The Uptake of Pyrroline 5-Carboxylate

GROUP TRANSLLOCATION MEDIATING THE TRANSFER OF REDUCING-OXIDIZING POTENTIAL*

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The cellular uptake of pyrroline 5-carboxylate (P5C) is of interest because this nutritionally responsive constituent of human plasma can mediate the transfer of oxidizing potential into cells and stimulate the production of phosphoribosyl pyrophosphate. Using a cloned line of Chinese hamster ovary cells, we found that the uptake of P5C was saturable, temperature-dependent, and sensitive to metabolic inhibitors. Furthermore, this uptake of P5C exhibited unusual features. It was independent of sodium ion and had an pH optimum of 6.4. The kinetics characteristics of P5C uptake included an apparent $K_m$ of 0.46 ± 0.04 mM and a $V_{max}$ of 19.6 ± 1.8 nmol/min/mg. Although the $V_{max}$ for P5C was comparable to those for certain other amino acids, e.g. leucine, it was significantly higher than that for $\alpha$-methylaminoisobutyric acid in these cells. Importantly, there was no interaction between these amino acids and the uptake mechanism for P5C. Twenty naturally occurring amino acids, each at a concentration of 5 mM, were without effect on the uptake of P5C. Interestingly, the uptake mechanism for P5C is unusual in that it is linked to the transfer of reducing-oxidizing potential. Over wide ranges of P5C concentration and duration of incubation, P5C entry is coupled to its conversion to proline and the concomitant oxidation of reduced pyridine nucleotide with stimulation of the pentose phosphate shunt. In fact, no free P5C derived from the medium could be detected in cells. Our interpretation of these findings is that P5C uptake occurs by its own unique mechanism, a group translocation that mediates the transfer of reducing-oxidizing potential.

The mechanism mediating the uptake of pyrroline 5-carboxylate (P5C)* is of increasing interest. Not only is P5C the immediate precursor of proline, but also as the common intermediate in the interconversions of proline, ornithine, and glutamate, it directly links the urea and tricarboxylic acid cycles. Although once considered principally as a cellular metabolite, P5C is now recognized as a nutritionally responsive constituent of human plasma and a potent metabolic stimulator. Although structurally similar to proline (Fig. 1), P5C, but not proline, can stimulate the activity of the pentose phosphate shunt and increase the production of 5-phosphoryl ribosyl 1-pyrophosphate (1, 2). The oxidation of NADPH accompanying the conversion of P5C to proline mediated by P5C reductase is presumed to be the underlying mechanism. The concentration-dependent metabolic effects of P5C occur in a variety of cells including erythrocytes, normal human fibroblasts, glial cells, Chinese hamster ovary cells, and isolated tissues such as lens (1-4). This is further illustrated when cells with asymmetric metabolism of P5C and proline are co-incubated. Hepatocytes can produce and release P5C because of their abundance of proline oxidase whereas erythrocytes lack proline oxidase but do have P5C reductase. The P5C released by the hepatocytes activates the pentose phosphate shunt in erythrocytes (5). The physiologic relevance of these effects of extracellular P5C has been supported by the identification of P5C as a constituent of human plasma (6). In response to meals, P5C levels in plasma may increase 10-15-fold (7). Thus, extracellular P5C can function as an intercellular communicator which, when taken up by peripheral cells, can stimulate intracellular metabolism. For these reasons we examined the uptake of P5C in cultured cells.

** MATERIALS AND METHODS**

Chemicals—$\alpha$-[U-14C]Methylaminoisobutyric acid (MeAIB; 53.5 mCi/mmol) and L-[U-14C]Serine (165 mCi/mmol) were purchased from Du Pont-New England Nuclear; L-[U-14C]Leucine (348 mCi/mmol) and D-[1-14C]glucose (45-60 Ci/mmol) were obtained from Amersham Corp. L-[U-14C]P5C (266 mCi/mmol) was prepared as described by Smith et al. (8). DL-P5C dinitrophenyhydrazon was purchased from Sigma and was hydrolyzed by the method of Mezl and Knox (9). As a final step of purification, DL-P5C was passed over a 0.5-ml bed volume column of AG-50-X8, hydrogen form, 200–400 mesh (Bio-Rad). The concentration of DL-P5C was determined colorimetrically using o-aminobenzaldehyde as described by Strecker (10). All other materials were purchased from Sigma.

Cells—Chinese hamster ovary cells (CHO-K1) are auxotrophic for proline. A cloned subline (R25), prototrophic for proline, was derived from CHO-K1s. Proline prototrophy was due to recovery of the ability to synthesize proline from glutamate. These cells were routinely grown in Eagle’s minimum essential medium supplemented with 10% fetal bovine serum (HyClone). For the experiments, cells were detached from a confluent monolayer by trypsinization and inoculated onto Costar 24-well trays (16-mm diameter wells) at a density of 5.0 × 10^4 in 1 ml of medium. Cells were allowed to grow for 3 or 5 days to confluency. One hour before the measurement of uptake, growth medium was aspirated and replaced by 1 ml of Krebs-Ringer phosphate buffer with glucose (5.5 mM) and CaCl_2 (0.2 mM) and the specified amino acid at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air.

Measurement of Uptake—The uptake of P5C and amino acids was measured using the cluster-tray method as described by Gazzola et al. (11). Cells were incubated in 0.5 ml of Krebs-Ringer phosphate buffer with glucose (5.5 mM) and CaCl_2 (0.2 mM) and the specified amino acid at 37°C in a shaking water bath in room air. After the incubation period, the medium was removed by inverting and shaking the plate, and each well was washed twice with 2 ml of cold phosphate-buffered saline. The residual phosphate-buffered saline was aspirated, and 250 μl of cold trichloroacetic acid was then applied to each well. After 30 min of equilibration, 200 μl was transferred from each well.
to a scintillation vial containing 12 ml of Hydrofluor (National Diagnostics Inc.), and the radioactivity was determined by liquid scintillation spectrometry.

**Sodium-free Medium**—Sodium-free medium was prepared by replacing the sodium chloride and sodium phosphate in Krebs-Ringer bicarbonate with their corresponding choline salts. Choline phosphate was prepared by titrating phosphoric acid to neutrality with choline hydroxide.

**Metabolism of P5C**—P5C and its metabolic derivatives were recovered by cation-exchange chromatography as previously described (12). After incubation and washing with phosphate-buffered saline, the monolayer was extracted with 0.3 ml of 10% trichloroacetic acid. An aliquot was treated with o-aminobenzaldehyde and then applied before the activity of the pentose phosphate shunt was measured by exposing the monolayer to radiolabeled P5C for 1 min. The activity of the pentose phosphate shunt was measured by applying the A, L, and ASC systems, respectively. The values for K, V, and V max for these three amino acids as well as for P5C are shown in Table I. The apparent K, for P5C is comparable to those for MeAIB and serine and higher than that for leucine. The V max for P5C is about the same as for serine and higher than those for leucine and MeAIB. Clearly, the uptake of P5C into R25, our clone of Chinese hamster ovary cells, is

**RESULTS**

**Characteristics of P5C Uptake**—We first characterized the uptake of P5C in R25 cells. With increasing duration of incubation with radiolabeled P5C at a concentration of 0.08 mM, radioactivity accumulated in the monolayer in a curvilinear fashion (Fig. 2). Uptake was linear for the first 5 min and approached a plateau by 20 min when the ratio of intracellular to extracellular radioactivity was 10.7. Based on the observed linearity over the first 5 min, we used 1-min incubations as a measure of initial velocities of P5C uptake. When initial velocities were examined with P5C concentrations varying over a 2000-fold range, uptake exhibited saturation consistent with a carrier-mediated system. Using the Lineweaver-Burk transformation of Michaelis-Menten kinetics, we calculated a value for the apparent V max of 0.46 ± 0.04 nmol/min/mg protein (Fig. 3). To determine whether this carrier-mediated uptake of P5C was energy-dependent, we treated cells with oligomycin or carbonyl cyanide m-chlorophenylhydrazone, an organic cyanide, at concentrations of 5 μg/ml and 20 μM, respectively. Cells were preincubated with or without these inhibitors for 15 min in Krebs-Ringer phosphate without glucose. The absence of glucose itself was without effect on P5C uptake, but oligomycin inhibited 57.1% and carbonyl cyanide m-chlorophenylhydrazone 65.1%. Thus, P5C uptake was not only carrier-mediated but also energy-dependent.

**Comparison of the Uptakes of P5C and Amino Acids in R25 Cells**—Although amino acid transport has been characterized for Chinese hamster ovary cells, we studied several amino acid transport systems in R25 cells as a basis of comparison for our studies of P5C uptake (13). MeAIB, leucine, and serine were studied because their uptakes are mediated principally by the A, L, and ASC systems, respectively. The values for K, V, and V max for these three amino acids as well as for P5C are shown in Table I. The apparent K, for P5C is comparable to those for MeAIB and serine and higher than that for leucine. The V max for P5C is about the same as for serine and higher than those for leucine and MeAIB. Clearly, the uptake of P5C into R25, our clone of Chinese hamster ovary cells, is

**FIG. 1.** Structural formulas of P5C and glutamic γ-semialdehyde, tautomeric forms that are in spontaneous equilibrium. At physiologic pH, P5C is the predominate form. Proline is also shown for reference.

**FIG. 2.** Time course of P5C uptake in Chinese hamster ovary cells. The initial concentration of P5C in the medium was 0.08 mM. Values shown represent means ± S.D. of four determinations from two experiments.

**FIG. 3.** Michaelis-Menten kinetics of P5C uptake. P5C uptake was determined over a 2064-fold range of P5C concentrations. The duration of uptake was 1 min. The nonsaturable diffusion component was subtracted from each point. Values shown represent means ± S.D. of six determinations from three separate experiments. The inset shows Lineweaver-Burk transformations of the data.

**TABLE I**

| Amino Acid | V max (nmol/min/mg protein) | K m (mM) |
|------------|-----------------------------|---------|
| P5C        | 19.8 ± 0.18                 | 0.46 ± 0.04 |
| MeAIB      | 1.15                        | 0.30    |
| Ser        | 16.0                        | 0.27    |
| Leu        | 5.60                        | 0.08    |
of a magnitude at least as great as for major amino acid transport systems.

*Amino Acids Do Not Inhibit the Uptake of P5C*—Even though the kinetic parameters for P5C uptake are comparable to those for other amino acid transport systems, the system for P5C uptake is not shared with other amino acids. At a concentration of 10 mM, amino acids of the L system (leucine, phenylalanine), ASC system (serine), and A system (AIB, MeAIB, proline) as well as amino acids with their own special transport systems were without effect on the uptake of P5C (Table II). Of special interest, proline, with its structural similarity to P5C, was without effect. In addition, all 20 naturally occurring amino acids were tested, and they were without significant effect on the uptake of P5C (data not shown).

*Sensitivity to p-Chloromercuribenzosulfonate—*Sensitivity of uptake to inhibition by p-chloromercuribenzosulfonate (pCMBS) has been used as evidence supporting the existence of specific carrier proteins for amino acid transport (14-16). pCMBS enters cells poorly, making possible the argument that its effects are mediated by interacting with surface sulfhydryl groups. We found that P5C uptake was very sensitive to inhibition by pCMBS. In cells preincubated for 10 min with 0.1 mM pCMBS, the uptake of P5C was essentially abolished. Under similar conditions, the uptake of MeAIB was inhibited 18%.

*The Effect of Sodium and pH on P5C Uptake*—Because sodium ion is necessary for the transport of many amino acids as well as other small molecules, we considered whether P5C uptake was sodium-dependent. Interestingly, P5C uptake appeared sodium-independent (Fig. 4). Even when sodium ion in the medium was completely replaced by choline, the uptake of P5C was not significantly changed. The uptake of MeAIB served as a control since it is well known that the uptake of this analogue by the A system is completely sodium-dependent. Indeed, the uptake of MeAIB was completely abolished when sodium ion was replaced by choline.

The A system is sensitive to small decreases in pH, whereas the ASC system is less sensitive (17-19). The characterization of the pH optimum for the uptake of P5C may provide insight into relevant mechanisms. In contrast to the A and ASC systems, the uptake of P5C increases with decreasing pH (Fig. 5). In fact, a sharp pH optimum was observed at pH 6.4. Preliminary results indicate that the apparent affinity of P5C for its carrier is increased as the buffer becomes more acid, i.e., the \( K_a \) decreases with decreasing pH. Presumably this effect is due to the protonation of P5C or its carrier.

*Metabolic Fate of P5C*—Because the effector functions of P5C added to incubated cells are linked apparently to its metabolism, we carefully examined the metabolic fate of P5C under the conditions for P5C uptake. We varied the duration of incubation between 0.5 and 20 min (Fig. 6) and used concentrations of P5C between 2 \( \mu M \), a concentration within the physiologic range for human plasma, and 0.64 mM, a concentration well above the apparent \( K_a \) for uptake (Table III). Surprisingly, over the entire range of conditions we recovered no radiolabeled P5C intracellularly. Instead, we found only radiolabeled proline; proline accounted for the total intracellular accumulation of P5C-derived radioactivity. Since the conversion of P5C to proline is catalyzed by P5C reductase, we tried to dissociate P5C uptake from its conversion to proline by inhibiting the enzyme. P5C reductase is known to be sensitive to inhibition by proline (20, 21), but preincubation of cells for 30 min with 5 mM proline or, indeed, with 1 mM P5C had no effect on uptake and did not dissociate the uptake of P5C from its conversion to proline. Since P5C reductase is also sensitive to inhibition by NADP+ (21, 22), we preincubated cells with 1 mM hydrogen peroxide in an attempt to alter NADP+/NADPH ratios but found no effect either on uptake or P5C metabolism. Thus, the conversion of...
column chromatography as described under "Materials and Methods." Values for total uptake are expressed as percent of total radioactivity recovered from the column effluent. The amount of radioactivity obtained from incubations at 4°C was used as a blank and subtracted from the value for total radioactivity.

| Time (min) | % recovered as proline at 0.002 mM | % recovered as proline at 0.064 mM |
|------------|----------------------------------|----------------------------------|
| 0.5        | 101.2 ± 0.26                     | 100.8                            |
| 2          | 101.8                            | 110.0 ± 0.82                     | 98.5                            |
| 5          | 100.3                            | 95.4 ± 0.22                      | 97.5                            |
| 10         | 100.4                            | 38.93 ± 6.47                     | 96.7                            |
| 20         | 100.6                            | 47.66 ± 1.44                     | 102.0                           |

The Uptake of Pyrroline 5-Carboxylate

and L (13); the uptake of P5C is not mediated by any of these systems. First, the A and ASC systems were excluded because they were sodium-dependent whereas P5C uptake was not. Although the L system was independent of sodium, amino acids transported by the L system were without effect on the uptake of P5C. In fact, 20 naturally occurring amino acids were without effect on P5C uptake, thus making it unlikely that P5C uptake occurred through any known or yet to be defined transport mechanism for amino acids.

Since the uptake of P5C was not mediated by established amino acid transport systems, the characterization of the uptake system for P5C became the major focus. Though exhibiting saturable kinetics and energy dependence, the uptake of P5C did not establish a concentration gradient, the sine qua non for an active transport mechanism. To the contrary, cells incubated with radiolabeled P5C over a wide range of experimental conditions accumulated no P5C but only proline; quantitatively, proline accounted for all the radioactivity taken up by cells. A trivial explanation for these findings is that P5C entered by passive diffusion and then was converted to proline by P5C reductase. However, the observed kinetics for uptake, if due to saturation of P5C reductase, would occur only if intracellular P5C approached the concentration of P5C in the medium. Such was not the case. Even under conditions where saturation was approached, no intracellular P5C was found.

More likely, a specific carrier mechanism for P5C mediates its intracellular entry, and interpretation is supported by two lines of evidence. First, the unusual pH optimum suggests that protonation of the amino moiety of P5C or of amino groups on a carrier is important for the cellular entry of P5C. But more importantly, the inhibition of P5C uptake by pCMBS, an organic mercurial compound that enters cells slowly, if at all, suggests the functional importance of sulfhydryl groups on the cell surface. Similar findings have been used to support the identity of a carrier for the A system in isolated hepatocytes (14).

Nevertheless, the entry of P5C mediated by its putative carrier was closely associated with its conversion to proline. On a functional level, these two processes could not be dissociated even with manipulations designed to inhibit P5C reductase, the enzyme catalyzing the conversion of P5C to proline. An attractive hypothesis is that the uptake of P5C is mediated by a mechanism similar to the "group translocation"
described by Kundig and Roseman (23, 24). In anaerobic bacteria, the high energy phosphate group from phosphoenolpyruvate is transferred to a low molecular weight heat-resistant protein and then to an accepting sugar as it enters the cell. Thus, phosphoenolpyruvate serves as an internal source for phosphorylating extracellular substrates. Although P5C is not the recipient of a specific group, e.g. high energy phosphate, it does have the ability to accept electrons, concomitant to its cellular entry.

Thus, P5C serves as an external source for oxidizing intracellular molecules, e.g. pyridine nucleotides. To our knowledge, this uptake mechanism for P5C is the first "group translocation" for reducing-oxidizing potential described for mammalian cells.

We are intrigued by the possible molecular mechanisms mediating this group translocation for P5C. Among the possibilities a close physical association between the putative membrane carrier and P5C reductase is included. Although cytosolic, P5C reductase migrates with plasma membranes on sucrose density gradients. Thus, a membrane-associating domain on the enzyme may allow physical association with the putative P5C carrier. Alternatively, the carrier itself may catalyze the reduction of P5C. Reduced pyridine nucleotides either free or membrane-associated or even a yet undefined source could serve as electron donors. Although cell homogenates cannot convert P5C to proline in the absence of reduced pyridine nucleotides, this does not rule out the possibility if vectorial asymmetry is critical.

Finally, the discovery of this "group translocation" for P5C uptake provides an explanation for the novel effects of exogenous P5C as a potent source of oxidizing potential (25). The lack of overlap with amino acid transport mechanisms is not surprising if the uptake of P5C is to transfer reducing-oxidizing potential rather than provide substrates for protein synthesis. Together with the finding of circulating P5C in human plasma, we can hypothesize that this uptake mechanism may represent a previously unappreciated mechanism for transmembrane signaling. The stimulatory effects of P5C synergistic to those produced by certain growth factors provide support for this hypothesis. Indeed, P5C may mediate physiologically the redox effects observed on mitogenesis in tissue culture with certain pharmacologic agents (26, 27).

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