Substrate-specificity of glutamine transporters in membrane vesicles from rat liver and skeletal muscle investigated using amino acid analogues

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We investigated the effects of glutamine and histidine analogues on glutamine transport processes in membrane vesicles prepared from rat liver (sinusoidal membrane) and skeletal muscle (sarcolemma). L-[¹⁴C]Glutamine is transported in these membranes predominantly by Systems N/N^m (liver and muscle respectively), and to a lesser extent by Systems A and L (e.g. about 60, 20 and 20% of total flux respectively via Systems N, A and L at 0.05 mM-glutamine in liver membrane vesicles). The glutamine anti-metabolites 6-diazo-5-oxo-L-norleucine and acivicin were relatively poor inhibitors of glutamine uptake into liver membrane vesicles (< 25% inhibition at 20-fold excess) and appeared primarily to inhibit System A activity (i.e. *N*-methylaminoisobutyric acid-inhibitable glutamine uptake). In similar experiments azaserine (also a glutamine anti-metabolite) inhibited approx. 50% of glutamine uptake, apparently by inhibition of System A and also of System L (i.e. 2-amino-2-carboxybicyclo[2,2,1]heptane-inhibitable glutamine uptake). Glutamate γ -hydroxamate, aspartate β -hydroxamate, histidine and *N*^m-methylhistidine were all strong inhibitors of glutamine uptake into liver membrane vesicles (> 65% inhibition at 20-fold excess), but neither homoglutamine nor *N*^m-methylhistidine produced inhibition. L-Glutamate- γ -hydroxamate was shown to be a competitive inhibitor of glutamine transport via System N (*K*_i ~ 0.6 mM). Glutamine uptake in sarcolemmal vesicles showed a similar general pattern of inhibition as in liver membrane vesicles. The results highlight limits on the substrate tolerance of System N; we suggest that the presence of both an L- α -amino acid group and a nitrogen group with a delocalized lone-pair of electrons (amide or pyrrole type), separated by a specific intramolecular distance (C₂–C₄ chain equivalent), is important for substrate recognition by this transporter.

INTRODUCTION

Glutamine is the most abundant free amino acid in mammalian plasma and in the cytosol of liver and skeletal muscle. It is a major carrier between tissues of α -amino nitrogen, a major fuel of rapidly dividing cells such as intestinal mucosa, lymphocytes and tumour cells, and a nitrogen donor in reactions involved in a variety of cell functions (for reviews see Abumrad *et al.*, 1989; Rennie *et al.*, 1989; Christensen, 1990; Häussinger, 1990). Glutamine is transported against a concentration gradient into liver and skeletal muscle cells by Na⁺-coupled transport mechanisms (Kilberg *et al.*, 1980; Hundal *et al.*, 1987). The principal Na⁺/glutamine transporters in the two cell types have many common features, notably a narrow substrate range (both are restricted to glutamine, asparagine and histidine among the natural amino acids), and are likely to be variants of the same system, denoted System N in liver cells [in which it was first described (Kilberg *et al.*, 1980; Handlogten *et al.*, 1982)] and System N^m in skeletal muscle (Hundal *et al.*, 1987; Ahmed *et al.*, 1990). The three other major pathways for uptake of neutral amino acids in mammalian cells are Systems A, ASC (both Na⁺-coupled) and L (for review, see Christensen & Kilberg, 1987). The substrate-specificity of Systems N/N^m is narrower than that of Systems A, ASC and L, indicating an unusual degree of selectivity at the presumed binding site for substrate amino acids on the transport protein.

The importance of glutamine in tumour metabolism is emphasized by the fact that a variety of glutamine analogues have been tested experimentally as anti-tumorigenic agents (e.g.

Prajda, 1985; Lachance *et al.*, 1987; Huber *et al.*, 1988). It is generally assumed that these chemicals exert their effect within the cell, and possible effects on glutamine transport into cells are frequently overlooked. Indeed, the specific mechanisms by which these analogues enter cells is uncertain, although recent evidence indicates that some are substrates of Systems A and L (Huber *et al.*, 1988; Sastrasin & Sastrasin, 1988; Segel *et al.*, 1989). The glutamine anti-metabolites 6-diazo-5-oxo-L-norleucine (DON) and *O*-diazoacetyl-L-serine (azaserine) are of particular interest, as they contain a potentially photoreactive diazo group in a position corresponding to that of the glutamine amide. These compounds have been used as photoaffinity probes for glutamine-binding enzymes (e.g. Hartman, 1963; Clark *et al.*, 1982), which raises the possibility that they may be used to label membrane-bound glutamine transporters (Segel *et al.*, 1989), whose biochemical purification and molecular characterization may thus be facilitated. This is an attractive possibility, because there is negligible information available on the structure of any amino acid transporter in mammalian cells, although at least thirteen such systems have been discriminated by kinetic criteria (Christensen & Kilberg, 1987).

We have therefore investigated the nature of the inhibition of glutamine transport, in muscle and liver membrane vesicles, by a variety of commercially available glutamine analogues, in order to (1) gain more information on the substrate-specificity of transport systems (i.e. the structural tolerances of their amino-acid-binding sites), and (2) identify potential covalent ligands for glutamine transporters.

Abbreviations used: MeAIB, *N*-methylaminoisobutyric acid; BCH, 2-amino-2-carboxybicyclo[2,2,1]heptane; DON, 6-diazo-5-oxo-L-norleucine; azaserine, *O*-diazoacetyl-L-serine; acivicin, α -amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid; Cho, choline.

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EXPERIMENTAL

Materials

L-[14 C]Glutamine (specific radioactivity ~ 10 GBq/mmol) was obtained from Amersham International (Amersham, Bucks., U.K.). All other chemicals, including glutamine and its analogues, were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.) unless stated.

Animals and dissection procedures

Tissue (liver, hindlimb muscle) was obtained from ~ 200 g female Wistar rats (Bantin & Kingman, Hull, U.K.) under terminal pentobarbitone anaesthesia (60 mg/kg body weight, intraperitoneal; Sagatal; May & Baker). Overnight-fasted rats were used unless otherwise specified.

Preparation of membrane vesicles

(a) Liver sinusoidal membrane. Excised liver was homogenized in 0.25 M-sucrose buffer (0.01 M-EDTA, 5 mM-Tris/Hepes pH 7.6) using a motorized Teflon-glass homogenizer (15 strokes). Liver homogenate was fractionated by a method based on that described by Wisher & Evans (1975). Nuclear debris and mitochondria were removed by differential centrifugation (2000 g for 10 min and 35000 g for 10 min respectively), and then the crude membranes were pelleted from the supernatant (85000 g_{av} , 1 h). The membrane pellet was resuspended in 51% sucrose buffer using a tight-fitting Dounce homogenizer (12 strokes), applied to a discontinuous sucrose-density gradient [10, 26, 34 and 51% (w/w) sucrose], and centrifuged for 2.5 h at 39000 rev./min using a Kontron TFT 50.38 rotor (140000 g_{av}). Sinusoidal membranes were obtained from the 26/34% sucrose interface, washed by dilution in buffer and pelleted (190000 g_{av} , 1 h). Membranes were resuspended in buffer (0.4 M-sucrose, 5 mM-MgCl₂, 0.2 mM-CaCl₂, 10 mM-Hepes, 5 mM-Tris, pH 7.4) using a glass/glass homogenizer to a final concentration of 1 mg of membrane protein/ml.

(b) Skeletal muscle sarcolemma. Sarcolemma was obtained from dissected hindlimb muscle by differential and sucrose-density-gradient centrifugation using methods described previously (Ahmed *et al.*, 1990). The isolated sarcolemma was resuspended in buffer as described above for liver membrane.

Assessment of membrane yield and purity

Protein was assayed by the bicinchoninic acid method (Smith *et al.*, 1985). Membranes were examined by transmission electron microscopy (Ahmed *et al.*, 1990) and membrane purity was assessed by assay of marker enzyme activities: K⁺-stimulated phosphatase (*p*-nitrophenyl phosphate substrate), glucose-6-phosphatase, Ca²⁺-stimulated ATPase, Na⁺/K⁺-stimulated ATPase and succinate dehydrogenase activities were measured by standard methods as described previously (Ahmed *et al.*, 1990).

Measurement of glutamine transport in membrane vesicles

L-[14 C]Glutamine uptake into vesicles was measured using a rapid gel-filtration method (Penefsky, 1977; Ahmed *et al.*, 1990). Individual experiments were performed in 1.5 ml microcentrifuge tubes. Uptake was initiated by mixing 20 μ l of vesicle suspension with an equal volume of double-strength transport buffer containing radiotracer; each reaction tube contained ~ 20 μ g of membrane protein/1.5 kBq of radioactivity in 40 μ l of buffer (pH 7.4) containing 100 mM-NaCl or choline chloride (ChoCl), 5 mM-MgCl₂, 0.2 mM-CaCl₂, 10 mM-Hepes and 5 mM-Tris (final concentrations). The osmolality of buffers used in uptake experiments was about 375 mosmol/kg (Gonotec Osmomat 030). Uptakes were terminated by quenching with 0.4 ml of ice-cold

stopping buffer and the sample was applied to a Sephadex-G50 column (NICK columns; Pharmacia) which retained extra-vesicular radioactivity but allowed free passage of vesicles. The eluate containing the vesicles was mixed with scintillation fluor (Ready-Value; Beckman) and assayed for radioactivity by liquid scintillation counting using a Beckman LS1800 scintillation counter.

Metabolism of [14 C]glutamine during the short period of an uptake experiment was negligible ($< 2\%$ of total tracer), as assessed by the failure to detect radioactive products using ion-exchange chromatography (Low *et al.*, 1990).

Data analysis

Results are presented as means $\pm$ S.E.M. for n vesicle preparations. Experiments were performed in triplicate; the coefficient of variation of triplicate samples was $\sim 6\%$. Statistical analysis was performed using Student's t test. The kinetic characteristics of membrane transport processes were estimated from lines fitted by least squares to linear-transformed data (Hane's plot) using commercial software on an Apple IIe microcomputer (Barlow, 1982).

RESULTS

Yield and purity of liver and sarcolemmal membrane vesicles

Liver sinusoidal membranes and hindlimb sarcolemma were prepared with a 15–25-fold enrichment in the activity of plasma membrane marker enzymes, but with substantial relative depletion in activities of marker enzymes for other membranes (Table 1). Transmission electron microscopy revealed that recovered membranes were predominantly in the form of vesicles (results not shown).

Glutamine transport in membrane vesicles

The time course for radiolabelled L-glutamine uptake into liver sinusoidal membranes displayed an overshoot in NaCl transport buffer which is characteristic of ion-gradient-coupled transport processes in membrane vesicles (Fig. 1). The initial rate was estimated from 30 s of uptake. We have shown previously (Ahmed *et al.*, 1990) that the initial rate of glutamine uptake into sarcolemmal vesicles (estimated from 45 s of uptake) is also stimulated by an inwardly-directed NaCl gradient. Glutamine uptake was stimulated by Li⁺ to about the same extent as by Na⁺ in membrane vesicles from both liver (results not shown) and skeletal muscle (Ahmed *et al.*, 1990), indicating that Na⁺ could be replaced by Li⁺ as the cation that is co-transported with glutamine. We also demonstrated that glutamine uptake into liver vesicles was saturable, stereospecific and strongly inhibited by histidine (Table 2). The identification of Li⁺-supportable, histidine-sensitive, Na⁺/glutamine transport as a major route for glutamine entry into liver vesicles is consistent with previous work proposing this uptake component to be due to activity of the System N transporter (Kilberg *et al.*, 1980; Jacob *et al.*, 1986).

Glutamine is reported to be transported across liver cell membranes by Systems N, A and L in the rat (Handlogten *et al.*, 1982) and man (Mailliard & Kilberg, 1990). The System A specific inhibitor *N*-methylaminoisobutyric acid (MeAIB) shows concentration-dependent inhibition of transport of 0.05 mM-glutamine in liver vesicles (Table 2), with a maximum inhibition of about 16% in membrane vesicles isolated from post-absorptive rats and a MeAIB concentration for half-maximal inhibition (K_i) of the order of 0.1 mM (Table 2). MeAIB inhibition of glutamine transport increased to 27% if rats had been fasted for 60 h (Table 2), under which conditions total glutamine uptake was virtually doubled from 0.372 ± 0.027 nmol/min per mg (12 h-

Table 1. Yield and purification indices for plasma membrane fractions isolated from rat liver and skeletal muscle

Marker enzymes used were: plasma membrane, K⁺-stimulated phosphatase (muscle), Na⁺/K⁺-stimulated ATPase (liver); reticular membrane, Ca²⁺-stimulated ATPase (muscle), glucose-6-phosphatase (liver); mitochondrial membrane, succinate dehydrogenase. N.D., enzyme activity not detected in plasma membrane fraction.

Fraction	Protein yield (mg/g of tissue wet wt.)	Purification indices of marker enzymes		
		Plasma membrane	Reticular membrane	Mitochondrial membrane
Sarcolemma (skeletal muscle)	0.1 ± 0.005	15 ± 1.2	0.03 ± 0.004	N.D.
Sinusoidal membrane (liver)	0.95 ± 0.05	25 ± 6	2.1 ± 0.4	N.D.

Fig. 1. Time course of L-glutamine uptake by sinusoidal membrane vesicles from rat liver

Membrane vesicles were prepared from livers of 12 h-fasted rats. Uptake was initiated by addition of vesicle suspension (~ 20 µg of protein in 20 µl of 0.25 M-sucrose buffer) to 20 µl of double-strength transport buffer containing L-[¹⁴C]glutamine. Uptake was terminated at the times shown by dilution of the reaction mixture with 0.4 ml of ice-cold stopping buffer. The transport buffer included 0.05 mM-L-glutamine and 100 mM-NaCl (●) or ChoCl (▲). The results are mean values ± S.E.M. for uptake experiments performed with four different vesicle preparations.

Fig. 2. Effects of glutamine analogues (azaserine, DON, acivicin, L-glutamate γ-hydroxamate, S-carbamyl-L-cysteine) on L-glutamine uptake in liver sinusoidal-membrane vesicles

Membrane vesicles were prepared from livers of 60 h-fasted rats in order to increase the relative proportion of glutamine transport via System A. Uptake was measured over 30 s and the transport buffer included 0.05 mM-L-[¹⁴C]glutamine, 100 mM-NaCl and 1 mM of any test substance (analogue ± MeAIB and/or BCH); the test substances were omitted in parallel-running controls. The results are presented as percentage inhibition of control uptake (mean ± S.E.M. for uptake experiments performed with 3-4 different vesicle preparations). □, Analogue only; ■, + 1 mM-MeAIB; ▨, + 1 mM-BCH; ▩, + MeAIB + BCH.

fast, *n* = 9 rats) to 0.732 ± 0.031 nmol/min per mg (60 h-fast, *n* = 7 rats) at 0.05 mM-glutamine in NaCl medium; these findings are consistent with the fact that System A shows a greater degree

Table 2. Effects of amino acids (L-glutamine, D-glutamine, L-histidine) and system-specific transport inhibitors (MeAIB, BCH) on L-glutamine uptake in liver sinusoidal-membrane vesicles

Membrane vesicles were prepared from livers of 12 h-fasted rats unless specified otherwise. Uptake was measured over 30 s and the transport buffer included 0.05 mM-L-[¹⁴C]glutamine, 100 mM-NaCl or -ChoCl and the test substance, which was omitted in parallel-running controls. The results are presented as percentage inhibition of control uptake (means ± S.E.M. for uptake experiments performed with 3-5 different vesicle preparations). Inhibition significantly different from zero: **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

Test substance	Inhibition of 0.05 mM- [¹⁴ C]glutamine transport (%)
100 mM-NaCl medium	
1 mM-L-Glutamine	82 ± 9***
1 mM-D-Glutamine	11 ± 4
1 mM-L-Histidine	71 ± 7***
0.1 mM-MeAIB	7 ± 2*
1 mM-MeAIB	16 ± 3*
10 mM-MeAIB	18 ± 4*
1 mM-MeAIB (60 h-fasted rat)	27 ± 6*
1 mM-L-Leucine	16 ± 3*
100 mM-ChoCl medium	
1 mM-L-Glutamine	58 ± 15*
10 mM-L-Glutamine	47 ± 7**
1 mM-D-Glutamine	2 ± 7
1 mM-BCH	38 ± 6**
10 mM-BCH	45 ± 7**
1 mM-L-Leucine	23 ± 5*

of adaptive regulation than other glutamine transporters in liver cell membranes (Kelley & Potter, 1978; Handlogten *et al.*, 1982). The Na⁺-independent uptake of glutamine in liver sinusoidal vesicles (uptake in ChoCl medium) also included a major saturable and stereospecific component which was largely inhibited by the System-L specific inhibitor 2-amino-2-carboxy-bicyclo[2,2,1]heptane (BCH) at 1 mM (Table 2; transport inhibitions at 10 mM-glutamine or -BCH were not significantly different to those at 1 mM). We therefore decided to estimate glutamine influx via Systems A and L in liver membrane vesicles from the percentage inhibitions of 0.05 mM-L-glutamine transport by 1 mM-MeAIB and 1 mM-BCH respectively.

Effects of synthetic amino acid analogues on glutamine transport in liver membrane vesicles

The results summarized in Fig. 2 are from experiments performed using liver membrane vesicles isolated from 60 h-fasted rats. The resultant increase in the relative proportion of glutamine transport via System A enabled the specific effects of analogues on transport Systems A, N and L to be more clearly

Table 3. Effects of amino acid analogues on Na⁺-independent L-glutamine uptake in liver sinusoidal-membrane vesicles

Membrane vesicles were prepared from livers of 12 h-fasted rats. Uptake was measured over 30 s and the transport buffer included 0.05 mM-L-[¹⁴C]glutamine and 100 mM-ChoCl ± 1 mM of a test analogue. Some results are presented as percentage inhibition of glutamine uptake relative to inhibition by 1 mM-BCH in the same preparation (mean relative inhibition ± S.E.M.). Numbers in parentheses refer to total numbers of vesicle preparations. *Uptake significantly different from control ($P < 0.05$).

Test analogue (1 mM)	L-Glutamine uptake (nmol/min per mg of protein)	Transport inhibition relative to 1 mM-BCH (%)
Control	0.064 ± 0.010 (9)	—
BCH	0.037 ± 0.006 (5)*	—
L-DON	0.054 ± 0.005 (3)	38 ± 18 (3)
L-Azaserine	0.036 ± 0.004 (3)*	105 ± 14 (3)
L-Glutamate γ -hydroxamate	0.056 ± 0.005 (3)	34 ± 15 (3)
S-Carbamyl-L-cysteine	0.037 ± 0.005 (3)*	101 ± 16 (3)

distinguished. Glutamine uptake into liver vesicles was inhibited to differing extents by a 20-fold excess of the glutamine anti-metabolites azaserine, DON and acivicin (Fig. 2); azaserine was the most effective transport inhibitor. Experiments in which one of these glutamine anti-metabolites and one or both system-specific transport inhibitors (MeAIB, BCH) were tested simultaneously for their combined effects on glutamine transport in liver vesicles (Fig. 2) revealed that MeAIB, DON and acivicin all inhibited the same small component of glutamine uptake (presumably System A transport). Azaserine inhibition of glutamine transport was equivalent to the combined inhibitions by the paradigm System A and L substrates MeAIB and BCH; simultaneous application of all three analogues did not significantly increase inhibition, from which we infer that azaserine may be inhibiting both Systems A and L, but probably not System N. In a separate experiment (Table 3), we confirmed that azaserine was a strong inhibitor of System L in liver vesicles (equally as effective as BCH in inhibiting glutamine transport in ChoCl medium), whereas DON was a relatively poor inhibitor of this Na⁺-independent transporter.

Table 4. Effects of synthetic amino acid analogues on L-glutamine uptake in liver sinusoidal-membrane vesicles

Membrane vesicles were prepared from livers of 12 h-fasted rats. Uptake was measured over 30 s and the transport buffer included 0.05 mM-L-[¹⁴C]glutamine, 100 mM-NaCl and 1 mM of the test analogue; the analogue was omitted in parallel-running controls. The results are presented as percentage inhibition of control uptake (mean ± S.E.M. for uptake experiments performed with 3–4 different vesicle preparations). Inhibition significantly different from zero: * $P < 0.05$, ** $P < 0.01$.

Test analogue (1 mM)	Inhibition of 0.05 mM-glutamine transport (%)
L-Homoglutamine	10 ± 4
L-Glutamate γ -hydroxamate	65 ± 6**
L-Aspartate β -hydroxamate	50 ± 13*
L-Glutamate γ -hydrazide	7 ± 5
L-Methionine sulphoximine	25 ± 4*
L-Pyroglutamate	0 ± 3
L-Ornithine	1 ± 5
S-Carbamyl-L-cysteine	37 ± 10*
L-Histidinol	18 ± 7
DL-1,2,4-Triazole-3-alanine	1 ± 5
N ⁷ -Methyl-L-histidine†	65 ± 7**
N ⁷ -Methyl-L-histidine‡	3 ± 9

† 1-Methylimidazole-5-alanine.
‡ 1-Methylimidazole-4-alanine.

The effects of several other synthetic amino acid analogues on glutamine transport in liver sinusoidal vesicles are also shown in Fig. 2 and Table 4. L-Glutamate γ -hydroxamate, L-aspartate β -hydroxamate, N⁷-methyl-L-histidine and S-carbamyl-L-cysteine were the most potent transport inhibitors of the analogues tested but, surprisingly, the elongated glutamine homologue L-homoglutamine was a poor inhibitor. The glutamine analogues L-glutamate γ -hydrazide and methionine sulphoximine, and the histidine analogues N⁷-methyl-L-histidine, L-histidinol and 1,2,4-triazole-3-alanine were relatively poor transport inhibitors. The inhibitory effects of L-glutamate γ -hydroxamate, MeAIB and BCH on glutamine transport appeared to be additive (Fig. 2), indicating that the analogue was a relatively poor inhibitor of Systems A and L in liver vesicles, as proven to be the case for

Fig. 3. Effects of L-glutamate γ -hydroxamate (GHX) on the kinetic characteristics of Na⁺-dependent glutamine uptake by rat liver sinusoidal membranes

Membrane vesicles were prepared from livers of 12 h-fasted rats. Uptake was measured over 30 s and the transport buffer included L-[¹⁴C]glutamine, 1 mM-MeAIB and 100 mM-NaCl or 100 mM-ChoCl. Results presented are for MeAIB-insensitive Na⁺-dependent glutamine uptake (uptake in NaCl medium – uptake in ChoCl medium; nmol/min per mg); i.e. transport via System N. Each point is the mean value of 2–3 experiments performed in triplicate. (a) Hanes linear transformation of glutamine transport data; transport buffer included 0.1, 0.5, 1, 2.5 or 5 mM-L-glutamine (▲, control; ■, +0.1 mM-GHX; ●, +2 mM-GHX). Line slopes ($1/V_{max}$) were not significantly different from one another, indicating that GHX inhibition of System N is competitive. Kinetic characteristics for System N transport in control membrane preparations ($n = 3$) are: V_{max} , 3.9 ± 0.9 nmol/min per mg, K_m , 1.7 ± 0.4 mM. (b) Dixon plot of glutamine transport data in the presence of 0, 0.1, 1 or 2 mM-GHX. Glutamine concentrations were 0.1 mM (■), 0.5 mM (▲) and 1 mM (●). The K_i for GHX inhibition of glutamine transport was estimated (from the points of intersection) to be about 0.6 mM.

Table 5. Effects of amino acid analogues on L-glutamine uptake by sarcolemmal vesicles from rat hindlimb muscle

Membrane vesicles were prepared from hindlimb muscle of 12 h-fasted rats. Uptake was measured over 45 s and the transport buffer included 0.005 mM-L-[¹⁴C]glutamine, 100 mM-NaCl and 1 mM of any test substance (which was omitted in parallel-running controls). The results are presented as percentage inhibition of control uptake (means $\pm$ s.e.m. for uptake experiments performed with three different vesicle preparations). Control uptake of L-[¹⁴C]glutamine (45 s initial rate) was 6.5 ± 0.3 pmol/min per mg; 1 mM-L-glutamine inhibited this uptake by $83 \pm 3\%$. Inhibition significantly different from zero: * $P < 0.05$, ** $P < 0.01$.

Test analogue (1 mM)	Inhibition of 0.005 mM-glutamine transport (%)
L-DON	9 $\pm$ 3
L-Azaserine	69 $\pm$ 10*
L-Acivicin	10 $\pm$ 4
L-Homoglutamine	33 $\pm$ 5*
L-Glutamate γ -hydroxamate	89 $\pm$ 8**
S-Carbamyl-L-cysteine	66 $\pm$ 12*
DL-1,2,4-Triazole-3-alanine	10 $\pm$ 5

System L at least (Table 3). S-Carbamyl-L-cysteine appeared to share some inhibition of a glutamine transport component with BCH (Fig. 2), and indeed it proved to be a strong inhibitor of System L transport in liver vesicles (Table 3).

Overall, the results indicated that, of the amino acid analogues tested, L-glutamate γ -hydroxamate was the most specific inhibitor of System N. This inhibition appeared to be competitive, as judged from a Hane's analysis of System N transport kinetics for glutamine $\pm$ glutamate γ -hydroxamate (Fig. 3a); the K_i for glutamate γ -hydroxamate inhibition of System N transport was estimated to be about 0.6 mM (Fig. 3b).

Effects of amino acid analogues on glutamine transport in muscle membrane vesicles

At 200-fold excess, L-glutamate γ -hydroxamate, S-carbamyl-L-cysteine and azaserine were all strong inhibitors of glutamine transport in sarcolemmal vesicles (displaying inhibitions of a similar magnitude to that caused by glutamine itself), but homoglutamine, DON, acivicin and 1,2,4-triazole-3-alanine were relatively poor inhibitors (Table 5).

DISCUSSION

A major problem in interpreting data on the inhibition of amino acid transport lies in correctly attributing the fractions of total inhibition to the different transport systems which may be effecting amino acid uptake (Christensen, 1989). We have attempted to interpret our inhibitions in a transport-system-specific manner in order to minimize these problems. Several previous studies have examined the inhibitory effects of glutamine analogues on transport of neutral amino acids in a variety of mammalian cell types (e.g. Kilberg *et al.*, 1980; Sastrasinh & Sastrasinh, 1988; Hundal & Rennie, 1989; Segel *et al.*, 1989), but in most cases it was not known if the analogues tested were inhibiting more than one of the amino acid transporters present on the membrane under investigation, or if the inhibition actually reflected competition between analogue and natural substrate for transport. An important result of the present work is the description of inhibitory effects of glutamine analogues on each of the three transport systems identified as glutamine carriers in the sinusoidal membranes of rat liver (Systems A, N and L). The

proportion of total glutamine transport attributable to each system varies with the prevailing glutamine concentration (a kinetic effect) and with the nutritional state of the animal (due to adaptive regulation of transporter activity). Under normal physiological conditions (which include a plasma glutamine concentration of 0.5–1.0 mM), it appears that for rat liver System N is quantitatively the most important transporter (Handlogten *et al.*, 1982; Fafournoux *et al.*, 1983; Jacob *et al.*, 1986) and System A the least important (accounting for an estimated 70% and 5% of total glutamine influx to liver cells respectively). It is important to note that under the test conditions we adopted in the present study (i.e. measurement of 0.05 mM-glutamine uptake), glutamine flux through System A (a high-affinity, low-capacity, Na⁺/glutamine transporter in rat liver; Handlogten *et al.*, 1982) represents a much greater proportion of the total measured glutamine flux than under physiological conditions.

In the present study, several amino acid analogues were potent inhibitors of glutamine transport in liver and muscle membrane vesicles under the test conditions used (see Fig. 4 for chemical structures of amino acids and analogues). The demonstration of inhibition of transport of one amino acid by the presence of another is not, however, sufficient in itself to indicate sharing of a common carrier. In our present investigation of the inhibitions caused by the glutamine anti-metabolites DON, azaserine and acivicin on glutamine transport, and their relationship to inhibitions shown by paradigm substrates for specific amino acid transporters, we were able to obtain some direct evidence for assignation of inhibitors as putative substrates of particular transporters. In liver membrane vesicles DON, acivicin and azaserine showed distinct patterns of glutamine transport inhibition. DON and acivicin appeared to inhibit System A (as judged by their failure to add to MeAIB inhibition), whereas azaserine appeared to cause a more generalized inhibition of glutamine transport Systems A and L; there was no additional evidence to indicate that it strongly inhibits System N. The results for sarcolemmal vesicles, although not so extensive, show a similar pattern in that azaserine is the most potent of the three anti-metabolites tested in terms of its inhibition of glutamine transport.

Fig. 4. Molecular structures of the principal amino acids and analogues used in this study

The structures depicted are (a) asparagine ($n = 1$), glutamine ($n = 2$), homoglutamine ($n = 3$); (b) 6-diazo-5-oxo-norleucine; (c) acivicin; (d) histidine; (e) N^m-methylhistidine; (f) N^m-methylhistidine. Hydroxamates of amino acids depicted in (a) have the terminal amide $-C(=O)-NH_2$ replaced with $-C(=O)-NH-OH$. S-Carbamylcysteine is derived from (a) by replacement of $-[CH_2]_n-$ with $-CH_2-S-$. Azaserine is derived from (b) by replacement of $-CH_2-CH_2-$ with $-CH_2-O-$. 1,2,4-Triazole-3-alanine is derived from (d) by replacement of the CH group marked $\star$ with N.

The designation System N was given to the high-capacity Na^+ /glutamine transporter of liver cells because nitrogen atoms occur in the side-chains of all of its natural substrates (Kilberg *et al.*, 1980). The fact that histidine shares System N with glutamine and asparagine demonstrates that the carbonyl group *per se* is not necessary for the transporter to recognize its substrate. It has been argued (Kilberg *et al.*, 1980) that the major structural differences between glutamine and histidine might permit large modifications in structural analogues to be tolerated by System N, but the present results demonstrate clearly that this is not the case. We conclude that System N (and probably System N^m also) has highly specific requirements in terms of the chemical structure of its amino acid substrates, because only four out of fifteen structural analogues of glutamine and histidine which we tested (glutamate γ -hydroxamate, N^m -methylhistidine, azaserine and aspartate β -hydroxamate) showed strong (i.e. $\geq 50\%$) inhibition of glutamine transport in liver membrane vesicles, and only glutamate γ -hydroxamate was demonstrably an effective competitive inhibitor of this transporter. It is possible that N^m -methylhistidine and aspartate β -hydroxamate (a homologue of glutamate γ -hydroxamate) may also be useful System N inhibitors, but in the case of the latter analogue our results using vesicles contrast with a previous report using intact hepatocytes (Kilberg *et al.*, 1980), in which aspartate β -hydroxamate was found to stimulate glutamine uptake (although paradoxically, in the same study, glutamate γ -hydroxamate had an inhibitory effect); these apparently conflicting results may reflect the activity of glutamine transport processes on the bile-canalicular membrane, which would contribute to glutamine uptake in hepatocytes but not in sinusoidal membrane vesicles.

Features which appear to be important for recognition of substrate by the System N transporter include the L - α -amino acid group (as D-glutamine, L-pyrroglutamate and L-histidinol all failed to inhibit glutamine transport; see Table 4) and a side-chain containing a carbon-nitrogen bond with a delocalized lone-pair of electrons. The area of lone-pair delocalization does not appear to be critical, as this is spread over three atoms in glutamine and asparagine (the amide $\text{O}-\text{C}-\text{N}$), but over five atoms (the imidazole ring) in histidine. We are therefore in agreement with the general conclusions of Kilberg *et al.* (1980), who pointed out that the electronic resonances permitted by the side-chain groups of glutamine, asparagine and histidine must have 'decisive common features'. One important feature of these resonances is that they lower the basicity of nitrogen atoms in the side chain of System N substrates. As a comparison, ornithine [which differs structurally from glutamine only in that the terminal $-\text{C}(=\text{O})\text{NH}_2$ is replaced by $-\text{CH}_2\text{NH}_2$] possesses a highly basic side-chain nitrogen atom and in consequence is predominantly cationic at physiological pH and shows no interaction with glutamine transport across liver cell membranes (Table 4; Kilberg *et al.*, 1980). More specifically, glutamine, asparagine and histidine all share a resonance structure in which an sp^2 -hybridized nitrogen atom in the side-chain has its lone-pair of electrons delocalized by π -bonding, and the resultant 'pyrrole'-type $\text{N}-\text{H}$ group (e.g. Allinger *et al.*, 1971) may be one of the decisive features for recognition by the System N transporter. The strong inhibition of glutamine transport in liver membrane vesicles by N^m -methylhistidine indicates that substitution of $\text{N}-\text{CH}_3$ for $\text{N}-\text{H}$ in this group may be tolerated. The second nitrogen atom in the imidazole group of histidine carries an undelocalized lone-pair of electrons ('pyridine'-type N), and the presence of two 'pyridine'-type nitrogens to one pyrrole group on 1,2,4-triazole-3-alanine may account for its lack of interaction with System N.

For System N it appears that the maximum intramolecular distance between the nitrogen atoms of the two putative substrate

recognition sites (L - α -amino acid and pyrrole-N) should be equivalent to a C_4 chain, as homoglutamine [side chain $-(\text{CH}_2)_3\text{C}(=\text{O})\text{NH}_2$] is a very poor inhibitor of glutamine transport compared with asparagine and glutamine itself [side chains $\text{CH}_2\text{C}(=\text{O})\text{NH}_2$ and $(\text{CH}_2)_2\text{C}(=\text{O})\text{NH}_2$ respectively]. At this maximum distance, the size of groups attached to the side-chain nitrogen may increase in importance as a recognition feature; for example, it appears that a $-\text{CH}_3$ group is no longer tolerated, as N^m -methylhistidine (N atoms separated by a C_4 chain) does not inhibit glutamine transport, but N^m -methylhistidine (N atoms separated by a C_3 chain) does. This may reflect increased steric hindrance of binding to the amino-acid site on the transporter for analogues with wider intramolecular separation of recognition groups.

The apparent limitation on side-chain length of System N substrates may partly explain why DON and azaserine appear to be poorly recognized by this transporter, since both analogues contain an extra atom which tends to elongate the side-chain relative to glutamine, although the presence of nitrogen atoms with undelocalized lone-pairs of electrons in the side-chain groups of both analogues ($-\text{C}=\text{N}=\text{N}:)$ may also contribute to their lack of interaction with System N. What, therefore, are the likely routes of glutamine anti-metabolite entry into animal cells (assuming that substrate preferences of different transporters are similar in different tissues)? Our results indicate that DON is a strong inhibitor of System A (but not Systems L or N), and it may also be a substrate for this Na^+ -dependent system, as DON is known to be concentrated by rat liver (Jacquez, 1958) and mouse leukaemia cells (Huber *et al.*, 1988; in this case specifically by a high-affinity transport process). Acivicin may also be a substrate for System A, as it inhibits MeAIB-sensitive glutamine transport in liver vesicles as well as a specific low-capacity component of glutamine transport in renal basolateral membrane vesicles (Sastrasinh & Sastrasinh, 1988). Azaserine exerts a less specific inhibition of glutamine transport in liver membrane vesicles, which appears to affect Systems A and L. Azaserine is transported in T-lymphocytes by System L and an unidentified Na^+ -dependent transporter which is apparently not System A (Segel *et al.*, 1989); on the basis of our results, this Na^+ /azaserine transport component is unlikely to be System N, leaving System ASC as a likely candidate transporter for azaserine. Both azaserine and DON are competitive inhibitors of System L transport in T-lymphocytes, but azaserine has a stronger effect (lower apparent K_i) than DON (Segel *et al.*, 1989), a result which is consistent with our findings in liver membrane vesicles.

The results confirm recent reports (Low *et al.*, 1990; Pogson *et al.*, 1991) that L-glutamate γ -hydrazide is a poor inhibitor of glutamine uptake into isolated hepatocytes (possibly explained in part by the presence of undelocalized lone-pairs of electrons in the hydrazide group). However, since the hydrazide is a strong inhibitor of liver glutaminase, it has proved to be an extremely useful tool for investigating the relative importance of transport and metabolism in the control of glutamine breakdown by the liver (Pogson *et al.*, 1991).

The pattern of inhibition of glutamine transport by analogues was broadly similar in liver and muscle membranes, with glutamate γ -hydroxamate exerting a particularly strong effect in both preparations. The results therefore support the idea that Systems N and N^m are closely related to one another (at least in functional terms). In the perfused rat hindlimb, DON is transported by an Na^+ -dependent system and shows mutual transport inhibition with glutamine, but the evidence presented here using sarcolemmal vesicles indicates that DON is a poor inhibitor of System N^m . It is possible that the Na^+ -dependent DON uptake observed in the perfused hindlimb reflects activity of System A, which is present with a low activity in the sarcolemma (Ahmed

et al., 1990) and which DON appears to inhibit strongly in vesicle studies, or, conceivably, that rapid metabolism of DON in intact muscle tissue yields products which can interact with System N^m.

The proteins responsible for amino acid transport have not been identified and characterized, and present knowledge of these systems is largely limited to data obtained from phenomenological kinetic studies. One potential method for identifying the components of a transporter is to bind a photoreactive amino acid derivative to the transporter protein, and this possibility primed our interest in the study of glutamine analogue transport, because of our long-standing interests in isolating the System N/N^m transport proteins. Unfortunately, from this viewpoint, the photoaffinity analogues that we tested (DON, azaserine) do not appear to fulfil the basic structural requirements for System N/N^m substrates; therefore, any attempt to isolate these transporters via photoaffinity-labelling will require more careful 'design' of glutamine analogues. The present results provide a sound basis from which to work towards this goal.

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