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Spectrophotometric determination of peptide transport with chromogenic peptide mimetics

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Abstract

A spectrophotometric assay to determine peptide transport has been developed. Using two chromogenic peptide mimetics, L-phenylalanyl-L-2-sulfanilylglycine (PSG) and L-phenylalanyl-L-3-thiaphenylalanine (PSP), the peptide transport patterns in individual cell species can be evaluated effectively. After the addition of PSG to a HeLa cell suspension, sulfanilic acid accumulated progressively inside, but not outside, the cells, demonstrating that PSG was transported wholly intact. The addition of PSP to the same cell suspension was followed immediately by extracellular thiophenol production. Measurement of the rate of thiophenol release thereby provided direct determination of PSP transport. The thiophenol release was consistent with Michaelis–Menten kinetics, with a K_m of 0.016 mM and a V_{max} of 5.07 nmol/min (1×10^6 cells/ml, pH 7.4, 37 °C). The resulting kinetic constants estimated were in agreement with values determined by single-substrate enzyme kinetics. Using PSP, transport kinetics of various dipeptides was examined by competitive spectrophotometry. As a result, dipeptides tested could be ranked in order of kinetic power for their transport.

Keywords: Peptide transport; Peptide transport kinetics; HeLa cells; Chromogenic assay; Peptide transporter; Peptide mimetics; Detector peptide; Competitive spectrophotometry

During the past few decades, researchers have attempted to take advantage of the properties of microbial peptide transport systems that lack side chain specificity, also known as portage transport [1,2]. The existence of such transport systems, which are regarded as indispensable for assimilating natural peptides, is also apparent in mammalian cells [3,4]. Furthermore, peptide transporters in humans control the half-lives of various peptidomimetic drugs, and this may have substantial impact in vivo [5]. Current studies to evaluate the pharmacological efficacy of peptidomimetics, therefore, have focused on the level of peptide transport in appropriate cell lines transfected with relevant genes [6].

Radioactively labeled peptides have been widely used for peptide transport studies. However, there are some problems associated with interpreting the observed data. Along with the inconvenient cell extraction methods, measurement of intracellular radioactivity is particularly difficult. By most accounts, this procedure can result in decisive errors unless otherwise established individual criteria are used to corroborate the results. Furthermore, peptides may be broken down prior to transport, or rapid intracellular metabolism may significantly affect the observed data. For this reason, nonhydrolyzable peptides (e.g., glycylsarcosine, D-enantiomers) often are used as detectors [6,7]. Unfortunately, such unnatural peptides are not recommended for in-depth analyses because they are also unfavorable substrates for peptide transport systems.

Peptides bearing an amino acid residue with a substituted moiety (e.g., –S–, –NH–) at the β-position are stable. But these nucleophilic substituents are readily liberated due to the lone pair of electrons on the nitrogen atom after hydrolysis of the peptide bond right in front of the amino acid mimetics [8,9]. If the leaving groups are quantifiable

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(e.g., thiophenol, sulfanilic acid), such peptides can be useful as detectors. Turning our attention to their differential mobility across the cell membrane, we have explored their applicability to direct determination of peptide transport in intact cells. In the current study, L-phenylalanyl-L-2-sulfanilylglycine (PSG)[1] and L-phenylalanyl-L-3-thiaphenylalanine (PSP) were prepared and their suitability for kinetic studies of general peptide transport was evaluated.

Materials and methods

Source of chemicals

PSP and PSG were synthesized as described previously [8,9] with enzymatic stereoisomer separation. Briefly, after treatment of N- and C-terminal-protected PSP and PSG with bovine pancreas α-chymotrypsin (EC 3.4.21.1) from Sigma (St. Louis, MO, USA), *N*-carbobenzoxy-L,L-PSP and *N*-carbobenzoxy-L,L-PSG were precipitated, filtered, and then deprotected by HBr in acetic acid. PSP: ^{1}H NMR (5% D_2O/DMSO)—δ 3.2 (d, 2H), 4.3 (t, 1H), 5.6 (s, 1H), 7.2–7.8 (m, 10H). PSG: ^{1}H NMR (D_2O/DMSO)—δ 2.9–3.0 (d, 2H), 4.1 (t, 1H), 5.5 (s, 1H), 6.5–7.5 (m, 9H). Benzylchloroformate was obtained from Aldrich (Milwaukee, WI, USA), HBr (solution in 33% acetic acid) was obtained from Merck (Schuchardt, Germany), sulfosalysilic acid was obtained from Yakuri (Osaka, Japan), triethylamine was obtained from Zunsei (Tokyo, Japan), and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) and peptides were obtained from Sigma. All other reagents were commercial preparations of analytical grade.

Preparation of intact cells

HeLa cells were grown in a 5% CO_2 incubator at 37 °C using plastic flasks or 96-well titration plates with Eagle's modified minimal essential medium containing 10% fetal bovine serum. Cells were harvested by treatment with trypsin and then washed twice by resuspension in cold phosphate-buffered saline (PBS) and centrifugation (1800*g* for 10 min) [10]. The harvested cells were resuspended in assay buffer (20 mM Hepes containing 2% D-glucose, 1 mM EDTA, and 2 mM $MgCl_2$, pH 7.4) to approximately 1×10^7 cells/ml in an ice bath.

Peptide transport assay

Competitive spectrophotometry

A small volume of the cell suspension (usually 1 ml) was placed in a 15-ml Falcon tube containing assay buffer (8.5–8.7 ml) with 0.1 mM DTNB and kept in a water bath at 37 °C. The blank used for absorbance measurements contained cells and DTNB only. Transport was initiated by the addition of 0.01 ml of 10 mM PSP in the presence or absence of a competing agent to give a final volume of 10 ml at 37 °C. Aliquots (1.2 ml) of the reaction mixture were removed every 3 to 7 min and centrifuged at 4 °C. The resulting supernatants were dispensed into cuvettes (1 cm light path length), and the absorbance was measured at 412 nm. The progress curves obtained with competing peptides were compared with a normalized progress curve with PSP only, and the resulting time lag, Δt (min), at every local PSP concentration was measured with a ruler [11].

Single-substrate kinetics

Cells incubated with Ala-Phe were aliquoted at 1-min time intervals, washed with PBS, exposed to a thermal change (freezing–thawing), and then homogenized. After removing cell debris by centrifugation, the supernatant was treated with trichloroacetic acid and centrifuged (9000*g* for 10 min). The soluble fraction was neutralized and reacted with approximately 0.01 U (10 μg protein) of a phenylalanine ammonia lyase (Sigma) at pH 8.5 to quantify ammonia. Initial velocity was estimated by extrapolating the observed rates at 1- to 2-min intervals for 20 min.

Peptidase assay

HeLa cells (1×10^7 cells/ml) were homogenized in assay buffer containing phenylmethanesulfonyl fluoride (PMSF, pH 7.4) and centrifuged (1800*g* for 10 min). The resulting supernatant was precipitated with ammonium sulfate; when necessary, portions were centrifuged (9000 *g* for 10 min) and the precipitate was dissolved in 20 mM Hepes buffer (pH 7.4) for direct use as an enzyme sample. For PSP hydrolysis [8], 1 ml of the reaction mixture containing 0.1 mM PSP, 0.1 mM DTNB, and 50 mM Hepes–Tris (pH 7.4) was placed in a cuvette. The enzyme reaction was initiated by the addition of enzyme solution (~0.1 mM, with competitors being added as necessary). The absorbance at 412 nm was measured for 10 min and corrected for the small increase observed in the cell extract alone. PSG hydrolysis was determined by measuring the amount of sulfanilic acid liberated; the absorbance at 560 nm was measured for the diazo adduct formed by consecutive reaction with 0.2 N HCl, 0.02% Na nitrite, 0.01% ammonium sulfamate, and 0.1 mg/ml *N*-(1-naphthyl)ethylenediamine · 2HCl [9].

Statistical analysis

Unless stated otherwise, all data values given in this article represent the means of at least two separate determinations with a triplicate assay ± standard errors.

Results

Living systems assimilate small peptides either intact or in the form of amino acids, depending on the available

[1] *Abbreviations used:* PSG, L-phenylalanyl-L-2-sulfanilylglycine; PSP, L-phenylalanyl-L-3-thiaphenylalanine; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); PBS, phosphate-buffered saline; PMSF, phenylmethanesulfonyl fluoride.