Sensitivity of system A and ASC transport activities to thiol-group-modifying reagents in rat liver plasma-membrane vesicles

Evidence for a direct binding of N-ethylmaleimide and iodoacetamide on A and ASC carriers

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1. In the present study we have examined the sensitivity of A and ASC amino-acid-carrier activities in rat liver plasma-membrane vesicles to the thiol-group modifying reagents N-ethylmaleimide (NEM) and iodoacetamide (IA). To this end, the different Na^+-dependent entities involved in alanine transport were assessed. 2. NEM inactivated Na^+-dependent alanine transport as a result of the inhibition of both system A and ASC transport activities. The functional sensitivity of system A to NEM was greater than that of system ASC. 3. The presence of L-alanine (10 mM) during the exposure of vesicles to NEM afforded partial protection to system A, but not to the ASC, carrier. This effect was specific, since the presence of L-phenylalanine (10 mM) did not cause any protection. 4. Na^+ did not protect A or ASC carriers against NEM inactivation; however, the presence of Na^+ (100 mM-NaCl) and L-alanine (10 mM) during the exposure of the vesicles to NEM protected against inactivation of system A and ASC transport activities. The extent of protection was greater in the case of the system ASC transport activity than in the case of the A carrier. 5. IA also diminished Na^+-dependent alanine transport by inhibition of A and ASC transport activities. Sodium and L-alanine afforded protection to both A and ASC transport activities from the inhibitory action of IA. The extent of protection induced by substrates was similar for both carriers. 6. It is concluded that there is one, or several, free thiol groups in A and ASC carriers, the integrity of which is essential for transport activity. Sensitivity to thiol-group-specific reagents and the pattern of protection with substrates against inactivation is different in A and ASC carriers. That suggests the existence of topological dissimilarities regarding the thiol-group containing site(s) in A and ASC amino acid carriers.

INTRODUCTION

In hepatocytes, neutral amino acids are taken up primarily through systems A, ASC and L (Kilberg et al., 1981; Kilberg, 1982; Shotwell et al., 1983). Transport by system A is Na^+-dependent and its activity is greatly reduced at lowered extracellular pH. It transports short polar straight-chain amino acids, including the non-metabolizable amino acid analogue α-(methyl)aminoisobutyric acid (MeAIB). System A transport activity is subjected to hormonal regulation, trans-inhibition and adaptive modulation in a variety of cell types (Guidotti et al., 1978; Fehlmann et al., 1979; Shotwell et al., 1983; Handlogten & Kilberg, 1984; Zorzano et al., 1985, 1986; Bracy et al., 1986; Gumà et al., 1988). System ASC also shows Na^+-dependency and it is subjected to trans-stimulation (Christensen et al., 1967; Kilberg et al., 1981; Saier et al., 1988); this system carries neutral amino acids with small side chains, especially those with an -OH or -SH group, excluding N-methylated amino acid derivatives. Amino acids such as alanine or serine are transported by both system A and system ASC. For these amino acids, system ASC is functionally defined as that system responsible for the Na^+-dependent amino acid uptake not inhibited by high concentrations of MeAIB (Shotwell & Oxender, 1983; Saier et al., 1988).

There is paucity of data regarding the molecular properties of A and ASC amino-acid-transport systems. Recently a 120–130 kDa peptide has been identified as a component of system A transporter in Ehrlich ascites-cell plasma membrane (McCormick & Johnstone, 1988). There is evidence that the system A carrier is a glycoprotein that contains an N-linked oligosaccharide moiety (Barber et al., 1983; Kilberg et al., 1985) and shows affinity to concanavalin A (Quesada & McGivan, 1988). Furthermore, a 20 kDa protein has been tentatively identified as an essential component of alanine carriers (A or ASC) in plasma membrane from rat liver (Hayes & McGivan, 1983).

System A and ASC carriers in hepatocytes or plasma-membranes vesicles derived from rat liver are sensitive to thiol-group-modifying reagents (Kilberg et al., 1980; Sips & Van Dam, 1981; Hayes & McGivan, 1983; Chiles et al., 1988). In the present study, in order to gain further structural information on systems A and ASC, we have investigated the sensitivity of both carrier systems in rat liver plasma-membrane vesicles to chemical modification induced by the thiol-group-modifying reagents N-ethylmaleimide (NEM) and iodoacetamide (IA). To that end we have examined the alanine transport mediated through system A and system ASC. We show that there is one or several free thiol groups essential for the activity of A and ASC carriers. Both sensitivity to thiol-group-specific reagents and the pattern of protection obtained with substrates is different in A and ASC carriers, providing support to the existence of structural differences on the thiol-group-containing site(s) in A and ASC carriers. Furthermore, thiol-group-modifying reagents might be useful tools for future studies on the structure–function relationship of amino acid carriers.

Abbreviations used: NEM, N-ethylmaleimide; IA, iodoacetamide; MeAIB, α-(methyl)aminoisobutyric acid; $K_{0.5}$, concentration producing 50% inhibition; AIB, α-aminoisobutyric acid.
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MATERIALS AND METHODS

Membrane vesicle preparation

Plasma-membrane vesicles were prepared from the livers of 200–300 g male Wistar rats fed *ad libitum* by the method of Van Amelsvoort *et al.* (1978) as modified by Pastor-Anglada *et al.* (1987). In brief, rats were killed by decapitation without anaesthesia and livers were immediately excised and minced in 5 vol. of cold 0.25 M-sucrose/0.2 mM-CaCl$_2$/10 mM-Hepes buffer, pH 7.5 (buffer A). After homogenization and filtration through nylon cloth, homogenates were diluted 1:3 with 0.25 M-sucrose/0.2 mM-CaCl$_2$/1 mM-EDTA/10 mM-Hepes buffer, pH 7.5 (buffer B). After centrifuging at 30000 g for 20 min at 4 °C, pellets were resuspended in buffer B and centrifuged at 700 g for 10 min. The supernatant was collected and the pellet was resuspended in buffer B and centrifuged as described above at 700 g. The supernatants from the two 700 g centrifugations were pooled (typically 48 ml), mixed with 6 ml of iso-osmotic Percoll [90% (w/v) Percoll/0.25 M-sucrose, pH 7.5] and centrifuged at 30000 g for 20 min. Membranes migrating to the 1.038 g/ml density zone were collected. Membranes were washed with buffer A and centrifuged at 30000 g for 30 min. The final pellet was resuspended and rehomogenized in a volume of buffer A to give a final protein concentration of 3–5 mg/ml. Aliquots were rapidly frozen in liquid N$_2$ and stored at −80 °C.

Enzyme and protein assays

Protein was determined by the Bio-Rad Protein Assay, the Coomassie Blue-binding method of Bradford (1976) being used. The activities of 5′-nucleotidase (Aronson & Touster, 1974), glucose-6-phosphatase (Baginsky *et al.*, 1974), N-acetyl-β-D-glucosaminidase (Carroll, 1978) and cytochrome c oxidase (Yanetani, 1962) were measured in liver homogenate and in isolated membranes. The enrichment of plasma membrane in the vesicle preparation, as assessed by 5′-nucleotidase activity, was 7-fold (Table 1), comparable with previous reports (Van Amelsvoort *et al.*, 1978; Pastor-Anglada *et al.*, 1987). Under those conditions, the activity of glucose-6-phosphatase, a microsomal marker, was not significantly enriched (Table 1). Furthermore, activities of N-acetyl-β-D-glucosaminidase and cytochrome c oxidase, markers for lysosomes and mitochondria, showed a decrease in specific activity in the plasma-membrane-vesicle fraction as compared with the values for homogenate (Table 1). The yield of vesicle membranes was 1.8 mg of membrane protein/g wet weight of liver.

Table 1. Enzyme activities in homogenate and plasma-membrane fractions from rat liver

Results are means ± S.E.M. for six to eight observations per group. Specific activities are expressed as μmol of substrate consumed · h^{-1} · mg^{-1} of protein. Enrichment was calculated by dividing specific activity in plasma membrane by specific activity in homogenate.

Enzyme	Homogenate	Plasma-membrane-vesicle fraction	Relative enrichment
5′-Nucleotidase (n = 8)	2.0±0.1	13.8±1.0	6.9
Glucose-6-phosphatase (n = 8)	4.8±0.5	6.3±1.0	1.3
N-Acetyl-β-D-Glucosaminidase (n = 7)	1.3±0.3	0.4±0.4	0.3
Cytochrome c oxidase (n = 6)	46.8±12.4	22.9±6.8	0.5

Modification reactions

Frozen vesicles were thawed at room temperature and fractioned in accordance with the experimental needs. Chemical reactions were routinely performed at a protein concentration of 2–4 mg/ml in buffer B and at room temperature. Chemical reagents (NEM and IA) were prepared fresh immediately before use at 100-fold the desired final concentration. The reagent solution was then added to the vesicles at a 1:100 dilution and the reaction was allowed to proceed for the desired time. The reaction was quenched by the addition of a 3-fold excess of ice-cold buffer B supplemented with dithiothreitol (required to block the excess of thiol-group-modifying reagent that had not reacted). Vesicles were immediately centrifuged at 30000 g for 10 min. Vesicles were washed again in buffer B and centrifuged at 30000 g for 10 min. Vesicles were resuspended in buffer A and immediately used for transport measurements. The overall vesicle washing procedure accounted for a 1000-fold dilution of chemicals present during the modification reactions.

Transport assay

Vesicle aliquots and reaction mixture were preincubated at 26 °C for 5 min. In order to assay total alanine transport, membrane vesicles (10 μl, 20–40 μg of protein) were incubated in a medium (final volume, 40 μl) containing 0.25 M-sucrose, 0.2 mM-CaCl$_2$, 10 mM-MgCl$_2$, 100 mM-NaSCN, L-[2,3-^{3}H]-alanine (sp. radioactivity 0.126 μCi·nmol^{-1}), 10 mM-Hepes/KOH, pH 7.5. Alanine uptake was terminated by adding 1 ml of ice-cold 0.25 M-sucrose/0.1 mM-CaCl$_2$/100 mM-NaCl/10 mM-Hepes buffer, pH 7.5 (buffer C). The diluted membranes were immediately filtered through nitrocellulose filters (pore size 0.45 μm; diameter 25 mm). Filters were washed once with 4 ml of ice-cold buffer C and placed in scintillation vials containing 6 ml of scintillation cocktail. Radioactivity was measured with a liquid-scintillation spectrometer. Estimation of the Na$^+$-independent alanine transport plus the diffusion component was performed by measuring alanine uptake in the presence of 100 mM-KSCN instead of NaSCN. In this case, the stop solution was 0.25 M-sucrose/0.2 mM-CaCl$_2$/100 mM-KCl/10 mM-Hepes buffer, pH 7.5.

Data analysis

A and ASC transport activities were determined by measuring alanine uptake in the presence or absence of a 50-fold excess of MeAIB (Shotwell & Oxender, 1983; Saier *et al.*, 1988). We avoided the use of cysteine, a system-ASC-specific amino acid in hepatocytes (Kilberg *et al.*, 1981), owing to its reactivity with thiol-modifying reagents.

Total Na$^+$-dependent alanine transport was defined as the difference between alanine transport assayed in the presence of Na$^+$ (Na$^+$-dependent + Na$^+$-independent alanine uptake) and the alanine transported in the presence of K$^+$ (Na$^+$-independent carrier-mediated alanine uptake + diffusion). The system ASC-mediated alanine transport was assessed as the rate of Na$^+$-dependent alanine taken up in the presence of 10 mM-MeAIB, a concentration which was high enough to block uptake of 0.2 mM-alanine through system A. The system A-mediated alanine-transport rate was obtained by subtracting the ASC component from the total Na$^+$-dependent alanine transport. Statistical comparisons between groups were done by using the paired Student's t test.

RESULTS

System A- and ASC-mediated L-alanine transport in plasma-membrane vesicles

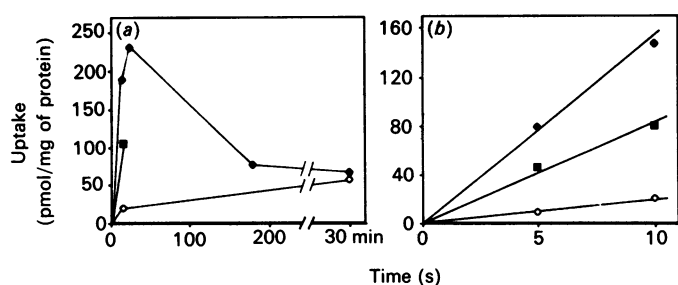The uptake of 0.2 mM-L-alanine by plasma membrane vesicles

Fig. 1. Time course of sodium-gradient-stimulated transport of L-alanine by plasma-membrane vesicles from rat liver

Rat liver plasma-membrane vesicles (20–40 μg of protein) were incubated in the presence of 0.2 mM-L-[2,3-^{3}H]alanine and 100 mM-NaSCN (●), 100 mM-NaSCN + 10 mM-MeAIB (■) or 100 mM-KSCN (○). For details, see the Materials and methods section. (a) Accumulation of alanine in vesicles as a function of time. (b) Initial uptake rate of alanine in membrane vesicles as a function of time. Each data point is the mean for triplicate samples from a representative experiment.

is showing in Fig. 1. After 10 s it had increased more than 10-fold in the presence of a NaSCN gradient as compared with that obtained in the presence of a KSCN gradient, in agreement with previous observations (Van Amelsvoort et al., 1978; Sips et al., 1980). This accumulation of L-alanine in the plasma-membrane vesicles was transient and, after 3 min of uptake, a state of equilibrium was attained (Fig. 1a). In the presence of 10 mM-MeAIB (50-fold excess compared with L-alanine), L-alanine transport after 10 s was substantially diminished owing to the blocking of system A L-alanine uptake; under these conditions, Na$^+$-dependent alanine uptake is a reflection of system ASC activity (Shotwell & Oxender, 1983; Pastor-Anglada et al., 1987). In keeping with previous data (Van Amelsvoort et al., 1978; Sips et al., 1980) the final amount of L-alanine associated with membrane vesicles after 30 min of uptake was independent of the presence of Na$^+$.

As shown in Fig. 1(b), vesicle alanine uptake increased linearly with time during 10 s of incubation in the presence of 100 mM-NaSCN, 100 mM-NaSCN + 10 mM-MeAIB or 100 mM-KSCN. Thus, in all subsequent studies, Na$^+$-dependent alanine uptake was assessed after 10 s of 0.2 mM-L-alanine addition. Under our conditions, alanine uptake mediated by system ASC accounted for 52±2% of total Na$^+$-dependent alanine uptake (n = 125), whereas alanine uptake mediated by system A represented 48% of total Na$^+$-dependent alanine transport. This agrees with previous observations carried out in isolated rat hepatocytes (Edmonson et al., 1979) and in rat liver plasma-membrane vesicles (Sips et al., 1980).

Inhibition of system A and ASC transport activities by NEM

It has previously been reported that NEM inhibits alanine uptake by plasma-membrane vesicles from rat liver (Sips & Van Dam, 1981; Hayes & McGivan, 1983). It has also been reported that the thiol-modifying reagent p-chloromercuribenzenesulphonate has a direct effect on α-aminoisobutyric acid (AIB) transport in several hepatoma cell lines (Dudeck et al., 1987). Thus in the present study we decided to examine whether both Na$^+$-dependent carriers, A and ASC, involved in alanine transport in rat liver, were subjected to inactivation by NEM. To that end, vesicles were treated with NEM, thoroughly washed to remove reagent that had not reacted and then system A and ASC activities involved in alanine transport were measured.

Fig. 2(a) shows that NEM inhibited Na$^+$-dependent alanine transport in a dose-dependent manner. The concentration of
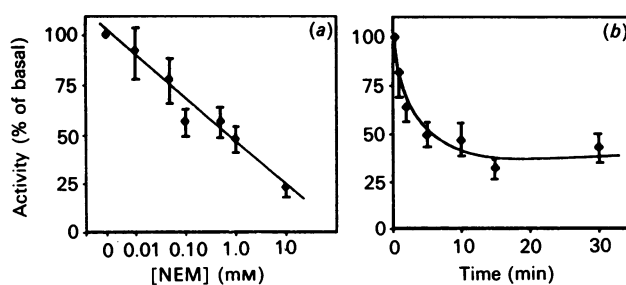

Fig. 2. NEM-induced inactivation of NA$^+$-dependent alanine transport by plasma-membrane vesicles from rat liver

(a) Dose–response curve for NEM-induced inhibition of Na$^+$-dependent alanine transport. Vesicles were preincubated for 30 min in the presence of different concentrations of NEM. (b) Time course of NEM-induced inactivation of Na$^+$-dependent alanine transport. Vesicles were preincubated for different times with 0.1 mM-NEM. The reaction was stopped by the addition of a 3-fold excess of ice-cold buffer B supplemented with dithiothreitol to block the excess of NEM. See the Materials and methods section for further details. Each data point is the mean±S.E.M. for three to seven vesicle preparations assayed in triplicate. Data are expressed as a percentage of transport in the absence of inhibitor (117±12 pmol/mg of protein).

NEM required for 50% inhibition ($K_{0.5}$) was 0.1 mM. Maximum inhibition (75%) occurred at 10 mM-NEM. Under our experimental conditions, exposure of vesicles to concentrations of NEM as high as 10 mM did not modify the apparent intravesicular volume of distribution (275 nl/mg of protein and 265 nl/mg of protein in control and NEM-treated vesicles respectively). NEM (0.1 mM) inactivated 50% of Na$^+$-dependent alanine transport within 5 min (Fig. 2b).

Subsequently, we investigated whether NEM-dependent inactivation of Na$^+$-dependent alanine transport was a consequence of modification of system A and/or ASC transport activities. Both carrier activities were indeed inhibited by NEM, and system A was more sensitive to inhibition than system ASC under all conditions investigated (Table 2). Kinetic analysis of the effects of NEM pretreatment was also performed (Fig. 3). Lineweaver–Burk plots revealed that NEM inhibited alanine uptake by promoting a decrease in V_{max} values without changes in the K_m for L-alanine. This occurred both in system A and

Table 2. Sensitivity of system A and ASC transport activities to inactivation by NEM

Vesicles were incubated, as described in Materials and methods section, for different times in the presence of different NEM concentrations. The reaction was quenched and the vesicles were washed. System A and ASC transport activities were measured as described in the Materials and methods section. Each value is the mean±S.E.M. for five to seven vesicle preparations assayed in triplicate. Data are expressed as a percentage of basal transport (49±5 pmol/mg of protein and 49±6 pmol/mg of protein for system A and ASC respectively). *Indicates a significant difference between system A and system ASC at $P < 0.05$.

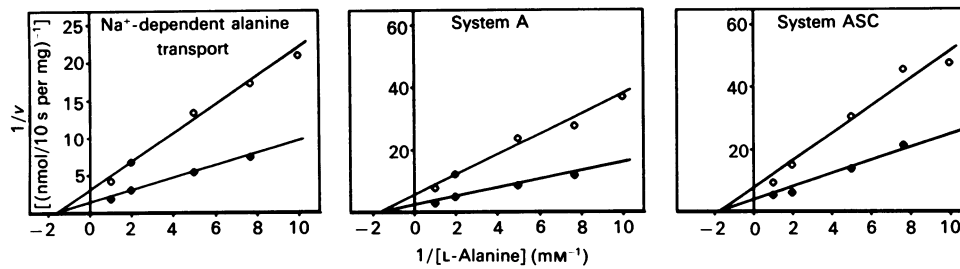| [NEM] (mM)	Time (min)	n	System...	Activity (% of basal)	
				A	ASC
0.1	2	7		50±6	76±7*
	5	7		50±7	70±9*
	30	6		41±6	56±7*
0.5	2	5		42±10	60±4*

Fig. 3. Kinetic characterization of NEM-dependent inactivation of Na^+-dependent, system A-mediated and system ASC-mediated alanine-transport activities

Vesicles were incubated for 2 min in the absence (◆) or presence (◇) of 0.1 mM-NEM. At that time, the reaction was stopped, the vesicles were washed and Na^+-dependent alanine transport, as well as system A and system ASC transport, activities were measured as described in the Materials and methods section. Each data point is the mean for triplicate samples from a representative experiment.

system ASC (Fig. 3). Thus $V_{max.}$ values for system A activity were 513 and 185 pmol/mg of protein in control and NEM-treated vesicles respectively; $V_{max.}$ values for system ASC were 326 and 160 pmol/mg of protein, in control and NEM-treated vesicles respectively. No modification in response to NEM was observed regarding K_m values for L-alanine, which were very similar for A and ASC systems.

To determine whether inactivation by NEM was the result of a direct carrier (system A or ASC) modification or due to non-specific plasma-membrane perturbations, we examined the ability of substrates (alanine and/or Na^+) to protect system A or ASC from NEM-dependent inactivation. To that end we initially performed experiments in which vesicles were preincubated in the presence of 10 mM-L-alanine + 100 mM-KCl, or just 100 mM-KCl. After 10 min, vesicles preincubated with or without L-alanine were subsequently incubated (or not) with 0.1 mM-NEM for 2 and 5 min. Vesicles were then washed, resuspended in buffer and immediately used for transport measurements. Incubation for 2 min in the presence of 0.1 mM-NEM caused a substantial inhibition of Na^+-dependent L-alanine transport by vesicles,

which was a consequence of inactivation of system A and system ASC transport activities (Table 3). Under these conditions, preincubation with 10 mM-L-alanine caused a substantial protection (about 47%) against NEM-induced inactivation of system A transport activity (Table 3); in contrast, 10 mM-L-alanine failed to protect system ASC transport activity (Table 3). An identical protection pattern was observed in experiments in which vesicles were exposed to NEM for 5 min (results not shown). To assess whether this protection was L-alanine-specific, we performed experiments similar to those specified above in which vesicles were preincubated in the presence of 10 mM-L-phenylalanine, an amino acid that is mainly transported by system L (Na^+-independent) and which does not inhibit Na^+-dependent L-alanine transport in rat liver plasma-membrane vesicles (Sips et al., 1980; Shotwell et al., 1983). Results shown in Table 3 indicate that 10 mM-L-phenylalanine did not protect Na^+-dependent L-alanine transport against inactivation induced by 0.1 mM-NEM.

In a further step, we evaluated the effect of the presence of Na^+ + alanine or Na^+ + phenylalanine during NEM treatment on

Table 3. Protection of A and ASC transport activities from NEM dependent inactivation by alanine

Vesicles were initially incubated for 10 min under different conditions: (a) 100 mM-KCl alone or 10 mM-L-alanine and 100 mM-KCl ('Control' and 'Alanine' groups) and (b) 100 mM-KCl alone or 10 mM-L-phenylalanine and 100 mM-KCl ('Control' and 'Phenylalanine' groups). At that time vesicles were incubated (or not) for 2 min in the presence of 0.1 mM-NEM. The reaction was quenched, the vesicles washed, and Na^+-dependent alanine transport, as well as system A and system ASC transport, activities were measured as described in the Materials and methods section. Each value is the mean ± S.E.M. for eight assays ('Protection with alanine') and three assays ('Protection with phenylalanine') from seven different vesicle preparations. Data are expressed either as pmol of alanine taken up/10 s per mg of protein or as a percentage of transport in the absence of inhibitor subjected to preincubation in the presence of L-alanine or L-phenylalanine or preincubated in the presence of potassium. *Represents statistically significant differences in the rates of transport activity between NEM-treated groups under protected or unprotected conditions at $P < 0.05$.

Conditions	Na^+-dependent alanine transport activity (pmol/10 s per mg)	(% of basal)	System A activity (pmol/10 s per mg)	(% of basal)	System ASC activity (pmol/10 s per mg)	(% of basal)
(a) Protection with alanine:						
Control	145±17		82±14		63±3	
NEM	79±6	53±4	35±5	43±10	44±5	69±7
Alanine	143±16		69±6		74±12	
Alanine+NEM	107±19	75±8*	54±6*	78±8*	53±10	71±11
(b) Protection with phenylalanine:						
Control	162±20					
NEM	104±11	64±7				
Phenylalanine	191±23					
Phenylalanine+NEM	126±14	66±7				

Table 4. Protection of A and ASC transport activities from NEM-dependent inactivation by alanine and Na⁺
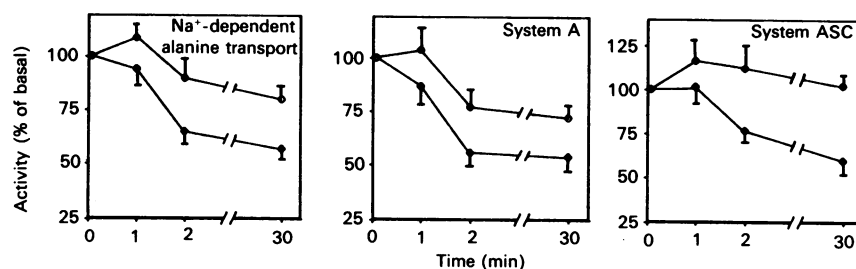
Vesicles were initially incubated for 10 min under different conditions: (a) 100 mM-KCl alone or 10 mM-L-alanine and 100 mM-NaCl ('Control' and 'Alanine + Na⁺' groups) and (b) 100 mM-KCl alone or 10 mM-L-phenylalanine and 100 mM-NaCl ('Control' and 'Phenylalanine + Na' groups). At that time, vesicles were incubated (or not) for 2 min in the presence of 0.1 mM-NEM. The reaction was quenched, the vesicles washed and Na⁺-dependent alanine transport, as well as system A and system ASC transport, activites were measured as described in the Materials and methods section. Each value is the mean ± s.E.M. for eight assays ('Protection with Na⁺ + alanine') and four assays ('Protection with Na⁺ + phenylalanine') from seven different vesicle preparations. Data are expressed either as pmol of alanine taken up.10 s per mg of protein or as a percentage of transport in the absence of inhibitor subjected to preincubation in the presence of Na⁺ + L-alanine or Na⁺ + L-phenylalanine or preincubated in the presence of K⁺. *Represents statistically significant differences in the rates of transport activity between NEM-treated groups under protected or unprotected conditions at $P < 0.05$.

Conditions	Na⁺-dependent alanine transport (pmol/10 s per mg)	(% of basal)	System A (pmol/10 s per mg)	(% of basal)	System ASC (pmol/10 s per mg)	(% of basal)
(a) Protection with Na⁺ + alanine:						
Control	154 ± 22		84 ± 17		70 ± 9	
NEM	98 ± 11	63 ± 3	47 ± 6	56 ± 6	51 ± 5	73 ± 7
Alanine + Na⁺	163 ± 21		81 ± 13		82 ± 11	
Alanine + Na⁺ + NEM	138 ± 10*	85 ± 7*	62 ± 6*	77 ± 7*	77 ± 10*	94 ± 8*
(b) Protection with Na⁺ + phenylalanine:						
Control	107 ± 4		59 ± 7		48 ± 6	
NEM	69 ± 8	65 ± 7	30 ± 6	51 ± 7	39 ± 2	81 ± 8
Phenylalanine + Na⁺	89 ± 4		45 ± 5		44 ± 3	
Phenylalanine + Na⁺ + NEM	54 ± 8	61 ± 6	26 ± 5	58 ± 6	27 ± 3	61 ± 8

system A and ASC transport activities. Thus the effect of preincubation of 100 mM-NaCl + 10 mM-L-alanine was assessed (Table 4). Incubation for 2 min in the presence of 0.1 mM-NEM caused a 44 % inhibition of system A and 27 % inhibition of system ASC transport activity (Table 4). However, prior incubation with Na⁺ + alanine only caused a 15 % inhibition of system A and 6 % inhibition of system ASC transport activity (Table 4). That is, Na⁺ + L-alanine protected both A and ASC carriers; however, whereas the extent of protection of system A (about 48 %) was very similar to the protection detected with prior incubation with L-alanine (see Table 3), the protection induced on system ASC was almost complete. In a separate set of experiments, vesicles were preincubated in the presence of 100 mM-NaCl + 10 mM-L-phenylalanine or in the presence of 100 mM-KCl for 10 min and subsequently subjected to NEM exposure. Under these conditions, Na⁺ + phenylalanine were

unable to protect system A or system ASC transport activities against inactivation induced by NEM (Table 4).

Experiments were also performed to investigate the protection exerted by Na⁺ + L-alanine on system A and ASC transport activities from vesicles incubated with NEM for different times. Results are expressed as a percentage of basal activity and are shown in Fig. 4. Under these conditions, system A displayed a higher sensitivity to time-dependent inactivation by NEM than did system ASC activity (Fig. 4) and, again, we found that the presence of substrates (L-alanine + NaCl) during the exposure of membranes to NEM protected A and ASC transport activities (Fig. 4). Furthermore, the extent of protection promoted by substrates on carrier activities was greater in system ASC compared with system A (Fig. 4) and, in fact, substrate-dependent protection was total in system ASC, even after 30 min of NEM treatment (Fig. 4).

Fig. 4. Alanine and sodium protect A and ASC transport activities from NEM-dependent inactivation

Vesicles were initially incubated for 10 min in the presence of 10 mM-L-alanine and 100 mM-NaCl (protected group, ○) or 100 mM-KCl (unprotected control group, ●). At that time the vesicles were incubated for different periods in the presence of 0.1 mM-NEM. The reaction was quenched, the vesicles were washed and Na⁺-dependent alanine transport, as well as system A and system ASC transport, activities were measured as described in the Materials and methods section. Each data point is the mean ± s.E.M. for eight assays (2 and 30 min of NEM treatment) and three assays (1 min of NEM treatment) from seven different vesicle preparations. Data are expressed as a percentage of transport in the absence of inhibitor subjected to preincubation in the presence of L-alanine and Na⁺ (protected group) or preincubated in the presence of K⁺ (unprotected group). Reference values for Na⁺-dependent alanine transport were 117 ± 12 pmol/mg of protein and 156 ± 20 pmol/mg of protein for control unprotected and control protected groups respectively.

Table 5. Alanine and Na⁺ protect A and ASC transport activities from inactivation by IA

Vesicles were initially incubated for 10 min in the presence of 100 mM-KCl alone or 10 mM-L-alanine and 100 mM-NaCl ('Control' and 'Na⁺ +alanine' groups). At that time, vesicles were incubated for an additional 10 min in the presence of 30 mM-IA. The reaction was quenched, the vesicles washed and Na⁺-dependent alanine transport, as well as system A and system ASC transport, activities were measured as described in the Materials and methods section. Each value is the mean±S.E.M. for four to six assays on separate vesicle preparations. Data are expressed either as pmol of alanine transported/10 s per mg of protein or as a percentage of transport in the absence of inhibitor. For details, see the Materials and methods section and the legends to Table 3 and Fig. 4. *Represents statistically significant differences in the rates of transport activity between IA-treated groups under protected or unprotected conditions at $P < 0.05$.

Conditions	Na⁺-dependent alanine transport (pmol/10 s per mg)	(% of basal)	System A (pmol/10 s per mg)	(% of basal)	System ASC (pmol/10 s per mg)	(% of basal)
Control	172±18		90±14		82±11	
IA	77±14	45±5	30±10	33±5	47±10	57±7
Na⁺ +alanine	216±27		96±24		120±17	
Na⁺ +alanine+IA	149±25*	69±10*	58±8*	60±6*	91±15*	76±8*

Inhibition of system A and ASC transport activities by iodoacetamide

In an attempt to gain further insight into the dependence of system A and ASC transport activities on thiol groups, experiments were also conducted using iodoacetamide (IA), another thiol-group-modifying reagent. IA inactivated NA⁺-dependent alanine transport and it did so in a dose-dependent manner. The $K_{0.5}$ for IA was approx. 10 mM, and maximum inhibition (100%) occurred at 100 mM-IA. Such a high concentration of reagent did not modify the integrity of vesicles, as assessed by the apparent intravesicular volume of distribution (results not shown). IA inhibited Na⁺-dependent alanine transport with a $t_{\frac{1}{2}}$ of 3 min, and maximum inactivation of 30 mM-IA was observed within 10 min. This effect of IA is consequence of the inactivation of system A and system ASC (results not shown).

We also addressed the question of whether IA inhibits alanine transport by reacting directly with amino acid carriers. Thus we performed experiments similar to the ones previously reported in Table 4 and Fig. 4 in order to investigate a possible protective effect of substrates (alanine + Na⁺) on the inhibition promoted by IA. In the presence of high concentrations of substrates (10 mM-L-alanine + 100 mM-NaCl), the inhibition of Na⁺-dependent alanine uptake induced by 10 min of exposure to 30 mM-IA was substantially slowed down (Table 5). Under these conditions, the presence of substrates during the exposure of membranes to IA partially protected system A and ASC transport activities (Table 5). The extent of protection induced by substrates was of similar magnitude in both A and ASC system carriers. Similar results were obtained by treatment during 5 min with 30 mM-IA (results not shown). The data are consistent with the view that the IA-dependent inhibition of system A and ASC is the result of direct chemical modification of these carrier proteins.

DISCUSSION

The results of our study demonstrate that NEM and IA, two thiol-modifying reagents, inhibit A and ASC amino acid transport activities in rat liver plasma-membrane vesicles. This inhibition of transport is a consequence of a direct binding of both thiol reagents to A and ASC carriers. This allows us to conclude that there is one or more thiol groups both in A and ASC carriers which are critical for transport activity. Whether these thiol groups are involved in the intrinsic mechanism of transport or in substrate binding remains unknown. Our data agree with

previous observations regarding the protective effect of L-alanine on NEM-dependent blocking of alanine transport in rat liver plasma membranes (Hayes & McGivan, 1983). In addition, a direct effect of p-chloromercuribenzene sulphonate, another sulphydryl-modifying reagent, has been reported on AIB transport (A + ASC) in membrane vesicles from H4 hepatoma cells (Dudeck et al., 1987).

Moreover, the utilization of thiol-modifying reagents has allowed the identification of several interesting differences between A and ASC carriers: (a) system A is more sensitive than ASC to inhibition by thiol-modifying reagents, as judged by dose–response studies with NEM or time–response studies with NEM and IA; and (b) the pattern of protection against NEM inactivation obtained by co-incubation with substrates markedly differs in A and ASC carriers; thus, whereas system A transport activity is partially protected by co-incubation with L-alanine or L-alanine + Na⁺, the ASC carrier is only protected with L-alanine + Na⁺. On the basis of these findings we suggest the site(s) modified by thiol-modifying reagents in A and ASC amino acid carriers present topological dissimilarities, and perhaps critical thiol groups might be located in a somewhat more accessible locus in the A carrier as compared with the ASC carrier. Our study represents one of the first attempts to differentiate structurally the A and ASC systems, carriers that share the property of co-transporting amino acids in a Na⁺-dependent manner. It should be mentioned that the information now available on the structure–function relationships of eukaryotic Na⁺-dependent co-transporters is very scarce and, in fact, the only Na⁺-dependent carrier so far cloned and sequenced is the Na⁺/glucose co-transporter (Hediger et al., 1987). Nevertheless, A and ASC carriers might not be structurally related to the intestinal Na⁺/glucose co-transporter, since they differ in their sensitivity to N-acetylimidazole, a tyrosine-specific reagent (Peerce & Wight, 1985; J. Bertran, A. Roca, E. Pola, X. Testar, A. Zorzano & M. Palacín, unpublished work).

Regarding the A carrier, we have set up conditions to protect it against inactivation by thiol-modifying reagents. Thus we have shown that preincubation with L-alanine during NEM exposure affords partial protection to system A, and no further protection is attained when both L-alanine + Na⁺ are present. Our data are in keeping with a protective effect against NEM-dependent inactivation of alanine transport (A + ASC) in the presence of 100 mM-L-alanine described by Hayes & McGivan (1983). Other authors have failed to show any protective effect of 5 mM-L-norleucine against NEM-dependent inactivation of AIB trans-

port in plasma-membrane vesicles derived from rat hepatocytes (Dudeck et al., 1987). However, the efficiency of L-norleucine as a substrate of system A has not been substantiated in liver (Oxender & Christensen, 1963). On a mechanistic level, data on system A protection might be explained if some thiol-group-containing site(s) were located near the L-alanine-binding site and their accessibility to NEM was only dependent on the presence of amino acid and independent of Na$^+$. However, other explanations based on the existence of conformational changes cannot be discarded.

In contrast with the situation in system A, the ASC carrier was only protected against inactivation of NEM when both L-alanine and Na$^+$ were present in the medium; nevertheless, the protection was almost complete under these conditions. That favours the existence of conformational changes induced by binding of alanine and/or Na$^+$ to the ASC carrier, making NEM-binding site/s much less accessible to attack by covalent reagents. In fact, the existence of conformational changes has been reported for a number of Na$^+$-dependent carriers, such as the erythrocyte membrane alanine carrier (Charalambous & Wheeler, 1985, 1987), the intestinal Na$^+$/glucose co-transporter (Peerce & Wright, 1984a,b) or the intestinal proline (imino) carrier (Wright & Peerce, 1984). However, our data might also be explained if the reaction with thiol groups was located at a site near to the alanine or Na$^+$ loci, with both loci lying close together, so when L-alanine and Na$^+$ bind to the ASC carrier the thiol group becomes inaccessible to NEM. In contrast with the protection studies performed with NEM, IA-dependent inactivation of ASC carrier was only partially protected by substrates (L-alanine + Na$^+$). This differential pattern of protection induced by L-alanine + Na$^+$ against NEM- or IA-dependent inhibition of system ASC activity suggests either that NEM and IA do not react with identical thiol groups in ASC carriers or that NEM and IA present differential accessibility properties to thiol-containing site(s) in the ASC carrier.

In summary, we have demonstrated that (a) NEM and IA directly bind to A and ASC carriers and (b) there are topological dissimilarities regarding the thiol-containing site(s) in the A and ASC amino acid carriers. In addition, this is the first study in which conditions have been set up to label A and ASC carriers selectively. In this regard, the selective targeting of A or ASC carriers with labelled probes might be worthwhile in future studies designed to investigate the structure–function relationship of both carriers, once functionally pure carriers are available in the way that other membrane proteins are.

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