pGlu-L-Dopa-Pro: A Tripeptide Prodrug Targeting the Intestinal Peptide Transporter for Absorption and Tissue Enzymes for Conversion

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Purpose. The purpose of this study is to investigate the characteristics of pGlu-L-Dopa-Pro as a prodrug of L-Dopa. ***Methods.*** pGlu-L-Dopa-Pro and L-Dopa-Pro were synthesized using the standard procedures of peptide synthesis. The conversion of pGlu-L-Dopa-Pro to L-Dopa was studied using pyroglutamyl aminopeptidase I and prolidase. With rats as the animal model, the stability of pGlu-L-Dopa-Pro in intestinal homogenates was determined, then the transport characteristics of pGlu-L-Dopa-Pro were studied using in-situ perfusion and Ussing chambers. ***Results.*** pGlu-L-Dopa-Pro, relatively stable in intestinal homogenates and intestinal fluid, had a dimensionless permeability of 1.8 at 0.04 mM. Its intestinal permeability was significantly inhibited by 20 mM captopril, by a mixture of dipeptides, 80 mM Gly-Gly and 5 mM Gly-Pro, and by 2 mM cephradine. Further, in Ussing chambers, its mucosal to serosal permeability decreased dramatically with concentration. Conversion studies showed that pGlu-L-Dopa-Pro was degraded by pyroglutamyl aminopeptidase I, an enzyme releasing the N-terminal pyroglutamic acid, with Vmax and Km of 0.6 μmole/min/g protein and 21 mM, respectively, and that L-Dopa-Pro was degraded by prolidase with Vmax and Km of 44 μmole/min/g protein and 0.48 mM, respectively. ***Conclusions.*** This tripeptide, a potential prodrug of L-Dopa, is absorbed by the intestinal peptide transporter, is relatively stable in the gut wall, and is converted to L-Dopa by peptidases with the cleavage by pyroglutamyl aminopeptidase I to L-Dopa-Pro as the rate limiting step.

KEY WORDS: L-Dopa; tripeptide prodrug; intestinal transporter.

INTRODUCTION

L-Dopa is the drug of first choice to control chronic Parkinson's disease, a disease in which patients deteriorate gradually if dopamine is not continuously supplied in sufficient amount. The major problems of oral-L-Dopa include low water solubility and extensive degradation in the gut wall (1). Consequently, though absorbed rapidly and completely, L-Dopa has low oral bioavailability. Eventually, only a small portion of the dose reaches the brain. Coadministration of carbidopa decreases gut wall and peripheral decarboxylation resulting in improved oral bioavailability; however, erratic plasma levels still cause the patients to experience wearing-off and on-off phenomena (2,3). To minimize decarboxylation in the gut wall, another alternative is to dose L-Dopa as prodrugs which prevent decarboxylation of L-Dopa.

Many prodrugs of L-Dopa have been studied in the past twenty years, including recent efforts on ester prodrugs of L-Dopa (1,4-6). Since for L-Dopa, gut-wall metabolism is much higher than hepatic first-pass effect, prodrugs of L-Dopa have to minimize decarboxylation of L-Dopa. Further, the prodrug has to be absorbed efficiently in the intestine. Without fulfilling these desired requirements, prodrugs may not offer any advantage. An early, rapid release of L-Dopa from the prodrugs in the lumen and epithelium will result in a similar extent of gut-wall first pass metabolism. Thus, desirable prodrugs should not release L-Dopa until absorbed into the circulation. Since the intestinal peptide transporter has a broad substrate specificity and does not require a free N-terminal α-amino group and since a di-/tripeptide lacking an N-terminal α-amino group and having Pro at the C-terminus is relatively resistant to intestinal proteolytic degradation (7,8), it is proposed to design a prodrug of L-Dopa which is absorbed by the transporter, is stable in the lumen and gut wall, and is converted to L-Dopa after absorption into the circulation. Under this principle, pGlu-L-Dopa-Pro was designed.

After absorbed into the circulation, pGlu-L-Dopa-Pro is supposed to be degraded by successive actions of pyroglutamyl aminopeptidase I and prolidase to release L-Dopa. Pyroglutamyl aminopeptidase (E.C.3.4.19.3) is an enzyme releasing N-terminal pyroglutamic acid from polypeptides and is distributed in the liver, kidney, and the brain with negligible activity in the plasma and intestine (9). Prolidase is an cytosolic enzyme degrading dipeptides with C-terminal proline, and is present in the intestine, kidney, liver, and brain (8). In this report, the characteristics of this tripeptide prodrug is detailed.

MATERIALS AND METHODS

Materials

EDTA, DTT (dithiothreitol), Tris Base, Tris/HCl, L-Dopa, carbidopa, $MnCl_2$, pyglutamate aminopeptidase (calf liver), Gly-Gly, Gly-L-Pro, Cephradine, PEG-4000, pentobarbital, and porcine kidney prolidase (suspended in 2.7 M, pH 8 $(NH_4)_2SO_4$ solution were obtained from Sigma Chemical Co. (St. Louis, MO). Pro-OBzl.HCl was obtained from United States Biochemical Co. (Cleveland, OH). Captopril was a gift from Squibb & Sons (Princeton, NJ). Acetonitrile was of HPLC grade; other chemical reagents and buffer components were of analytic grade. All chemicals were used as obtained. All the peptides used in this study consist of L-amino acids.

Synthesis of pGlu-L-Dopa-Pro and L-Dopa-Pro

pGlu-L-Dopa-Pro. L-Dopa reacted with di-tert-butyldicarbonate to form tert-butyloxycarbonyl-L-Dopa (Boc-L-Dopa); then the phenol groups of Boc-L-Dopa were protected by reacting with benzylchloride. Boc-L-Dopa$(Bzl)_2$-OH was coupled to prolyl benzyl ester by the DCC method. The product was treated with HCl and then coupled with benzyloxycarbonyl-pGlu using the same method. The final product was added to a suspension of

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$Pd/BaSO_4$ in acetic acid and hydrogenated under 50 psi H_2 for 15 hr. The mixture was filtered, purified and crystallized. Mp: 155°, ^{1}HNMR(300 MHz, DMSO-d_2, spectrum indicates the presence of rotamers about the L-Dopa-Pro-amide bond) δ 1.57-2.25 (m, 8 H, pGlu β-CH_2 and γ-CH_2, Pro β-CH_2 and γCH_2), 2.58 (dd, 1H, Dopa β-CH_2), 2.77 (dd, 1H, Dopa β-CH_2), 3.42-3.52 (m, 1H, Pro δ-CH_2), 3.55-3.70 (m, 1H, Pro δ-CH_2), 3.95 and 4.02 (dd, 1H, Pro α-CH_2), 4.23(dd, 1H, Dopa α-CH), 4.48-4.59 (m, 1H pGlu α-CH), 6.48-6.66 (m, 3H ArH), 7.71 and 7.76 (s, 1H, pGlu NH), 8.00 and 8.75 (d, 1H, Dopa NH), 8.68 (br, s, 1H, COOH). FAB-MS, m/e 406 $(MH)^+$. Calcd. C, 52.77; H, 6.06; N, 9.71; Found C, 53.74; H 6.31; N 9.31.

L-Dopa Pro. Its synthesis was as described above. Boc-L-Dopa$(Bzl)_2$-OH was coupled to prolyl benzyl ester by the DCC method; the mixture was acidified, mixed with a suspension of $Pd/BaSO_4$ in acetic acid, and hydrogenated under 50 psi H_2. The final product was filtered, purified and crystallized. ^{1}HNMR(300 MHz, D_2O, spectrum indicates the presence of rotamers about the L-Dopa-Pro-amide bond) δ 1.55-1.70 (m, 1.3H, Pro γ-CH_2), 1.85-2.00 (m, 2H Pro β-CH_2 and γ-CH_2), 2.20-2.34 (m, 0.7H, Pro β-CH_2), 2.8-3.01 (m, 1H, Dopa β-CH_2), 3.05-3.15 (m, 1H, Dopa β-CH_2), 3.16-3.65 (m, 2H, Pro δ-CH_2), 4.10 (dd, 0.7H, α-CH), 4.36-4.45 (m, 1.3H, Pro α-CH and Dopa α-CH_2), 6.65-6.9 (m, 3H, ArH).

Animals

Male Sprague-Dawley rats, 250-300 g, were used as the animal model. Rats were fasted for 18 hr prior to perfusion and Ussing chambers studies.

Ussing Chambers

Ussing chambers with a voltage clamp, borrowed from Dr. Stephen Berge (3M Pharmaceuticals, St. Paul, MN), were used to study *in vitro* jejunal permeability of pGlu-L-Dopa-Pro (10,11). After the serosa and muscularis propris were removed, mucosal sheets were mounted in the Ussing chambers in non-short-circuited state at 37° C and gassed with 95 % oxygen-5% carbon dioxide. The Krebs buffer of pH 7.4 was used to prepare pGlu-L-Dopa-Pro of various concentrations.

Determination of transepithelial potential difference (mV) and short-circuit current (I) was used to determine tissue viability throughout the experiments. Mucosal to serosal transport of ^{3}H-D-glucose against a concentration gradient was observed for all jejunal tissues. Steady state flux was determined from the linear regression of the accumulative amount transported/time profile, and membrane permeability was determined from the steady state flux using the equation $J = P\ (C_d - C_r)$ where C_d and C_r are donor and receiver concentrations of pGlu-L-Dopa-Pro, respectively. The donor concentration of pGlu-L-Dopa-Pro remained constant throughout the experiments.

Methods

In situ *Single-Pass Perfusion*

In situ jejunal perfusion of pGlu-L-Dopa-Pro or L-Dopa was studied and its dimensionless membrane permeability was calculated as published previously (7). The perfusion solution consisted of a phosphate buffer (20 mM, pH 7), pGlu-L-Dopa-Pro or L-Dopa (0.04 mM) with or without an inhibitor, NaCl, and trace amount of ^{14}C-PEG-4000 and PEG-4000. ^{14}C-PEG-4000 is a water flux maker. The perfusate was subjected to HPLC assay.

Conversion of pGlu-L-Dopa-Pro by Peptidases

pGlu-L-Dopa-Pro by Pyroglutamyl Aminopeptidase. A pH 7.5 Tris buffer (20 mM) containing 125 m*M* NaCl was used. Dithiothreitol (2 mM) and EDTA (2 mM) were used to activate calf-liver pyroglutamyl aminopeptidase I before pGlu-L-Dopa-Pro was added (9, 12). Reaction proceeded at 37° C; 0.01 M HCl was used to stop reactions. The specific action of pyroglutamyl aminopeptidase I (EC 3.4.19.3) is to release the N-terminal pGlu from peptides (9,12). In the control group, boiled enzyme did not hydrolyze the prodrug. Hydrolysis of pGlu-L-Dopa-Pro was studied only up to 20 mM because of its limited aqueous solubility.

L-Dopa-Pro by prolidase: Hydrolysis of L-Dopa-Pro by porcine kidney prolidase was studied at 37° C as published previously (13).

Stability of pGlu-L-Dopa-Pro in Intestinal Homogenate and Intestinal Lumenal Content

Intestinal homogenate was prepared in a pH 7.5 Tris buffer (20 mM) containing 125 mM NaCl as published previously. Stability of pGlu-L-Dopa-Pro (0.04 mM) in homogenate was determined at 37° C with or without dithiothreitol (DTT) (2 mM) and EDTA (2 mM). A pH 7.5 Tris buffer (20 mM) containing 125 mM NaCl was perfused through the intestine and collected. Stability of pGlu-L-Dopa-Pro in this blank perfusate was evaluated at 37°C. All reactions were stopped using 0.01 M HCl.

Assay

The HPLC system consisted of SIL autoinjector, LC-600 pump, SPD-6A UV spectrophotometric detector, and CR 601 recorder (Shimadzu Corporation, Kyoto, Japan). Samples of pGlu-L-Dopa-Pro were assayed using a C8 Beckman column (5 μ, 4.6 mm X 15 cm), a mobile phase of 0.05% TFA (trifluoroacetic acid): acetonitrile (ACN) = 90:10, and UV wavelength of 220 nm. pGlu-L-Dopa-Pro eluted at 14 min at a flow rate of 0.5 ml/min. L-Dopa-Pro was eluted by a mobile phase of 0.05% TFA: ACN = 95:5 at 15 min at a flow rate of 0.5 ml/min; L-Dopa eluted at 6 min by this mobile phase.

Data Analysis

Initial hydrolysis rates were obtained from the first 15 to 20% hydrolysis. The Michaelis-Menten parameters, Vmax and Km, were obtained by nonlinear regression using eq. 1.

$$V = [(Vmax/Km)/(1 + ([S]/Km))]*[S] \quad (1)$$

RESULTS AND DISCUSSION

Without the presence of activators, EDTA and DTT, stability of pGlu-L-Dopa-Pro in intestinal homogenate and

blank perfusate was not different from that in buffer, with more than 95% left after 1.5 hr incubation. In the presence of EDTA and DTT, pGlu-L-Dopa-Pro was degraded slowly by intestinal homogenates with hydrolysis rates of 0.005 ± 0.002 μmole/min/g protein and 0.006 ± 0.002 μmole/min/g protein by duodenal and jejunal homogenates, respectively. However, rates of its hydrolysis by jejunoileal and ileal homogenates in the presence EDTA and DTT were not significantly different from zero. The small intestine has no detectable activity of pyroglutamyl aminopeptidase I (12). It is unknown what enzymes in homogenates attacked pGlu-L-Dopa-Pro and why only the homogenates from the proximal intestine slowly attacked this tripeptide. Altogether, pGlu-L-Dopa-Pro is stable in intestinal lumen and is relatively stable in epithelial homogenates. Further, during Ussing chambers studies, no detectable breakdown products of pGlu-L-Dopa-Pro, i.e., L-Dopa-Pro, L-Dopa and pGlu-L-Dopa, were detected.

Hydrolysis of pGlu-L-Dopa-Pro by pyroglutamyl aminopeptidase I was determined up to 20 mM. Because of its limited aqueous solubility, saturation of pyroglutamyl aminopeptidase I was not achieved. From nonlinear regression, its hydrolysis had Vmax and Km of 0.6 ± 0.03 μmole/min g enzyme and 21 ± 1.6 mM, respectively (Fig. 1a). Hydrolysis of L-Dopa-Pro by prolidase was much faster with Vmax and Km of 40 ± 3 μmole/min g enzyme and 0.48 ± 0.15 mM, respectively (Fig. 1b). These results reveal that L-Dopa is released from pGlu-L-Dopa-Pro by successive actions of pyroglutamyl aminopeptidase I and prolidase (Fig. 2). The release of L-Dopa-Pro from the tripeptide prodrug by pyroglutamyl aminopeptidase I is much slower than prolidase.

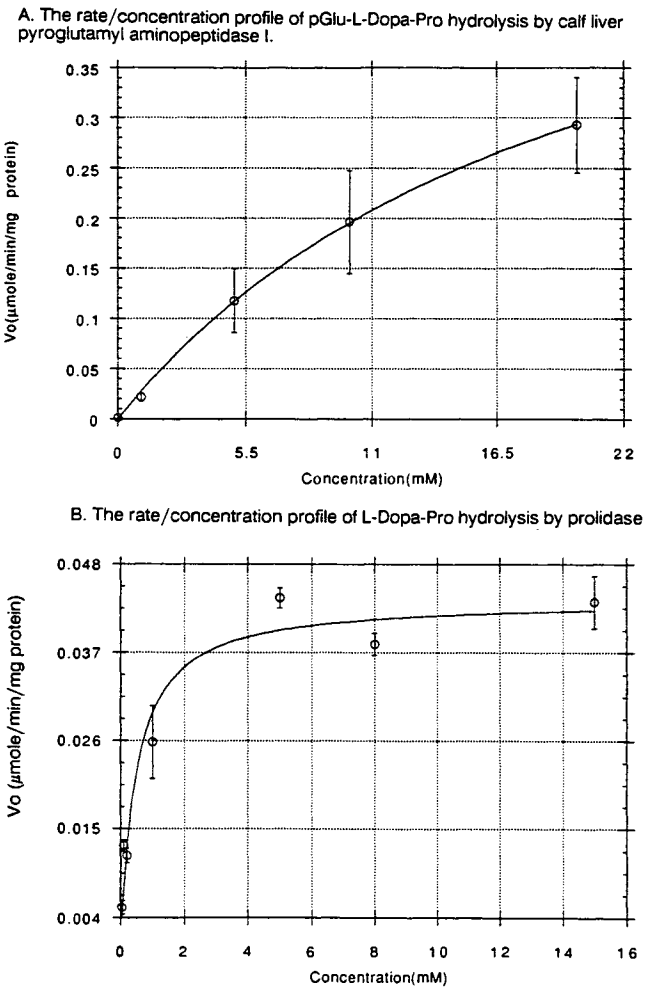

Fig. 1. Hydrolysis rate/concentration profiles of prodrug conversion: (A) pGlu-L-Dopa-Pro by calf liver pyglutamyl aminopeptidase I and (B) L-Dopa-Pro by prolidase.

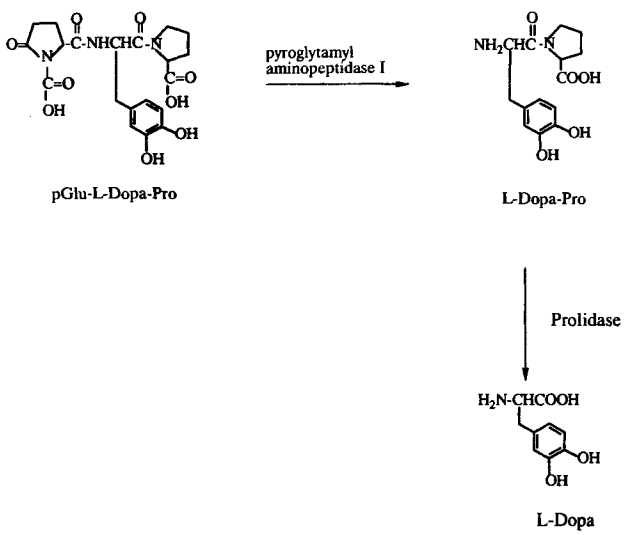

Fig. 2. Release of L-Dopa from pGlu-L-Dopa-Pro by Successive actions of proteolytic enzymes.

Intestinal absorption of pGlu-L-Dopa-Pro was efficient, with a dimensionless permeability of 1.81 ± 0.25 at 0.04 mM. Its permeability is not significantly different from that of Phe-Pro (7). According to a theoretical analysis, dimensionless intestinal permeability of greater than 1 projects complete absorption (14). Hence, it is expected that intestinal absorption of pGlu-L-Dopa-Pro would be complete. L-Dopa at 0.04 mM had a permeability of 3.01 ± 0.7. The concentration of 0.04 mM was studied because the lumenal concentration of L-Dopa would be 0.04 mM if an oral dose of L-Dopa is completely mixed with 250 ml of gastric fluid or content. There is no significant difference between the permeabilities of 0.04 mM L-Dopa and 0.04 mM pGlu-L-Dopa-Pro at a significant level of 0.05. It is concluded that intestinal absorption of pGlu-L-Dopa-Pro is efficient.

In situ single-pass perfusion studies also showed that permeability of pGlu-L-Dopa-Pro was significantly reduced by 20 mM captopril ($p < 0.005$), by 2 mM cephradine ($p < 0.01$), and by a mixture of dipeptides ($p < 0.01$), 80 mM Gly-Gly and 5 mM Gly-Pro (Fig. 3). Cephradine and captopril are both absorbed by the intestinal di/tripeptide transporter with a Km of 1.5 mM and 5.9 mM, respectively (8). Hence, the results demonstrate that pGlu-L-Dopa-Pro, though without an N-terminal free α-amino group, is absorbed through the intestinal peptide transporter. Transport by this carrier explains why its intestinal absorption is efficient.

Ussing chambers, a unique tool to determine the mucosal to serosal transport, was used to determine whether pGlu-L-Dopa-Pro was absorbed to the other side of the membrane and whether its absorption was concentration de-

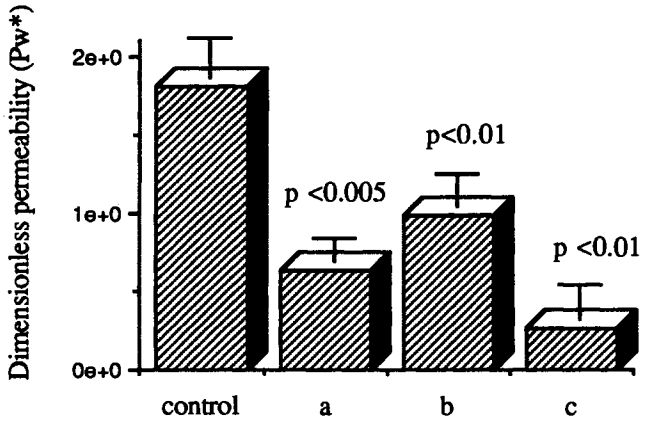

Fig. 3. Effects of inhibitors (a) 20 mM captopril, (b) 80 mM Gly-Gly and 5 mM Gly-Pro, and (c) 2 mM cephradine on the in situ intestinal permeability of pGlu-L-Dopa-Pro.

pendent. Its mucosal to serosal permeability was the highest at 0.005 mM and decreased as its concentration increased to 10 mM (Tab. I). At 10 mM, its permeability was only 0.1% of that at 0.005 mM, suggesting that passive permeation contributes much less to its absorption than carrier-mediated transport.

In summary, pGlu-L-Dopa-Pro is absorbed efficiently by the intestinal peptide transporter, is relatively stable in intestinal epithelium and is converted to L-Dopa by successive actions of pyroglutamy aminopeptidase I and prolidase. pGlu-L-Dopa-Pro has the desired properties to ensure good absorption and minimal decarboxylation in the gut wall. Further, pGlu-L-Dopa-Pro can also reduce the extent of decarboxylation of L-Dopa in the liver since during its residence in the liver L-Dopa will not be exposed to decarboxylase until released by peptidases.

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Table I. The Concentration-Dependence of Apparent Permeability of pGlu-L-Dopa-Pro Obtained from in Vitro Ussing Chambers

Concentration	Permeability (cm/min)	N
10 mM	0.00012 (0.00008)	3
1 mM	0.00075 (0.0002)	3
0.05 mM	0.028 (0.005)	4
0.005 mM	1.24 (0.36)	5

Mean (S.E.)

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