The Transport of Alanine, Serine, and Cysteine in Cultured Human Fibroblasts*

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The transport of L-alanine, L-serine, and L-cysteine has been studied in skin-derived diploid human fibroblasts in culture. Competition analysis, mathematical discrimination by nonlinear regression, and conditions varying the relative contribution of the various mediations have been used to characterize the systems engaged in the inward transport of these amino acids. All the adopted criteria yielded results showing that L-alanine, L-serine, and L-cysteine enter the cell by two Na⁺-dependent systems, System A and System ASC, and by a Na⁺-independent route, whose major component has been identified as System L. The apparent affinity of L-alanine, L-serine, and L-cysteine for the putative carrier was higher for System ASC than for System A. The transport $V_{\text{max}}$ for System A increased in response to cell starvation; after 12 h, its values were similar or higher than those exhibited by System ASC. At amino acid concentrations approaching those present in human plasma, System ASC appeared to be the primary mediation for the inward transport of L-alanine, L-serine, and L-cysteine in human fibroblasts. The contribution of System A was negligible in nonstarved cells and became appreciable under conditions of cell starvation. The Na⁺-independent System L made no substantial contribution to the uptake of L-alanine and L-serine and accounted for approximately one-fourth of the total uptake of L-cysteine.

In previous reports, we described the inward transport of neutral amino acids in cultured human fibroblasts and characterized some of the regulatory mechanisms acting thereon (1,2). Among these amino acids, L-alanine exhibited a distinctive feature. It entered the cell by more than one transport system and, depending upon the system considered, it underwent opposite controls by trans-effects (1) and by nutritional adaptation (2). The largest fraction of its uptake was by a Na⁺-dependent route and two components of it were discriminated by competition using the analogue MeAIB,1 a Site A-reactive, transport-specific substrate. This inhibition analysis was adequate to assess the activity of System A, whereas the identification of a second component relied upon a negative criterion, all the Na⁺-dependent migration not inhibited by MeAIB and by cysteic acid (a specific substrate for the anionic amino acid route in human fibroblasts, cf. Ref. 3) being assigned to System ASC. Recently, two criteria (inhibition analysis using N-methylalanine and a kinetic approach) were used by Feneant et al. (4), who confirmed the occurrence of two Na⁺-dependent systems for L-alanine uptake in human fibroblasts.

In order to characterize System ASC in cultured human fibroblasts, we have further extended the criteria of identification to include competition analysis, mathematical discrimination by nonlinear regression, and conditions in which the activity of System A was minimized by repression; we have also extended the study to three putative substrates of System ASC, namely L-alanine, L-serine, and L-cysteine. L-Serine is known to be a preferential substrate of System ASC. Recently, two criteria (inhibition analysis using N-methylalanine and a kinetic approach) were used by Feneant et al. (4), who confirmed the occurrence of two Na⁺-dependent systems for L-alanine uptake in human fibroblasts.

In the present paper, some indication of the physiological role of System ASC has been derived from the study of the transport of L-alanine, L-serine, and L-cysteine at external concentrations miming those present in human plasma. The mutual interactions between System ASC and System A (as the preponderant mediations for the Na⁺-dependent uptake of these amino acids by human fibroblasts) have been investigated by varying the nutritional state of the cells (starvation versus amino acid refeeding) which is known to alter the activity of System A (12, 13).

We report here a formal characterization of System ASC in cultured human fibroblasts, its relevance to the uphill transport of neutral amino acids, and its participation in determining the physiological level at which amino acids are held intracellularly.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Human fibroblasts, obtained from skin biopsy explants as described previously (1), were routinely grown in 10-cm diameter dishes (Costar, Cambridge, MA) in Medium 199 containing 10% fetal calf serum. Conditions of culturing were: pH 7.4; atmosphere, 5% CO₂ in air; temperature, 37 °C. All measurements of amino acid transport were made on fibroblast subcultures resulting from 4 × 10⁴ cells seeded onto 2-cm² wells of disposable multiwell trays (Costar) and incubated for 96 h in 1 ml of growth medium (always renewed 24 h before the experiment). At this time, the cells were confluent, as estimated visually with an inverted phase microscope, and slowly growing, as assessed by [methyl-3H]thymidine incorporation and conventional autoradiography of human fibroblasts exposed to [3H]thymidine pulses (14, 15).

Human fibroblasts were used between the 10th and the 20th passages; in this interval, they retained a normal complement of chromosomes, as assessed by a trypan-Leishman banding technique (16).

**Incubations and Uptake Assay**—In all experiments described in this paper (depletion, derepression), cell monolayers were incubated in Earle’s balanced salt solution always containing 10% dialyzed fetal calf serum (1,2). Depletion of intracellular amino acid pools lasted 90
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min before uptake assay (cf. Ref. 1). Amino acid uptake was assessed under conditions approaching initial entry rates in 10-s incubations at 37 °C using the cluster-tray method for rapid measurement of solute fluxes in adherent cells described by Gazola et al. (17). During this period, serum was omitted. In designated experiments, the medium contained nonmetabolizable amino acid analogues (MeAIB or BCH) at the desired concentrations and/or choline replaced Na+ in the sodium salts of the Earle’s mixture; in the latter case, cell monolayers were incubated for 10 min in the Na+-free medium prior to uptake assay. When L-cysteine was the tracer substrate for transport, the uptake medium contained 10 mM dithiothreitol to ensure complete reduction of the amino acid. Assays were terminated by rapidly rinsing the cell monolayers with ice-cold 0.9% NaCl. Acid-soluble pools were extracted with 10% trichloroacetic acid and counted in a liquid scintillation spectrometer. The cells were dissolved in 1 N NaOH and assayed for protein directly in the wells using a modified Lowry procedure (18) as described previously (17). All samples out of the range 20 to 30 μg of protein/cm² were discarded.

Cell Water and Calculations—The intracellular fluid volume was estimated from the difference between total water (l'H2O) and extracellular fluid volume (l'H2O) as described previously (18). The rates of amino acid uptake, corrected for unsaturable components (19), were expressed as micromoles/ml of cell water/min. Kinetic parameters were determined by a computer (Hewlett-Packard 9845S) using the Marquardt’s algorithm, a general procedure for least squares estimation of nonlinear parameters (20).

Materials—All sera, growth media, antibiotics, and trypsin solution were from Gibco, L-[2,3-3H]alanine (37 Ci/mmol), L-[3-3H]serine (14 Ci/mmol), L-[35S]cysteine (33 mCi/mmol), [methyl-3H]hymtidine (5 Ci/mmol), and [14C]urea (59 mCi/mmol) were obtained from American. [methoxy-3H]linulin (102.5 mCi/g) was purchased from New England Nuclear. 2-Methylaminopiperidine-4-carboxylic acid (BCH, isomeric form of System L) was from Aldrich. 2-Aminobicyclo(2,2,1)heptane-2-carboxylic acid (BCH, isomeric form of System L) was from Aldrich. 4-amino-1-guanidine-4-carboxylic acid (NH) and 4-amino-1-guanidine-4-carboxylic acid were generous gifts from Professor Halvor N. Christensen, University of Michigan, Ann Arbor. Sigma was the source of all unlabeled amino acids and other chemicals.

RESULTS

Na+ Dependence of L-Alanine, L-Serine, and L-Cysteine Uptake by Human Fibroblasts—Fig. 1 shows that L-alanine and L-serine are primarily taken up by a Na+-dependent route in cultured human fibroblasts. This observation confirms previous results for L-alanine (1, 4). L-Cysteine also entered the cell largely by a Na+-dependent mediation, but a Na+-independent component contributed substantially to its total uptake. As shown in Fig. 2, the latter component was strongly inhibited by BCH, as expected for a System L-mediated uptake, and much less by 4-amino-1-guanylpiperidine-4-carboxylic acid, a transport-specific substrate of the Na+-dependent System y+ (for cationic amino acids) in human fibroblasts (21). The inhibition of the Na+-independent entry of L-cysteine by BCH proved to be competitive in a Dixon plot (results not shown).

Discrimination of the Na+-Dependent Transport Systems—The study of the reactivity of L-alanine, L-serine, and L-cysteine for the Na+-dependent systems required proper conditions to pull the activity of these systems toward levels susceptible to unambiguous discrimination and to avoid the interference of trans-effects (trans-inhibition, trans-stimulation) by internal amino acid pools (1). These requirements were met by measuring amino acid uptake before and after a 12-h starvation of the cell monolayers in Earle’s balanced salt solution (2) and by depleting nonstarved fibroblasts of their cellular amino acid pools by a 90-min preincubation before uptake assay (1). Discrimination among components mediating the inward transport of the three amino acids has been approached by measuring their uptake (under intervals short enough to approximate initial entry rates) in the absence and in the presence of excess MeAIB, a transport-specific substrate of System A in human fibroblasts (MeAIB/natural amino acid ratio, 10:1) and by programming a digital computer to read out the total transport velocity from the summation of independent Michaelis-Menten expressions.

The analysis of the results presented in Figs. 3, 4, and 5 indicates that: (i) when the transport of L-alanine, L-serine, and L-cysteine was determined in nonstarved cells (parts a),
were measured in starved cells in the presence of excess MeAIB to obstruct transport by System A, indicating that more than one system of mediation was active. Consequently, the uptake of each amino acid was calculated by subtracting Na+-independent fractions from total uptake (as measured in the presence of Na+). Data from experiments with nonstarved cells were fitted by linear regression analysis

The near linearity of the $v$ versus $v/S$ plots ($r = -0.99$ for L-alanine, $r = -0.99$ for L-serine, and $r = -0.97$ for L-cysteine) suggested that a single mediation was primarily involved in their uptake; (ii) starvation enhanced the uptake of each amino acid, the increase being more pronounced at high than at low external amino acid concentrations (parts a). As a consequence, the $v$ versus $v/S$ plots became curvilinear, indicating that more than one system of mediation was active under these conditions. The well known increase in activity of transport System A upon starvation (2) supports the involvement of this agency in causing the observed heterogeneity; (iii) when the uptakes of L-alanine, L-serine, and L-cysteine were measured in starved cells in the presence of excess MeAIB to obstruct transport by System A (parts b), the linearity of the plots (noninhibitable components) was restored and the contribution of System A to the total uptakes could be estimated by difference. The latter contribution was appreciable for all the amino acids studied, particularly at high external concentrations; under the latter conditions, it became predominant when L-cysteine was the tracer substrate; (iv) the largest fraction of the Na+-dependent uptake of L-alanine, L-serine, and L-cysteine, not inhibited by excess MeAIB, is attributable to System ASC. This component encompassed almost completely the Na+-dependent uptake of all these amino acids in nonstarved cells and its absolute value was comparable in starved and nonstarved fibroblasts (parts a and b); (v) when the total Na+-dependent uptake of L-alanine, L-serine, and L-cysteine was resolved into components relevant to each component were fitted by linear regression analysis ($r = -0.99$ for MeAIB-noninhibitable component; $r = -0.98$ for MeAIB-inhibitable component). The combined curve was obtained by the parameters of the 2 regressions, fitted to total Na+-dependent uptake ($r^2 = 0.996$). The $Na^+$-dependent fraction of starved cells (●) best fitted by computer ($r^2 = 0.999$) and subdivided into components (straight lines) by computer.
where discrimination was attained by competition analysis. Two fractions identified above in starved cells (iii and iv), than the corresponding value for L-cysteine as obtained by computer analysis. Their kinetic properties were strikingly similar to those of the enzymes identified in nonstarved cells (parts a), and by nonlinear regression analysis using a computerized procedure based on the Marquardt's algorithm (parts c).

The values of each kinetic parameter were remarkably similar irrespective of the method adopted for their determination. The only exception was $K_m$ for L-cysteine uptake by System A, whose value calculated by computer analysis was almost double that derived from competition analysis. The average $K_m$ values for the transport of Lalanine and L-serine by System A were comparable (0.6–0.7 mM) and a little lower than the corresponding value for L-cysteine as obtained by inhibition analysis (0.96 mM); those for the transport by System ASC were approximately 5 to 6 times lower for L-alanine and L-serine and 18 times lower for L-cysteine (when compared to the $K_m$ value for System A as obtained by inhibition analysis). The average $V_{max}$ values for the transport of L-alanine and L-serine by System ASC were similar, in a range of 3–4 µmol/min/ml of cell water; that for L-cysteine was 2 µmol/min/ml of cell water. The corresponding values for the transport of Lalanine and L-serine by System A in starved cells approximated those reported for System ASC and the $V_{max}$ value for the transport of L-cysteine by System A was definitely higher than that obtained for System ASC.

Uptake by Cultured Human Fibroblasts of L-Alanine, L-Serine, and L-Cysteine at Human Blood Plasma Levels—The relative role of the transport systems involved in the uptake of L-alanine, L-serine, and L-cysteine by cultured human fibroblasts was assessed by transport measurements (under conditions allowing discrimination) at external amino acid concentrations approaching those present in human plasma (22) (Fig. 6). The Na+–independent fraction of transport has been determined in a Na+–free medium and the relative contribution of System L to it was evaluated by the addition of increasing concentrations of BCH to the assay medium. The Na+–dependent fraction of transport was calculated by subtracting the value obtained in the Na+–free medium from total uptake as measured in the presence of Na+.

In contrast, the inward transport of L-alanine, L-serine, and L-cysteine by cultured human fibroblasts was assessed by transport measurements (under conditions allowing discrimination) at external amino acid concentrations approaching those present in human plasma (22) (Fig. 6). The Na+–independent fraction of transport has been determined in a Na+–free medium and the relative contribution of System L to it was evaluated by the addition of (L) components. Data relevant to each component were fitted by linear regression analysis ($r = -0.97$ for MeAIB-noninhibitable component; $r = -0.96$ for MeAIB-inhibitable component). The combined curve was obtained by the parameters of the 2 regressions, fitted to total Na+–dependent uptake ($r^2 = 0.999$). The Na+–dependent fraction of starved cells (parts a) best fitted ($r^2 = 0.999$) and subdivided into components (straight lines) by computer.

### Table I

| Amino acid | System A | System ASC |
|------------|----------|------------|
|            | $K_m$ (µmol/min/ml cell water) | $V_{max}$ (µmol/min/ml cell water) | $K_m$ (µmol/min/ml cell water) | $V_{max}$ (µmol/min/ml cell water) |
| L-Alanine  | Competition analysis | 0.64 | 3.2 | 0.10 | 3.8 |
|            | Computer derived cells | 0.54 | 5.9 | 0.08 | 3.2 |
|            | Unstarved cells | 0.10 | 4.1 |
| L-Serine   | Competition analysis | 0.72 | 3.4 | 0.16 | 4.1 |
|            | Computer derived cells | 0.74 | 4.9 | 0.13 | 3.1 |
|            | Unstarved cells | 0.16 | 4.2 |
| L-Cysteine | Competition analysis | 0.96 | 3.7 | 0.05 | 1.8 |
|            | Computer derived cells | 1.82 | 3.2 | 0.06 | 2.0 |
|            | Unstarved cells | 0.05 | 2.3 |
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UNSTARVED STARVED

![Graphs of amino acid transport](https://example.com/graphs)

**Inhibitor Concentration [mM]**

**Fig. 6. Pathways of L-alanine, L-serine, and L-cysteine transport at the amino acid concentrations present in human blood plasma (22).** Human fibroblast cultures, grown to confluence in Medium 199 (96 h), were either depleted of intracellular amino acids (unstarved cells, see Fig. 1), or incubated for additional 12 h in Earle's balanced salt solution (starved cells). The transport assays at the amino acid concentration indicated was made either in unstarved or in starved cells during 10 s at 37°C (a) in a Na+-containing medium in the absence and in the presence of increasing concentrations of MeAIB (2.5 to 10 mM) and (b) in a Na+-free medium in the absence and in the presence of BCH (2.5 to 10 mM). In each part, the curves are inhibition profiles of amino acid uptake by MeAIB in Na+-containing medium (upper curve, •) and by BCH in Na+-free medium (lower curve, □). At right, bars represent total uptake divided into 3 fractions contributed by System A (open), System ASC (shaded), and System L (black) as described in the text.

The results presented in this paper provide evidence that L-alanine, L-serine, and L-cysteine share the same transport pathways to gain entrance into cultured human fibroblasts. At low external amino acid concentrations (comparable to those present in human plasma), the major transport component corresponds to a Na+-dependent mediation endowed with a low $K_m$ value (Table I). This mediation accounts for almost the entire Na+-dependent amino acid transport in nonstarved cells. Its activity does not change upon either cell starvation or addition of excess MeAIB. Sensitivity to starvation and lack of inhibition by a typical Site A-reactive substrate indicate that this mediation can be identified with transport System ASC (cf. Refs. 7 and 13) known to operate in cultured human fibroblasts (1). A rigorous assessment of the relative contribution of System ASC to amino acid uptake using high concentrations of transport-specific substrates to obstruct other electrogenic mediations (i.e. MeAIB for System A) may be hampered by changes in membrane potential caused by the presence of the added inhibitor (23). The consequent alteration in the overall rate of Na+-dependent transport, however, must be of minor importance under our experimental conditions. In fact, at least three different criteria (competition analysis, mathematical discrimination by computer, and transport measurements in nonstarved, repressed cells) yielded comparable values for the kinetic parameters defining the activity of System ASC.

A second Na+-dependent mediation for L-alanine, L-serine, and L-cysteine became significant in cultured human fibroblasts upon starvation. Its activity increased steadily with the progress of starvation up to 20 h (results not shown) and was fully inhibited by the addition of MeAIB. These features allow us to ascribe this transport component to System A, the sole Na+-dependent mediation for neutral amino acids which exhibits adaptive regulation in cultured human fibroblasts (Ref. 2 and results not shown).

As judged from the relevant kinetic parameters of transport (Table I), the apparent affinity of L-alanine, L-serine, and L-cysteine toward the putative carrier is higher for System ASC than for System A. Among these amino acids, the lowest $K_m$ value for System ASC is that of L-cysteine. On the other hand, the apparent $K_m$ value for the uptake of these amino acids by System A is by no means physiologically insignificant, being of the same order of magnitude as that observed for such natural or model amino acid substrates of System A as L-proline and MeAIB in human fibroblasts (24). On this ground, we cannot confirm for our biological model the results reported by Kiberg and co-workers (10, 11) in cultured rat hepatocytes, indicating that L-cysteine could be considered a transport-specific substrate for System ASC. Only in un-
starved human fibroblasts, when System A is fully repressed (2), is the Na’-dependent uptake of L-cysteine attributable to System ASC, but even under these conditions, a Na’-independent fraction contributes substantially to the total uptake of this amino acid. Under the same circumstances, L-serine is perhaps a better substrate than L-cysteine and L-alanine, its entry into the cells by Systems A and L being almost negligible. L-Serine has been found the best substrate of System ASC also in Chinese hamster ovary cell line CHO-K1 (25).

Fig. 6 shows that, at amino acid concentrations close to those present in human plasma, L-alanine, L-serine, and L-cysteine are substrates for both System ASC and System A. The latter is typically an adaptive system. Its activity is subject to a fast modulation through trans-effects (trans-inhibition and its release) by the intracellular concentration of Site A-reactive amino acids (1) and to a long lasting modulation through adaptive regulation. This control mechanism correlates inversely the activity of System A with the extracellular concentration of Site A-reactive amino acids (2).

Provided that the behavior of Systems A and ASC in fibroblasts of human tissues in vivo parallels that reported here for cultured cells, one may speculate that these two mediations assist each other in regulating the uptake of common amino acid substrates: when the external substrate concentration decreases, the rate of their entry via System ASC should slow down and the increasing activity of System A (release from trans-inhibition, derepression) would tend to counteract this slackening; conversely, when the external substrate concentration increases, the enhanced rate of their entry via System ASC could be buffered by the progressive decrease of the activity of System A (trans-inhibition, repression).

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