations provide strong evidence that mevalonate is a substrate, albeit very ineffective, for the erythrocyte lactate transporter. It is difficult to assess the difference between transport rates for L-lactate and mevalonate, since lactate transport is too fast to measure under these conditions. However, an estimate can be made. Transport of 10 mM L-lactate in the presence of 2 mM CHC (calculated assuming a similar K_i value to that for rat erythrocytes to give approx. 93 % inhibition) was 50-fold faster than that of 10 mM mevalonate. Thus it can be estimated that at this concentration mevalonate transport is approx. 700-fold slower than that of L-lactate on the transporter.

DISCUSSION

The data we have presented here strongly suggest that the lactate carrier of rabbit erythrocytes is the rabbit equivalent of MCT1. The MCT1 protein has 12 putative transmembrane segments, with a large hydrophilic loop predicted between transmembrane segments 6 and 7. This topology is similar to that proposed for many sugar transporters (Griffith et al., 1992; Gould and Holman, 1993), but there is no homology between these proteins at the sequence level (Kim et al., 1992). On a search of the sequence databases (March 1994) the best overall sequence similarity we were able to find was with the ProP proline/betaine transporter from *Escherichia coli* (Culham et al., 1993), for which there is 16.4 % identity in a 390 amino acid overlap. This protein is proposed to have a similar membrane topology to that predicted for MEV. There were also limited regions of sequence similarity with some transporters, including some of the Na⁺- and Cl⁻-dependent neurotransmitter transporter family. The highest similarities are for the taurine (Jhiang et al., 1993) and GABA (Borden et al., 1992) transporters, for which there is approx. 30 % identity for a 62 amino acid overlap towards the N-terminus of these proteins.

There is additional information which strengthens the association of MCT1 with plasma membrane lactate transport in erythrocytes. Firstly, MCT1, like the erythrocyte lactate transporter, has an apparent molecular mass of 43 kDa on SDS/PAGE analysis. This higher-than-predicted mobility for a protein

of 494 amino acids is common for hydrophobic membrane proteins (Viitanen et al., 1986). Secondly, transport of pyruvate by MCT1 is sensitive to inhibition by inhibitors such as DIDS and derivatives of cyanocinnamate (Kim Garcia et al., 1994). Finally, Northern blots also revealed expression of MEV/MCT1-related RNA in spleen, a major erythropoietic tissue in rodents (Kim et al., 1992).

MEV, a mutant protein which transports mevalonate, is identical to MCT1 with the exception of a Phe to Cys substitution at position 360. This mutation appears to have a profound effect on substrate and inhibitor selectivity, since MEV transports pyruvate poorly if at all, and is much less sensitive to inhibition by derivatives of cyanocinnamate (Kim Garcia et al., 1994). It is not difficult to imagine that the substitution of a Phe with the less bulky Cys at position 360 might allow much more effective binding of the bulky mevalonate. It is of note that the apparent K_m for mevalonate transport by mammalian cells transfected with MEV is within the range found for many substrates of lactate transporters (estimates of 2–15 mM; Kim et al., 1992; Kim Garcia et al., 1994). However, transfection of MCT1/MEV-deficient breast carcinoma cells with MEV results in very much lower rates of transport of mevalonate than of pyruvate into the same cells transfected with MCT1. It would therefore appear that MEV has a rather low k_{cat} for mevalonate transport, presumably due to a low rate constant for translocation of substrate-loaded and/or -unloaded carrier (see Poole and Halestrap, 1993). It is perhaps not surprising that Kim Garcia et al. (1994) did not observe mevalonate transport mediated by MCT1 since they used a relatively insensitive technique in their studies. It should be noted that the inhibitor-sensitivity of mevalonate transport into erythrocytes appears to correspond to that of MCT1, not that of MEV.

The identification of the erythrocyte lactate transporter as MCT1 will aid future studies, since there is a wealth of information concerning the properties of the lactate transporter in these cells (kinetics, inhibition, chemical labelling) that will form a sound background for future studies of the relationship between the structure and function of MCT1, by heterologous expression of normal and mutant proteins. The erythrocyte also offers an ideal experimental system for certain studies to investigate topology of membrane proteins, since it is relatively easy to prepare sealed, leaky and inside-out preparations of membranes

to perform vectorial proteolysis and antibody binding. These studies, along with those of the near-purified protein which can now be prepared with ease, can be interpreted much more readily within the context of the sequence of MCT1 which encodes this protein.

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