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Glycine Uptake by Trout (*Salmo trutta*) Red Blood Cells

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Abstract. The present study demonstrates the presence of different amino acid carriers in the membrane of trout red cells. Most glycine is taken up through the Na^+-dependent system ASC, although the nearly specific Gly system is also active. Besides these carriers, glycine is taken up by means of Na^+-independent transporters, system L being the most important. A system asc of high affinity and low capacity has been found, and band 3 is unable to transport glycine under physiological conditions. These results suggest that although all these carriers are already present in primitive vertebrates, several differences exist in their properties with respect to those found in mammalian cells.

Key words: Glycine uptake — Trout (*Salmo trutta*) — Red blood cells

Introduction

Red blood cells (RBC) play an important role in amino acid metabolism, being implied, for example, in the biosynthesis of glutathione (Ellory, Jones & Young, 1981), interorgan transport (Christensen, 1982), and peptide degradation following amino acid efflux (Tucker, Young & Crowley, 1981). Some amino acids are involved in the regulation of cell volume, particularly in fish red blood cells (Goldstein & Boyd, 1978; Fincham, Wolowyk & Young, 1987).

Amino acids cross cell membranes by a variety of transport systems, whose molecular specifity varies considerably. Thus, the L system shows a broad spectrum, being able to interact with most neutral amino acids (Barker & Ellory, 1990). In contrast, the Gly system shows a narrow spectrum, carrying only glycine, and its N-methylated derivative, sarcosine (Barker & Ellory, 1990). Another kind of variability is based on the presence/absence of these carriers in different cell types. For example, the L system is practically ubiquitous, while system C is known only in sheep red cells, although asc, a related system which was initially found in horse erythrocytes by Fincham, Mason and Young (1985), has now been demonstrated in other systems, such as pigeon (Vadgama & Christensen, 1985) and Pacific hagfish (Fincham, Wolowyk & Young, 1990) erythrocytes, exocrine pancreatic cells (Norman & Mann, 1987) and transformed kidney cells (Kuhlmann & Vadgama, 1991), and also in different trout cell types (J.L. Albi, P. Canals, M.A. Gallardo and J. Sánchez, *submitted for publication*).

Studies on amino acid uptake by fish red cells have been largely limited to taurine, GABA and β-alanine, and related to changes in either cell volume or extracellular osmolarity (Goldstein & Boyd, 1978; Goldstein & Brill, 1990, 1991). Nevertheless, there are recent reports on the uptake of several amino acids by hagfish RBC (Fincham et al., 1990) and L-serine by trout red cells (Gallardo, Planas & Sánchez, 1992). The study of glycine uptake by red cells offers the advantage that it can be carried by several amino acid transport systems (Ellory et al., 1981), thus providing evidence of their presence and/or functional ability to transport amino acids in the cell type considered.

The present study shows that glycine is incorporated to trout red cells by means of different amino acid transport systems, but their relative importance differs from that described for human RBC, suggesting evolutionary variations.

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Materials and Methods

Brown trout (*Salmo trutta*) were obtained from a factory (Dept. Medi Ambient, Generalitat de Catalunya), located in the Pyrenees (Bagà, Spain), and were acclimatized to the laboratory conditions (a closed water circuit, filled with dechlorinated and decalcified water and with controlled $[NH_4^+]$ and $[O_2]$, maintained at 15°C) for at least one week before the experiments were performed. Body weight ranged between 200 and 400 g.

Blood was obtained by caudal puncture, diluted with heparinized RPMI 1640 (Sigma), rinsed several times and left overnight at 4°C to eliminate a possible catecholamine effect. Due to the high metabolic activity of white blood cells, these cells, as well as thrombocytes were removed by centrifugation with Histopaque-1077™ (Sigma), following the procedure suggested by the supplier, slightly modified to overcome the high viscosity of trout blood. Once separated, the RBC were rinsed four times with Cortland buffer (pH 7.4) (Houston et al., 1985) slightly modified (mM: NaCl 141, KCl 3.5, $MgSO_4$ 1, NaH_2PO_4 3, $CaCl_2$ 1, pyruvic acid 2, HEPES 10, bovine serum albumin 0.3%, glucose 3). Henceforth this buffer will be referred to as MCB. The osmolality was adjusted to 305 mosmol · Kg^{-1}. When cells were used for experiments using buffers with a different ionic composition, the whole rinsing procedure was performed using the final buffer.

(2^3H)-Glycine was obtained from New England Research Products (47.5 Ci.mmol^{-1}) and other chemicals were from Sigma. Both cells and solutions were pre-equilibrated at 15°C before the experiments were started by mixing (1 : 1, v/v) the RBC suspension with the glycine to obtain the desired concentrations and a final hematocrit of about 10%. (2^3H)-glycine (0.3 μCi) for each ml of cell suspension was added to the cold glycine solution before mixing with cells. Incubations were performed in a shaking bath at 15°C, using air as atmosphere.

Glycine uptake was stopped by diluting with MCB (1 : 9, v/v) containing a 10- to 50-fold excess of nonradioactive amino acid, and rinsing the cells with this solution (1 : 9, v/v) three times. RBC were separated each time by centrifugation (810 × *g* for 8 min at 4°C). Cell suspensions were deproteinized by adding sufficient ice-cold perchloric acid to obtain a final concentration of 6%. A clear supernatant was obtained by centrifugation (1,825 × *g* for 20 min at 4°C). The radioactivity in this supernatant was measured by a liquid scintillation counter.

Cell water content was determined gravimetrically and extracellular sodium measurements were performed by flame photometry following Mahé, García-Romeu and Motais (1985). Cl^- was determined spectrophotometrically by means of a colorimetric test (Colorfix, Menarini Diagnostics, Spain).

Curve-fitting of experimental data was performed by computerized nonlinear regression systems.

Results

Figure 1A shows a typical result of glycine accumulation by trout RBC. Half equilibrium time was 129 ± 7 min and the in/out concentration ratio obtained at equilibrium ranged between 0.6 and 1.5, according to seasonal variation (M.A. Gallardo and J. Sánchez, *unpublished data*). These values were slightly lower than those observed physiologically (about 4.5).

The concentration dependence on the rate of uptake is shown in Fig. 1*B*. The observed changes in the uptake when K^+ was used instead of Na^+, indicated that glycine was accumulated by trout RBC via at least two systems, one being Na^+-dependent, the other being Na^+-independent. However,

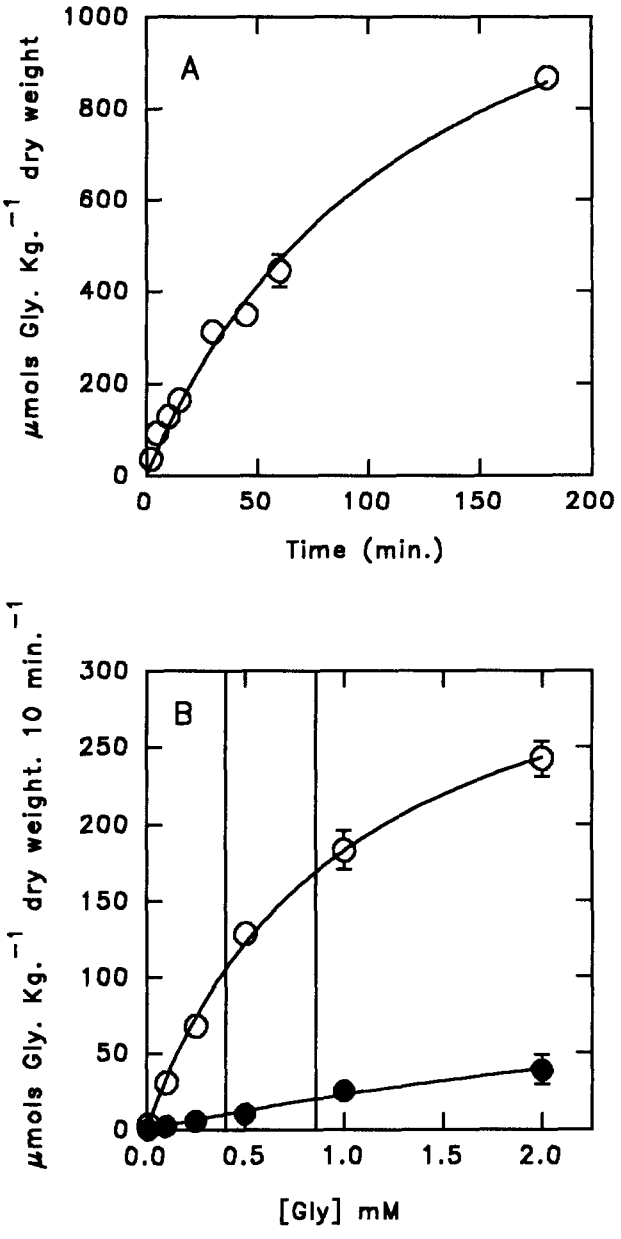

Fig. 1. (*A*) Time course of glycine uptake by trout RBC. Cells were suspended in MCB and incubated for different times in the presence of 500 μM glycine. (*B*) Concentration dependence of glycine uptake by trout RBC. Initial rates were measured in the presence of MCB containing either sodium or potassium as the main cation. Cells were incubated for 10 min before the uptake was stopped, as described in Materials and Methods. (○) Total uptake. (●) Sodium-independent uptake. Lines indicate the physiological range of plasma glycine concentrations. Each point is the mean of 3–5 individual experiments. Bars are standard deviation of the mean.

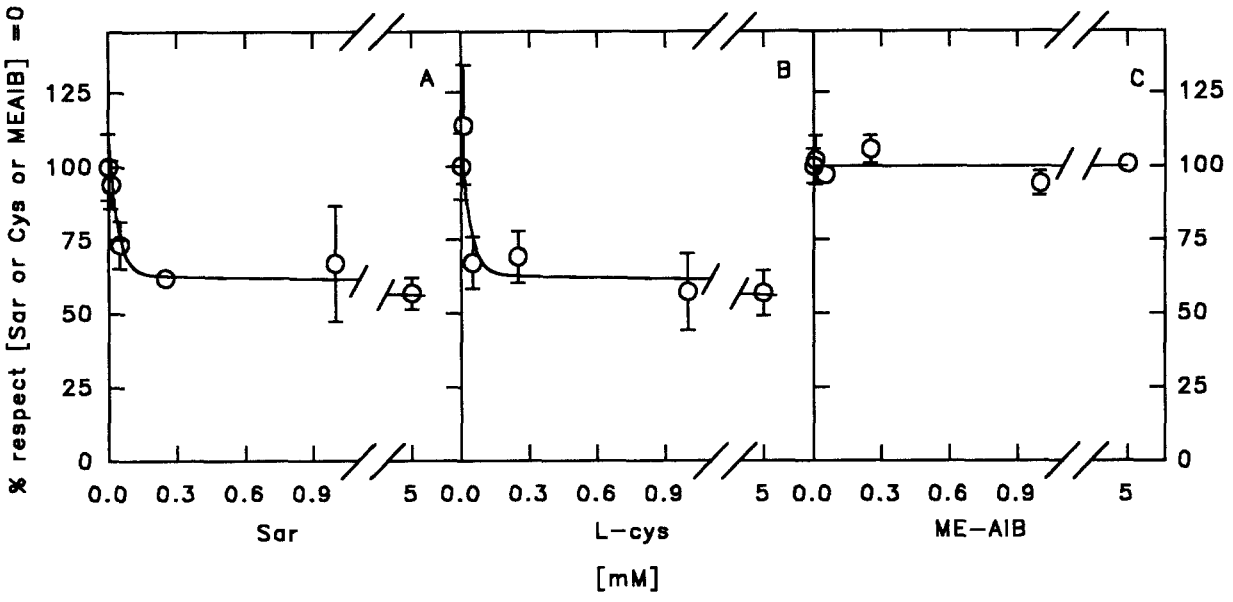

Fig. 2. Inhibition by different amino acids of the Na^+-dependent uptake of 10 μM glycine by trout RBC. Cells were incubated for 10 min. The uptake was measured in the presence of several concentrations (0.01–5 mM) of (*A*) Sarcosine; (*B*) L-cysteine; (*C*) MeAIB. Each point is the mean of three individual experiments. Bars are standard deviation of the mean.

to ascertain whether different Na^+-dependent transport systems could be involved in this uptake, "specific" inhibitors for several carriers were used. Figure 2 shows the effect of increasing concentrations of methyl-aminoisobutyric acid (MeAIB, for the A system), L-cysteine (for the ASC system) and sarcosine (for the Gly system) on the uptake of 10 μM glycine by trout RBC. The lack of inhibition by MeAIB confirms the absence of system A in these cells, while the inhibitory actions of either L-cysteine or sarcosine gave evidence that there are two Na^+-dependent transport systems for glycine uptake in trout RBC. The sum of uptakes in the presence of either L-cysteine or sarcosine accounts for almost all the Na^+-dependent glycine uptake.

In addition to the inhibitory activity of L-cysteine and sarcosine, the activity of these two Na^+-dependent systems was further characterized by means of *trans* effects as were the Na^+ and Cl^- dependences of the uptake.

Trans effects were measured by preloading cells with nonradioactive 500 μM glycine for different times, then tracer glycine was added externally and the uptake was developed for 10 min. At the times selected, different intracellular glycine concentrations were achieved and Fig. 3 shows how they modified the rate of tracer uptake, in the presence of either sarcosine or L-cysteine (5 mM). The ASC system became *trans*-stimulable, but the Gly system was not.

The slightly sigmoidal relationship obtained be-

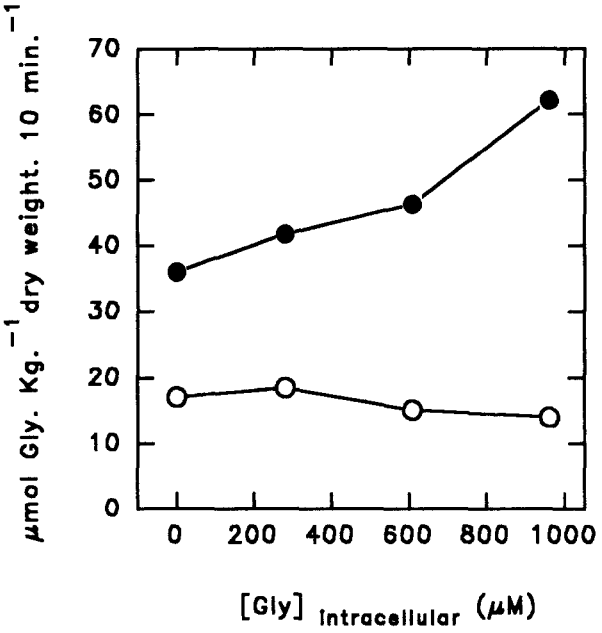

Fig. 3. *Trans*-stimulation of the Na^+-dependent uptake of glycine by trout RBC. Cells were preloaded with "cold" glycine for different times until different intracellular concentrations were achieved and then they were washed twice with cold MCB. The uptake of 500 μM labeled glycine was measured for 10 min. (○) Gly system (uptake through system ASC was inhibited by 5 mM L-cysteine) and (●) ASC system (uptake through system Gly was inhibited by 5 mM sarcosine). Each point is the mean of three individual experiments.

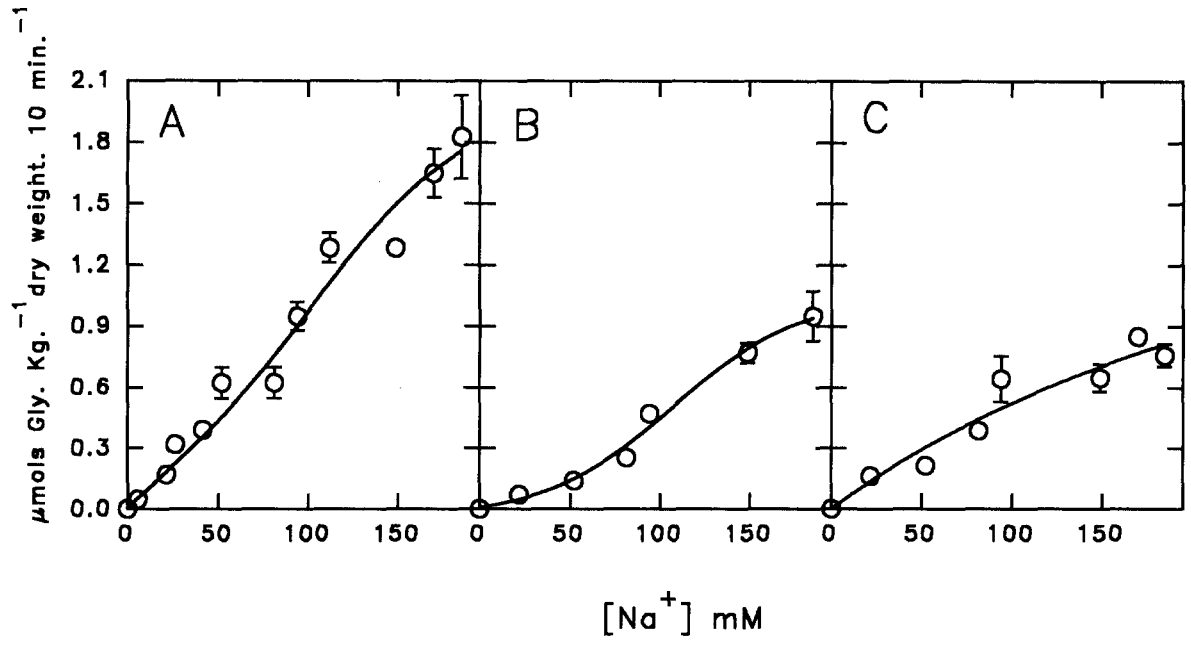

Fig. 4. Dependence of glycine uptake by trout RBC with respect to the extracellular Na^+ concentration. (*A*) Total uptake dependence. (*B*) Uptake through the Gly system. (*C*) Uptake through the ASC system. The glycine concentration used was 10 μM. Potassium was used throughout to replace sodium and osmolality was maintained constant (305 mosmol Kg^{-1}). The rates were measured after 10 min of incubation. Each point is the mean of 3–5 individual experiments. Bars are standard deviation of the mean. Kinetic constants for the uptake are given in text.

tween 10 μM glycine uptake rate and extracellular Na^+ concentration (Fig. 4*A*), was split into two curves (uptake through Gly and ASC systems) by means of the inhibition with L-cysteine or sarcosine. Glycine uptake through the Gly system clearly rose sigmoidally with respect to the extracellular Na^+ concentration (V_{max} = 1.0 ± 0.2 μmol Gly · Kg^{-1} dry weight · 10 min^{-1}; $[Na^+]_{0.5}$ = 107 ± 13 mM; n = 1.8 ± 0.2) (Fig. 4*B*), while a hyperbolic relationship for the uptake through the ASC system appeared (V_{max} = 1.1 ± 0.1 μmol Gly · Kg^{-1} dry weight · 10 min^{-1}, apparent K_m = 86.3 ± 15.6 mM) (Fig. 4*C*). Moreover, when Li^+ was used instead of Na^+, the uptake through both Na^+-dependent systems was abolished (the actual rates of the 10 μM glycine uptake in the presence of Na^+, Li^+ and K^+ were 2.8 ± 0.2, 0.6 ± 0.2 and 0.7 ± 0.07 μmol Gly · Kg^{-1} dry weight · 10 min^{-1}, respectively).

Besides the specific inhibition by sarcosine and the sigmoidal relationship between Gly uptake and extracellular Na^+ concentration, more data confirming the presence of system Gly in trout RBC were obtained when Cl^- was replaced by other anions, since this system was dependent on the presence of Cl^- in the medium. Both acetate and sulfate nearly abolished the incorporation through system Gly (Fig. 5), although they also had a small inhibitory effect on the activity of system ASC.

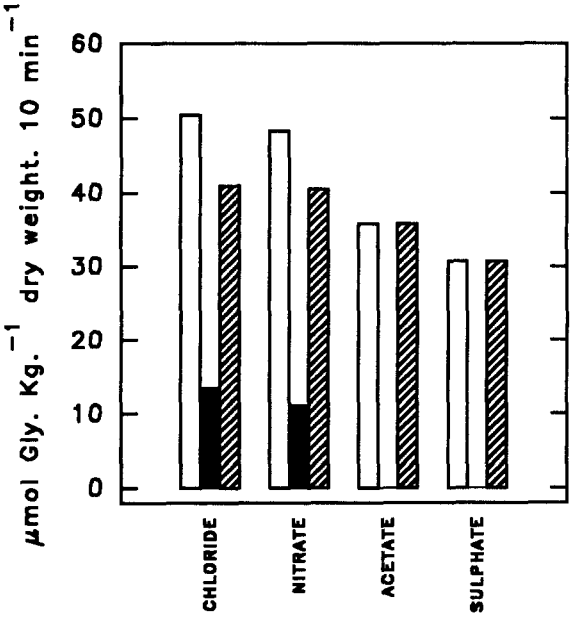

Fig. 5. Anion dependence of glycine uptake. Cells were equilibrated with the different media and incubated for 10 min before the uptake was stopped. Total Na^+-dependent glycine uptake (□), system Gly (■) and system ASC (▨). Each point is the mean of three individual experiments.

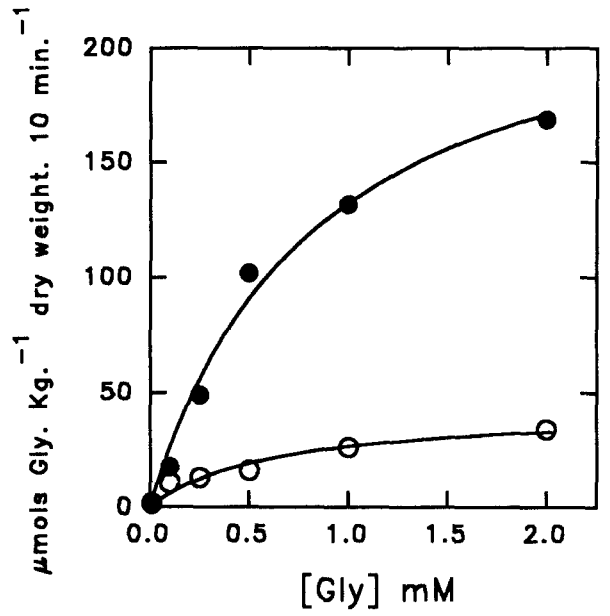

Fig. 6. Dependence of the Na^+-dependent glycine uptake on glycine concentration, through system Gly (○) and system ASC (●). Rates were measured after 10 min of incubation. Each point is the mean of 3–5 individual experiments. Kinetic constants for the uptake are given in the text.

Once the existence of these two systems had been established, L-cysteine and sarcosine were used to study how the Na^+-dependent uptake of glycine was distributed between the two carriers. Results shown in Fig. 6 indicated a higher capacity of system ASC (V_{max} = 242 ± 10 µmol Gly · Kg^{-1} dry weight · 10 min^{-1}), than system Gly (V_{max} = 43 ± 3.4 µmol Gly · Kg^{-1} dry weight · 10 min^{-1}). However, their K_m had similar values (0.8 mM for system ASC and 0.6 mM for system Gly).

As seen in Fig. 1*B*, glycine is mostly taken up through Na^+-dependent systems, although there is a component of the total uptake of glycine by trout RBC that is Na^+-independent. The possibility that it may be taken up through more than one Na^+-independent system was also tested by means of the inhibitory action of several amino acids. Figure 7 shows the effect of these amino acids on the uptake of either 10 or 500 µM glycine. There were two groups of amino acids able to inhibit this uptake. One group corresponds to good system asc substrates, while the other is formed by good system L substrates. The inhibitory pattern differed at low glycine concentration with respect to the high concentration. Thus at a concentration of 10 µM, glycine was better taken up through system asc, while at higher (physiological) concentrations the uptake through system L was greater (Fig. 7*A* and *B*).

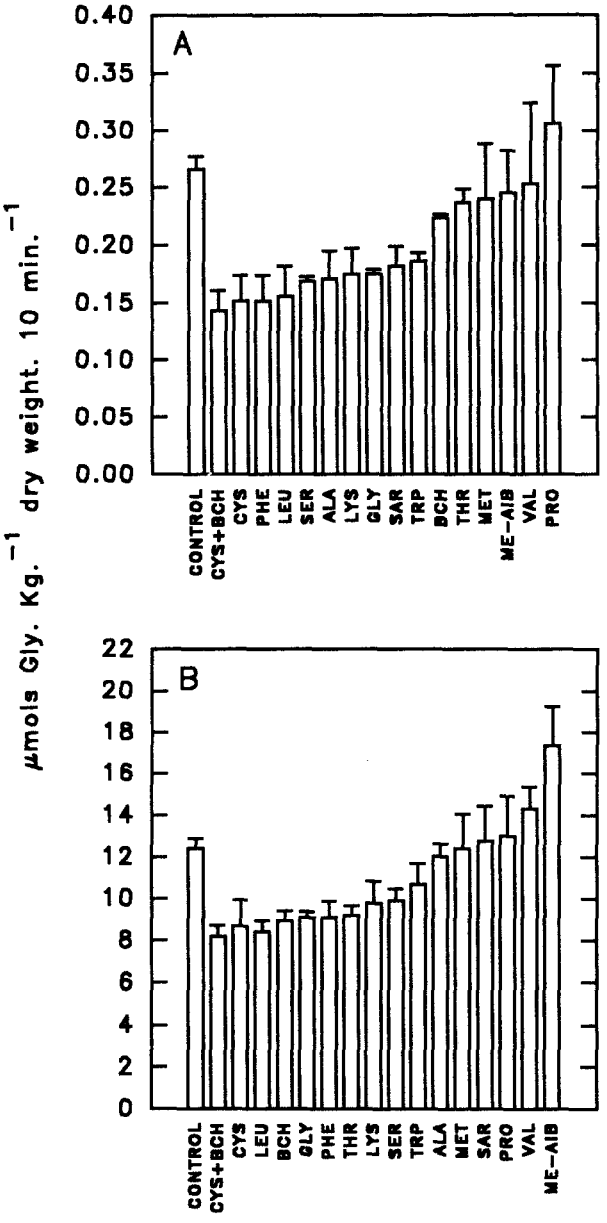

Fig. 7. Inhibition by different amino acids (5 mM) of the Na^+-independent uptake of 10 µM (*A*) and 500 µM (*B*) glycine by trout RBC. Cells were incubated for 10 min in a Na^+-free medium. Each point is the mean of three individual experiments. Bars are standard deviation of the mean.

At 500 µM glycine, the simultaneous presence of BCH and L-cysteine did not show an additive effect on the uptake. However, both molecules behaved as competitive inhibitors of the Na^+-independent glycine uptake with apparent K_i (mM) values of 6.1 ± 0.5 for BCH and 5.4 ± 0.3 for L-cysteine (Fig. 8). To test the possibility that L-cysteine may be acting through more than one carrier, an Eadie-Hofstee plot of the Na^+-independent L-cysteine-

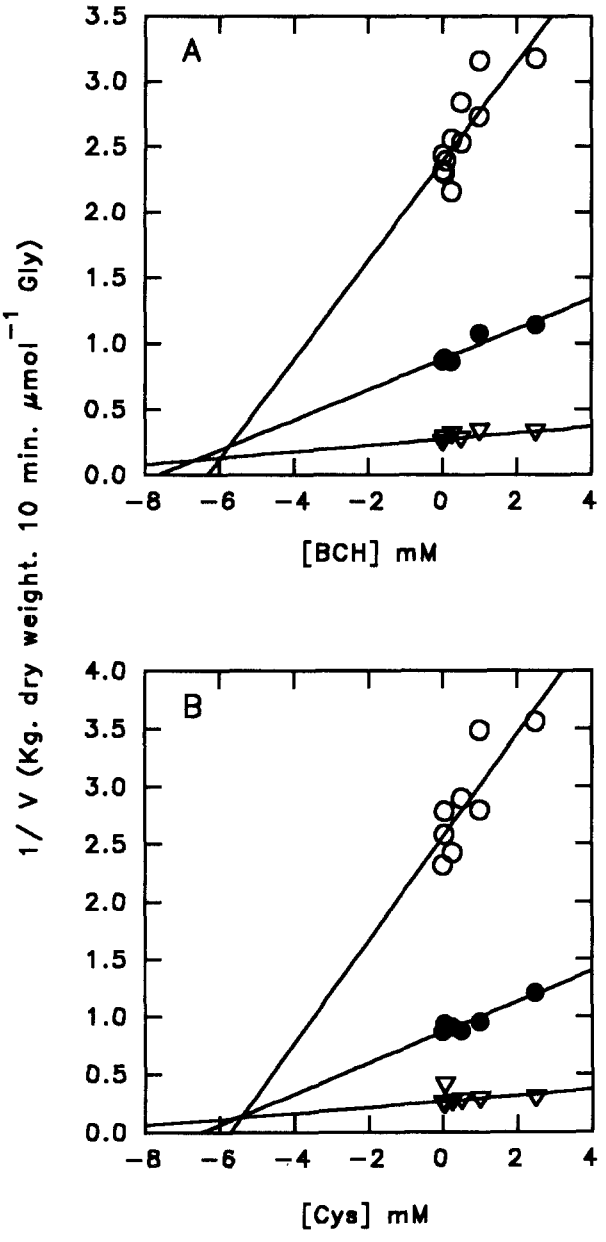

Fig. 8. Kinetics of the effect of BCH (*A*) and L-cysteine (*B*) on the Na^+-independent glycine (10, 25 and 100 μM) uptake by trout RBC. Experimental conditions as in Fig. 7. The K_i values are given in the text.

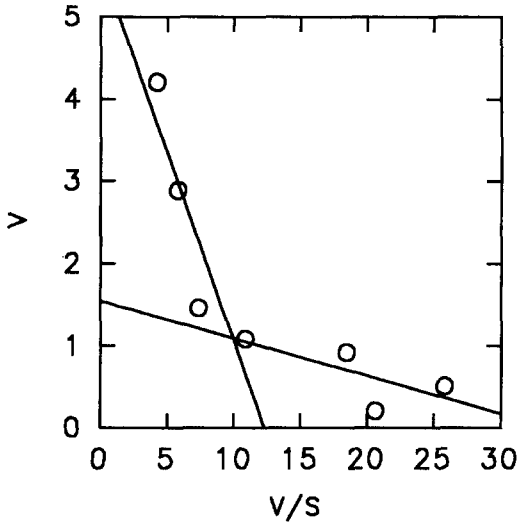

Fig. 9. Eadie-Hofstee plot of the Na^+-independent L-cysteine-sensitive glycine uptake. v = uptake rate (μmol Gly · Kg^{-1} dry weight · 10 min^{-1}); s = glycine concentration (mM).

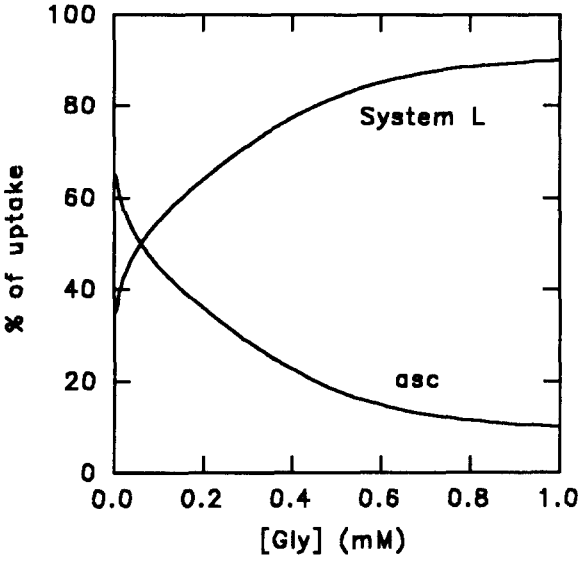

Fig. 10. Distribution of the Na^+-independent carrier-mediated glycine uptake between systems L and asc.

sensitive glycine uptake was obtained. A biphasic uptake appeared (Fig. 9), one component being of high capacity and low affinity, while the other was of high affinity and low capacity. Kinetic constants for the first component were V_{max} = 5.6 μmol Gly · Kg^{-1} dry weight · 10 min^{-1} and K_m = 0.2 mM and for the second V_{max} = 1.6 μmol Gly · Kg^{-1} dry weight · 10 min^{-1} and K_m = 0.02 mM. As a consequence, not all the Na^+-independent glycine uptake that is sensitive to inhibition by L-cysteine should be ascribed to the asc system in trout RBC.

To discriminate which system was of high or low affinity, the Na^+-independent uptake of glycine was tested at different concentrations of glycine in the presence of BCH alone or BCH plus L-cysteine. Figure 10 shows how the Na^+-independent uptake of glycine is distributed between systems L (measured as total Na^+-independent uptake minus Na^+-independent uptake in the presence of 5 mM BCH)

and asc (measured as Na^+-independent uptake BCH-inhibitable minus Na^+-independent uptake L-cysteine-inhibitable). It can be seen that as glycine concentration rose, system L became most important. Thus, the high affinity component cited above can be associated with system asc, while the low affinity carrier is the L-system.

The amino acid carrying ability of band 3 in human RBC was established by Young, Jones and Ellory (1981) by means of the specific inhibition of glycine uptake by SITS in a Na^+-free medium. This protein has been recently implicated in a taurine movement to obtain a regulatory volume decrease in skate RBC (Goldstein & Brill, 1991). Since this protein is present in the membrane of trout red cells (Romano & Passow, 1984; Michel & Rudloff, 1989), studies to measure its role as a glycine transporter in these cells were carried out. Figure 11 shows the effect of different SITS concentrations on the Na^+-independent glycine uptake in the presence of two different anions, Cl^- and SO_4^{2-}. When chloride was the main extracellular anion, there was no inhibition of the uptake (Fig. 11*B*) (in fact, there was a 10% increase), while in the presence of SO_4^{2-} SITS inhibited the uptake (Fig. 11*A*) and a half-maximal inhibitory concentration of 4 μM was obtained.

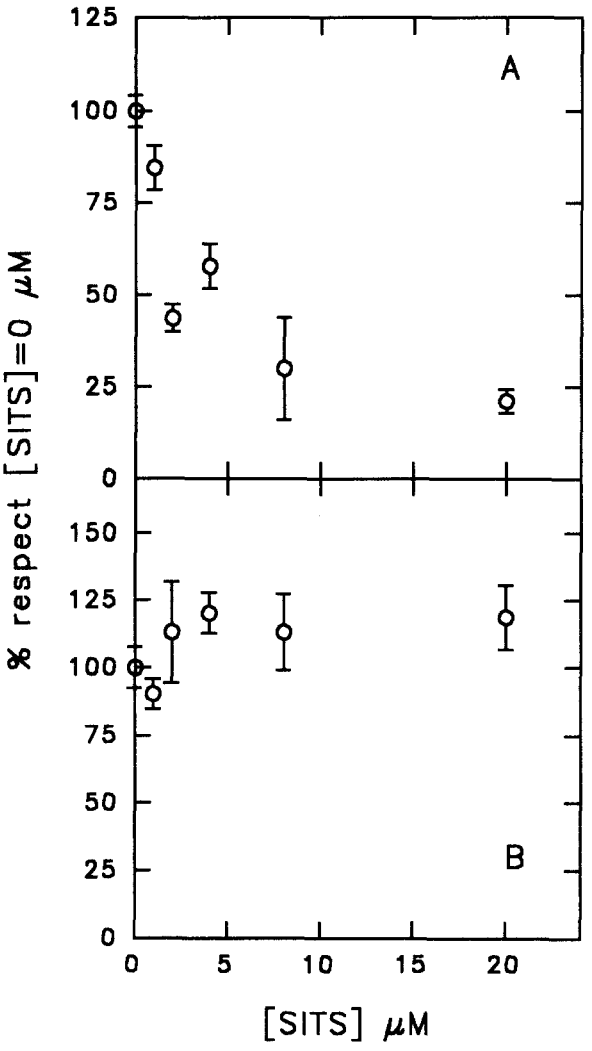

Fig. 11. Effect of SITS on the Na^+-independent glycine uptake by trout RBC. The initial rates were measured after 10 min of incubation. (*A*) Relative (referred to 0 μM SITS) uptake values when SO_4^{2-} instead of Cl^- was used as the main extracellular anion. (*B*) As in *A* but with Cl^- used as the main extracellular anion. Each point is the mean of 3–5 individual experiments. Bars are standard deviation of the mean.

Discussion

In the present study we have demonstrated the ability of trout RBC to take up glycine by means of different amino acid transport systems, some of them being Na^+-dependent (ASC and Gly), while others are Na^+-independent [an asc-type system and L system]. Band 3, which has been proposed as a glycine carrier for human red cells does not have a similar role in trout RBC. The present study confirms previous results about the existence of a transport system belonging to the ASC system family in trout RBC (Gallardo et al., 1992) while system A is lacking in these cells.

Despite the presence of these carriers, one of them almost specific for glycine, both half equilibration time and in/out ratios at equilibrium differ considerably from values for some related amino acids. Thus, $t_{1/2}$ for L-alanine uptake was 17 min (*unpublished data*), whereas for L-serine uptake it was 70 min in these cells (Gallardo et al., 1992). With respect to the in/out ratios, these two amino acids became three times concentrated inside trout RBC at equilibrium, while with glycine only a maximum value of 1.5 for this ratio has been achieved. This is a usual situation when working with this amino acid. Thus, both Na^+-dependent and Na^+-independent systems for glycine uptake operate in hagfish RBC, but in/out ratios of only about 7.5 are obtained in vitro (compared with an in/out ratio of 32 for conditions in vivo) (Fincham et al., 1990). Moreover, there is a great diversity in the concentrative ability of glycine uptake between RBC from different species, and an impairment in this capacity, associated with cell maturation, has been described (Winter & Christensen, 1965). A scarcely concentrative transport of glycine in vitro is also present in rabbit (Wheeler & Christensen, 1967), rat (Felipe, Viñas & Remesar, 1990) and guinea pig (Fincham, Willis & Young, 1984) mature RBC, while their immature forms are able to concentrate glycine under the same experimental conditions.

Because of its small size, glycine is a potential substrate for most of the membrane amino acid carriers, and Ellory et al. (1981) established the different routes for glycine influx in human erythrocytes. In the present study a different picture appears—the ASC system being the main carrier responsible for the Na^+-dependent glycine uptake, due to its high capacity. However, the apparent K_m for glycine uptake through this system is about 0.8 mM, far from the K_m value for L-serine uptake by these cells (about 0.03 mM; Gallardo et al., 1992). A more obscure finding, is that K_m values for the uptake through the Gly system are about 0.6 mM, far from K_m values in human (Ellory et al., 1981) or pigeon (Eavenson & Christensen, 1967; Imler & Vidaver, 1972) RBC and, except for species specificities, there are no clear explanations for these differences. In any case both K_m values are within the physiological range of plasma glycine.

Although trout RBC take up less glycine than human RBC through Na^+-independent systems (Ellory et al., 1981), the inhibition obtained with BCH indicates that system L is involved in the Na^+-independent glycine uptake. At first, this is not surprising because of the postulated wide scope of substrates that can be carried by this system. System L has not been fully characterized in the present study, but the ability of L-cysteine to inhibit the uptake of glycine through it was a surprise. L-cysteine has been considered as a "nearly" pure ASC/asc substrate (Barker & Ellory, 1990) and although a good substrate may not be a good inhibitor (Christensen, 1989), this amino acid has been thoroughly used to delimitate the uptake through these systems. Furthermore, the different inhibitory ability of BCH and L-cysteine at low or high glycine concentrations besides the inhibition by different amino acids and kinetic data indicate that there is another Na^+-independent carrier, namely the asc system. Due to its substrate preference, this system was originally related to the ASC system, although its activity is independent of extracellular Na^+ (Fincham et al., 1985). At present, there are indications that this analogy may be limited to horse RBC. For example, the asc system found in BSC-1 cells differs considerably from their ASC system, in substrate preference, *trans*-response and pH dependence (Kuhlmann & Vadgama, 1991). From our results (*to be published elsewhere*), there are at least two functionally different asc systems in trout cells. The carrier present in trout RBC highly resembles the asc system present in BSC-1 cells in both substrate preferences and pH dependence. However, it appeared as *trans*-inhibitable, while the kidney system does not show *trans*-effects. In any case, this transporter is able to carry glycine, although in the physiological range of plasma glycine concentration for this species, it is probably saturated.

Band 3 is another Na^+-independent carrier that has been implicated in glycine uptake by human RBC (Ellory et al., 1981; Young et al., 1981; King & Gunn, 1989) by means of a glycine anion and H^+ cotransport (King & Gunn, 1991). However, our results do not support a similar role in trout RBC. In the presence of Cl^- as the main extracellular anion, SITS does not inhibit glycine uptake, while it can act when SO_4^{2-} is used instead of Cl^-. In human red cells, SITS inhibits glycine uptake in the presence of either anion (Young et al., 1981). These results indicate differences in the properties of band 3 from these species, which may be related with the existence of structural differences between the two proteins (Michel & Rudloff, 1989). On the other hand, results from Goldstein and Brill (1991) show that sulfate does inhibit movements of taurine through band 3 under hyposmotic conditions, suggesting that both molecules share the same transporter. Unfortunately, there are no indications about this interaction under isosmotic conditions, when the interaction between this carrier and some other proteins (ankyrin, spectrin, . . .) is not modified.

Finally, the presence of all these amino acid carriers in the RBC of a relatively primitive group of fish such as salmonids, indicates their usefulness along the evolution of vertebrates. Despite this, some changes may be observed because their properties are not fully shared between systems of different species. On the other hand, the question as to the actual role for these different transporters, which carry nearly the same substrates, remains to be answered, although interesting hypotheses have been advanced (Christensen, 1982).

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