Competitive Inhibitors of Renin. Inhibitors Effective at Physiological pH†

James Burton, Knud Poulsen, and Edgar Haber*

ABSTRACT: Previously we reported the development of competitive inhibitors of renin effective at pH 5.5 (Poulsen, K., Burton, J., and Haber, E. (1973), *Biochemistry 12*, 3877). At physiologic pH (7.5), the inhibitory constants (K_i) increased and solubility decreased to the point that inhibition could not be demonstrated with these peptides. Modification of the octapeptide sequence, His-Pro-Phe-His-Leu-Leu-Val-Tyr, either by addition of serinol to the carboxyl terminus or by replacement of valine-7 with an isosteric threonyl residue failed to yield peptides active at pH 7.5. Attachment of polyproline sequences to the amino terminus increased solubility from threefold to tenfold and decreased K_i so that competitive inhibition was demonstrable at physiologic pH. In addition, if leucine-6 was replaced in these peptides with a phenylalanyl or tyrosyl residue, K_i decreased (3–12 μM) to give effective competitive inhibitors at physiologic pH in both buffer and in plasma.

We previously reported (Poulsen et al., 1973) that six analogs of the octapeptide sequence described by Skeggs et al. (1957, 1968), His-Pro-Phe-His-Leu-Leu-Val-Tyr, representing a segment of renin substrate, were effective competitive inhibitors of proteolysis of the tetradecapeptide substrate, Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser by renin. Several compounds had an inhibitory constant one order of magnitude smaller than that of the model octapeptide. In addition, those in which either component of the leucylleucine bond was replaced by the D isomer were not cleaved by renin. At pH 7.5, K_i increased and solubility decreased so that inhibition could not be demonstrated with these octapeptides. To obtain inhibitors that might be effective in vivo, the octapeptide sequence was modified in the following three ways to increase its solubility: (1) the C-terminal carboxyl was replaced by a hydroxyl (Kokubu et al., 1968), (2) an isosteric, hydrophilic residue, threonine, was substituted for the hydrophobic residue, valine, and (3) one or more prolines were added to the amino terminus of the octapeptide. Of these methods, only the last was effective in both increasing solubility and maintaining a low K_i at pH 7.5.

Peptide Synthesis

tert-Butoxycarbonylamino acids were synthesized by the method of Schnable (1967) or were purchased from Beckman Instruments, Inc. Side chain protecting groups employed in the solid phase syntheses are: histidine, N^{τ}-tosyl; threonine, *O*-benzyl; and tyrosine, *O*-benzyl. Dichloromethane was distilled from CaH_2 (Perrin et al., 1966), and triethylamine was stored over KOH and distilled from naphthyl isocyanate (Durham et al., 1963). Dimethylformamide was stored over molecular sieve (4 Å) and, after filtration, it was distilled at reduced pressure (bp 63–65°). Dicyclohexylcarbodiimide was distilled at reduced pressure (bp 135°) and transferred in 3.09-g aliquots to 0.5-oz bottles which were stored at −20°. Before use, the bottles were defrosted and diluted with 15.45 g of CH_2Cl_2, which gave 15.0 ml of a 1 *M* solution. Anisole was stored over $CaSO_4$, distilled at reduced pressure (bp 55°), and stored in 10-ml aliquots. N^1-Hydroxybenztriazole was synthesized (Nietzki and Braunschweig, 1894). The solid support that was used for the syntheses was Lab Systems Merrifield Resin (1% cross-linked, 200–400 mesh, 0.75 mmol of Cl^-/g) or Bio-Beads SX-1 (200–400 mesh, 0.69 mmol of Cl^-/g). Other chemicals were of reagent grade and were used without further purification.

Absorbance was routinely measured at 280 n*M* on a Gilford 240 spectrophotometer equipped with a 2443-A rapid sampler. For absorbance at both 280 and 230 n*M*, a Cary-15 spectrophotometer was used. Effluent refractive index was measured with a Model 1101L refractometer from Laboratory Data Control.

Reactions were performed in a vessel constructed by fitting a 50-ml polypropylene syringe with a polyethylene frit (70-μ porosity) as is shown in Figure 1. Pressurized reagents were dispensed through Teflon tubing which was held in place by a Teflon cap which also served as a bearing for a turbine agitator (Ace Glass Inc.). Reagents were drained from the reaction vessel through a Teflon valve equipped with a 12-gage Teflon needle (Hamilton Co.) that was attached to a waste bottle.

Completeness of coupling reactions were tested with the ninhydrin reagents developed by Kaiser et al. (1970). A 50-μl sample of reaction mixture (3 mg of peptidyl-resin) was placed in a 1-ml polypropylene syringe with a polyethylene frit (70-μ porosity) and attached to a vacuum source. The resin was washed well with ethanol and dichloromethane and the syringe was then inserted through a rubber collar into a 0.8 × 7 cm test tube (Figure 2). Three drops of each of the ninhydrin reagents was added to the tube and the assembly was warmed at 105° for 5 min. Hot air was forced through the frit where it mixed the peptidyl-resin and reagents and caused color development. The assembly was set aside to cool at the end of the reaction period. The

†From the Cardiac Unit, Department of Medicine, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts 02114 (J.B. and E.H.), and the University Institute for Experimental Medicine, Norre Alle 71, Copenhagen 2100, Denmark (K.P.). *Received January 22, 1975.* This work was supported by a Hypertension-SCOR Grant from the NHLI (14150) and by the Danish Heart Association and the Danish State Medical Research Council. A preliminary version of this paper was presented at a meeting of the Pan-American Hypertension Society in Mendoza, Argentina, Aug 1974.

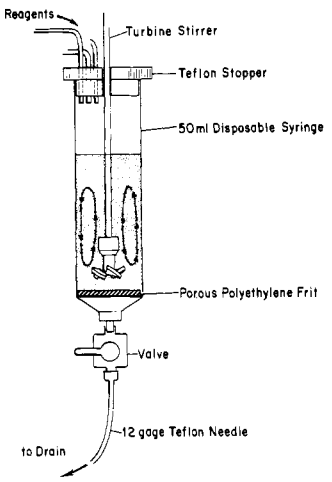

FIGURE 1: Disposable 50-ml polypropylene reaction vessel for solid phase peptide syntheses.

partial vacuum that is formed in the outer chamber draws the reagents through the frit into the bottom of the tube. Completeness of reaction may be judged by examining the color of the filtrate and of the resin retained on the frit.

Completeness of the coupling reaction was also determined in a parallel experiment using the Fluram test protocol suggested by Felix and Jimenez (1973). The 1-ml fritted syringe was used as a reaction vessel. Concentration of Fluram in dichloromethane was 0.3% (w/v). Fluorescence may be viewed in the syringe directly. After capping, the sample remains fluorescent for several months.

Amino acid analyses were performed on samples of peptidyl-resin that were dried to constant weight and hydrolyzed for 24 hr at 110° in a mixture of acetic acid–concentrated HCl (1:1).

Chromatographic and electrophoretic systems that were used to analyze peptides were reported previously (Poulsen et al., 1973) with the exception of T8, which has the composition: butan-2-one–acetic acid–water (30:90:15).

Solubility was determined by adding excess dry peptide as the acetate salt to a small volume of pH 7.5, 0.1 *M* phosphate buffer containing 0.01% thimerosal. pH was readjusted to 7.5 with 0.1 *N* NaOH and the suspension was stirred at 20°. At 24 and 48 hr, 1-ml aliquots were filtered and the clear filtrate was submitted for amino acid analysis.

Histidylprolylphenylalanylhistidylleucylleucylvalyltyrosine (*Octapeptide*) (**1**). Synthesis and characterization of **1** were reported previously (Poulsen et al., 1973; Skeggs et al., 1968).

Prolylhistidylprolylphenylalanylhistidylleucylleucylvalyltyrosine (*Pro-octapeptide*) (**2**). Boc-Tyr(Bzl)-resin was prepared by using standard techniques (Merrifield, 1963). Analysis showed 0.145 mmol of tyrosine/g of resin, which corresponds to 22.2% esterification of the chloromethyl groups on the polymer.

The operations and the protected derivatives reported in the first part of this study (Poulsen et al., 1973) were employed in these syntheses. A fivefold excess of protected amino acid was, however, added as the dry powder to the neutralized washed resin followed by the minimum amount of CH_2Cl_2, which gave a suspension that could be stirred. After 10 min, an equimolar amount of 1 *M* *N,N*-dicyclohexylcarbodiimide in CH_2Cl_2 was added and the mixture was stirred 30 min after which tests for completeness of reaction were performed. If warranted, the resin was washed, reneutralized, and coupled a second time using similar conditions. The Fluram test was usually more sensitive than the modified ninhydrin test we used, though this was not invariably so. When the synthesis was completed, the peptidyl-resin was dried to constant weight in an Abderhalden apparatus maintained at 35° and 0.1 Torr. Dried resin (0.296 g) was treated with HF–5% anisole as outlined by Sakakibara and Shimonishi (1965). The product was extracted three times with 1 *M* acetic acid (AcOH). Subsequent extraction with 0.01 *N* HCl did not yield additional peptide. Combined extracts were filtered and lyophilized to yield 37 μmol of crude Pro-octapeptide (100%).

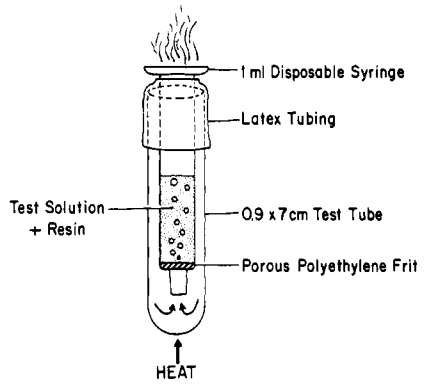

FIGURE 2: Vessel for ninhydrin test.

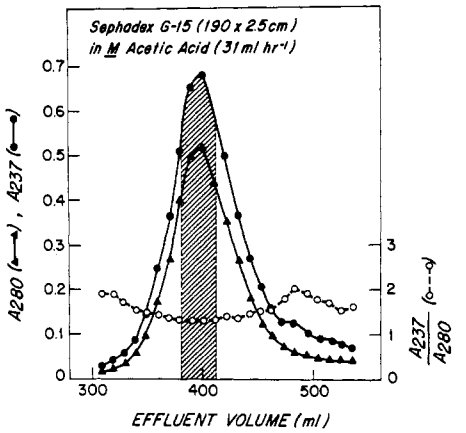

FIGURE 3: Chromatography of 34.6 μmol of crude $(Pro)_3$-octapeptide on Sephadex G-15 (2.5 × 190 cm) eluted with 1 *M* acetic acid at 31 ml hr^{-1}. Volume indicated by shaded area (32%) shows a constant ratio between A_{237}/A_{280} and was used for further purification.

Crude **2** (44.8 mg) was dissolved in 10 ml of 0.01 *N* HCl and chromatographed on Bio-Gel P-2 (2.5 × 196 cm) in 0.01 *N* HCl. The fraction eluting between 650 and 706 ml was pooled, lyophilized, and relyophilized from 1 *M* AcOH to yield 17.6 μmol of homogeneous peptide (73%): His, 1.97; Pro, 1.82; Phe, 0.97; Leu, 2.13; Val, 0.98; Tyr, 0.91;[1] R_f(T3) 0.61; R_f(T4) 0.15; R_f(T6) 0.61; R_{Leu}(E1) 3.75 $[M]^{16}_{578\ nm}$ − 1415° (1.35 m*M*, 1.00 *M* AcOH).

Prolylprolylprolylhistidylprolylphenylalanylhistidylleucylleucylvalyltyrosine (*(Pro)$_3$-octapeptide*) (**3**). Boc-Tyr(Bzl)-resin was prepared by using conditions described previously to yield a product containing 0.084 mmol of tyrosine/g of sample (12.6%). After the completion of synthetic operations, 0.670 g of peptidyl-resin was treated with HF–5% anisole for 1 hr at 0° to yield, after work-up, 41.6 μmol of crude **3** (83%). Chromatography of 34.6 μmol of crude **3** on Sephadex G-15 (2.5 × 190 cm) in 1 *M* AcOH yielded a product whose elution profile is shown in Figure 3.

[1] Value not included in averaging procedure.

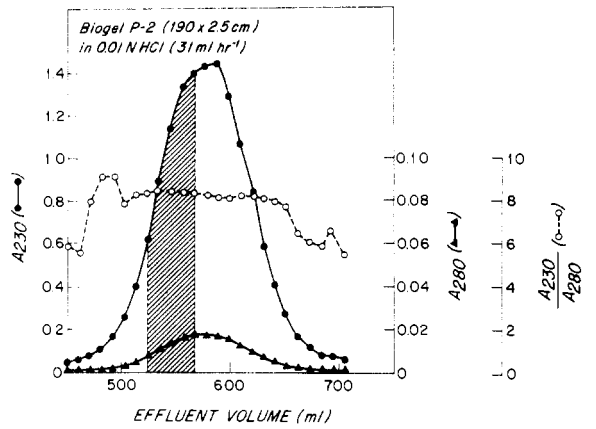

FIGURE 4: Chromatography of partly purified $(Pro)_3$-octapeptide on Bio-Gel P-2 (2.5 × 190 cm) eluted with 0.01 *N* HCl at 35 ml hr^{-1}. Volume indicated by shaded area (28%) shows a constant ratio between A_{230}/A_{280} and was lyophilized to yield $(Pro)_3$-octapeptide.

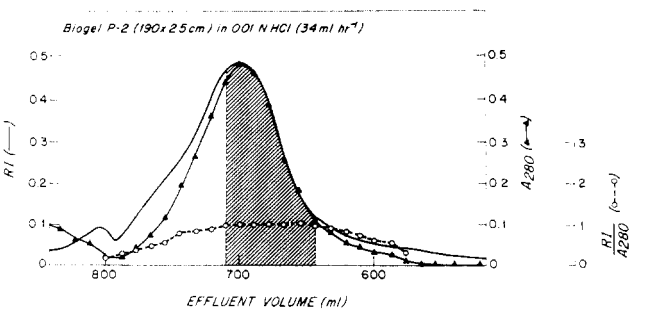

FIGURE 5: Chromatography of 38.7 μmol of crude Ile-octapeptide on Bio-Gel P-2 (2.5 × 190 cm) eluted with 0.01 *N* HCl at 35 ml hr^{-1}. Volume indicated by shaded area (80%) shows a constant ratio between refractive index and A_{280} and was lyophilized to yield Ile-octapeptide.

Only the center fraction eluting between 382 and 412 ml shows a constant ratio between A_{230} and A_{280}. This fraction was lyophilized to yield 11.1 μmol (26.7%) of peptide. Amino acid analyses of each fraction were acceptable though the average values of leucine in the three fractions decreased (2.13, 1.91, 1.81) and those of proline increased (4.08, 4.11, 4.25) with the order of elution. All fractions were heterogeneous on thin-layer chromatography. The center fraction was chromatographed on Bio-Gel P-2 (190 × 2.5 cm) in 0.01 *N* HCl and the eluate divided into two major fractions (Figure 4), which were lyophilized and relyophilized from 1 *M* AcOH. The fraction eluting between 524 and 568 ml had a constant A_{230}/A_{280} ratio and was homogeneous by all criteria (3.03 μmol, 27%): Tyr, 0.96; Val, 1.00; Leu, 2.00; His, 2.05; Phe, 0.99; Pro, 4.18; R_f(T3) 0.50; R_f(T4) 0.65; R_f(T8) 0.76; R_{His}(E1) 0.45; $[M]^{18}_{578\ nm}$ −3018° (0.0106 m*M*, pH 7.50, 0.1 *M* phosphate buffer). Edman degradation showed only proline (94%) as the N-terminal residue. The fraction eluting between 568 and 608 ml had a low R_f component on thin-layer chromatography in T3.

Prolylprolylprolylprolylprolylhistidylprolylphenylalanylhistidylleucylleucylvalyltyrosine (*(Pro)*$_5$*-octapeptide*) (**4**). Boc-Tyr(Bzl)-resin was prepared by using conditions described previously to yield a product containing 0.145 mmol of tyrosine/g of resin (22.1%). After the completion of synthetic operations, 2.015 g of peptidyl-resin was treated with HF–5% anisole at 0° for 90 min to yield, after work-up, 89 μmol of **4** (37.7%). Chromatography of 29.0 mg of crude **4** on Bio-Gel P-2 in 0.01 *N* HCl (2.5 × 196 cm) yielded a fraction that eluted between 504 and 575 ml, which was pooled, lyophilized, and relyophilized from 1 *M* acetic acid to yield 4.2 μmol of homogeneous **4** (38%): Tyr, 0.70;[1] Val, 0.97; Leu, 2.08; His, 2.03; Phe, 0.92; Pro, 6.40; R_f(T3) 0.56; R_f(T6) 0.59; R_{Leu}(E1) 3.09; $[M]^{16}_{578\ nm}$ −4207° (*c*, 0.813 m*M*, 1.00 *M* AcOH).

Isoleucylhistidylprolylphenylalanylhistidylleucylleucylvalyltyrosine (*Ile-octapeptide*) (**5**). Boc-Tyr(Bzl)-resin was prepared by using conditions described previously to yield a product containing 0.175 mmol of tyrosine/g of resin (27.0%). After completion of the synthesis, 1.395 g of peptidyl-resin was treated with HF–5% anisole for 1 hr at 0° to yield, after work-up, 104 μmol of crude **5** (51.7%). A 13.0-ml solution of 0.01 *N* HCl containing 38.7 μmol of crude **5** was chromatographed on Bio-Gel P-2 (190 × 2.5 cm) to yield a fraction that eluted between 646 and 710 ml and had a constant value when the refractive index (RI) was divided by A_{280} (Figure 5). Inconstancy of the ratio between refractive index and absorbancy shows the heterogeneity of the later fractions. The desired fraction was pooled, lyophilized, and relyophilized from 1 *M* AcOH to yield 30.9 μmol of homogeneous **5** (79.8%): Tyr, 0.98;[1] Val, 1.00; Leu, 2.00; His, 2.00; Phe, 1.11; Pro, 1.01; Ile, 0.88; R_f(T3) 0.62; R_f(T6) 0.68; R_f(T8) 0.80; R_{His}(E1) 0.58; $[M]^{18}_{578\ nm}$ −633° (*c*, 5.15 m*M*, 1.0 *M* AcOH).

*Histidylprolylphenylalanylhistidylleucyl-*D*-leucylvalyltyrosine* [D-*Leu*^{6}]*Octapeptide*) (**6**). Synthesis and characterization were reported previously (Poulsen et al., 1973).

*Prolylhistidylprolylphenylalanylhistidylleucyl-*D*-leucylvalyltyrosine* (*Pro-*[D-*Leu*^{6}]*octapeptide*) (**7**). Boc-Tyr(Bzl)-resin was prepared by using conditions described previously to yield a product containing 0.202 mmol of tyrosine/g of resin (31.5%). After the completion of synthetic operations, 0.712 g of peptidyl-resin was treated with HF–5% anisole for 1.5 hr at 0° to yield, after work-up, 56.4 μmol of crude **7** (49.5%). The peptide was dissolved in 10 ml of 1 *M* AcOH to yield a clear solution which gelled when it was cooled. The sample was warmed, diluted to 20 ml with 1 *M* AcOH, and chromatographed on G-15 (2.5 × 196 cm) in the same solvent to yield a fraction that eluted between 350 and 430 ml which, when lyophilized, yielded 32.8 μmol (58%) of peptide. This was then dissolved in 10 ml of 0.01 *N* HCl and chromatographed on Bio-Gel P-2 (2.5 × 194 cm) in 0.01 *N* HCl. The fraction that eluted between 682 and 735 ml had a constant A_{280}/RI ratio and was pooled, lyophilized, and relyophilized from 1 *M* AcOH to yield 3.0 μmol of homogeneous peptide (9.1%): Pro, 2.29; His, 2.05; Phe, 0.98; Leu, 2.00; Val, 0.97; Tyr, 0.90;[1] R_f(T3) 0.49; R_f(T4) 0.03; R_f(T6) 0.60; R_{His}(E1) 0.17; $[M]^{18}_{578\ nm}$ −1316° (*c*, 30.4 μ*M*, 1 *M* AcOH).

*Prolylprolylprolylhistidylprolylphenylalanylhistidylleucyl-*D*-leucylvalyltyrosine* (*(Pro)*$_3$-[D-*Leu*^{6}]*octapeptide*) (**8**). Boc-Tyr(Bzl)-resin was prepared by using techniques described previously to yield a product containing 0.042 mmol of tyrosine/g of resin (6.2%). After the completion of synthetic operations, 1.10 g of peptidyl polymer was treated with HF–5% anisole for 1.5 hr at 0° to yield, after work-up, 23.2 μmol (53%) of crude **8**. The material was dissolved in 10 ml of 1 *M* AcOH, filtered to remove a brown precipitate, and chromatographed on Sephadex G-15 (190 × 2.5 cm). The fraction that eluted between 285 and 350 ml was lyophilized to yield 9.3 μmol (40.1%) of partly purified peptide. The entire sample was totally dissolved in 4.8

ml of 0.01 *N* HCl and chromatographed on Bio-Gel P-2 (2.5 × 190 cm) in the same solvent. The ratio of A_{280}/A_{230} was constant between 506 and 587 ml and this fraction was pooled, lyophilized, and relyophilized from 1 *M* AcOH to yield 5.05 μmol (59%) of homogeneous **8:** Pro, 4.00; His, 2.19; Phe, 1.00; Leu, 1.95; Val, 0.92; Tyr, 0.73;[1] R_f(T3) 0.55; R_f(T6) 0.64; R_f(T4) 0.06; R_{His}(E1) 0.69; $[M]^{18}_{578\ nm}$ −3018° (*c*, 515 μ*M*, 1 *M* AcOH).

Histidylprolylphenylalanylhistidylleucyllleucylthreonyltyrosine (*[Thr⁷]Octapeptide*) (**9**). Boc-Tyr(Bzl)-resin was prepared by using techniques described previously to yield a product containing 0.207 mmol of tyrosine/g of resin (32.3%). Synthetic operations were completed in the standard manner. Peptidyl-resin (2.00 g) was treated 1.5 hr at 0° with HF–5% anisole to yield 145 μmol (43%) of crude **9**. Chromatography of 84 μmol of peptide on Sephadex G-15 (190 × 2.5 cm) in 1 *M* AcOH yielded a fraction that eluted between 319 and 374 ml which, when lyophilized, gave 44.7 μmol of white powder (53%). This was dissolved in 0.01 *N* HCl and chromatographed on Bio-Gel P-2 (190 × 2.5 cm) in the same solvent to give a major fraction eluting between 725 and 809 ml, which was lyophilized and relyophilized from 1 *M* AcOH to yield 36.7 μmol (82%) of homogeneous product: His, 2.22; Pro, 0.94; Phe, 0.88; Leu, 1.93; Thr, 0.94; Tyr, 0.87;[1] R_f(T3) 0.60; R_f(T6) 0.66; R_f(T8) 0.76; R_{His}(E1) 0.67; $[M]^{18}_{578\ nm}$ −924° (*c*, 2.91 m*M*, 1 *M* AcOH).

Histidylprolylphenylalanylhistidylleucyllleucylvalyltyrosylserinol (*Octapeptidylserinol*) (**10**). Boc-Tyr(Bzl)-resin was prepared by using conditions described previously to yield a product containing 0.13 mmol of tyrosine/g of resin (20.3%). After the completion of the standard synthetic cycle, 3.1 g (360 μmol) of protected peptidyl-resin was obtained. One-half of the resin was placed in a glass vial along with 10 ml of dimethylformamide and 10 mmol of serinol. The remaining resin was placed in an identical vial containing 10 ml of dimethylformamide, 10 mmol of serinol, and 2 mmol of hydroxybenztriazole. The vessels were shaken for 103 hr on a mechanical shaker (Schwarz/Mann) after which each suspension was independently filtered and the precipitate washed well with dimethylformamide. The combined filtrate and washings from the displacement that was done in serinol–dimethylformamide were evaporated, diluted to 1 ml, and chromatographed on Sephadex LH-20 (1.2 × 95 cm) in dimethylformamide–0.25 *M* toluene at a flow rate of 18.5 ml/hr. The elution profile was measured with a differential refractometer; fractions eluting between 48 and 61 ml were pooled and evaporated to yield 33.8 μmol (18.8% displacement) of the protected peptide. Similar treatment of the crude mixture that displaced from the resin in the presence of hydroxybenztriazole yielded 89.1 μmol of peptide (49.5% displacement) after chromatography on LH-20. Protected octapeptidylserinol (57.0 μmol), after evaporation of dimethylformamide on a Buchi VE-50 attached to an oil pump, was treated with HF–5% anisole for 1.5 hr at 0° to yield, after extraction with 0.01 *N* HCl, 32.3 μmol (56.7%) of peptide. This was chromatographed on Bio-Gel P-2 (2.5 × 194 cm) in 0.01 *N* HCl and the fraction eluting between 550 and 660 ml was lyophilized and relyophilized to yield 24.6 mg (47%) of product: His, not calculated; Pro, 1.06; Phe, 0.96; Leu, 2.00; Val, 0.92; Tyr, 0.92; R_f(T3) 0.63; R_f(T4) 0.12; R_f(T6) 0.63; R_{Leu}(E1) 4.25; $[M]^{18}_{578\ nm}$ −969° (*c*, 258 μ*M*, 1.00 *M* AcOH).

Prolylhistidylprolylphenylalanylhistidylleucylphenylalanylvalyltyrosine (*Pro-[Phe⁶]octapeptide*) (**11**). Boc-Tyr(Bzl)-resin was prepared by using conditions previously described to yield a product containing 0.13 mmol of tyrosine/g of resin (20.4%). After the completion of synthetic operations, 3.30 g of peptidyl-resin was treated with HF–5% anisole at 0° to yield, after work-up, 237 μmol of **11** (62.5%). Chromatography of 40.1 μmol of crude **11** dissolved in 9.5 ml of 1 *M* acetic acid on Sephadex G-15 (2.5 × 194 cm) in the same solvent yielded a fraction that eluted between 412 and 442 ml, which was pooled and lyophilized to yield 26.4 μmol of peptide (66%). The partly purified peptide (14.5 μmol) was dissolved in 6.5 ml of 0.01 *N* HCl to give a clear solution which was chromatographed on Bio-Gel P-2 in the same solvent. Fractions eluting between 795–865 ml have identical values when A_{280} is divided by the RI and, after pooling, lyophilization, and relyophilization from 1 *M* acetic acid, yielded 12.0 μmol of homogeneous **11** (83%): Pro, 1.83; His, 2.00; Phe, 1.99; Leu, 1.04; Val, 1.15; Tyr, 0.88;[1] $\epsilon_{280\ nM}$ 1353 (*c*, 0.269 m*M*, 0.01 *N* HCl); R_f(T2) 0.59; R_f(T4) 0.06; R_f(T8) 0.77; R_{His}(E1) 0.52; $[M]^{18}_{578\ nm}$ −461° (*c*, 412 μ*M*, pH 7.5, 0.1 *M* phosphate).

Prolylhistidylprolylphenylalanylhistidylleucyltyrosylvalyltyrosine. (*Pro-[Tyr⁶]octapeptide*) (**12**). The cesium salt of Boc-Tyr(Bzl) was prepared by using techniques reported by Gisin (1973) for other amino acid derivatives. Bio-Beads SX-1 0.75 mmol of Cl^-/g of resin (10.7 g) and Boc-Tyr(Bzl)-OCs (3.71 g) were suspended in 85 ml of dimethylformamide and stirred for 12 hr at 55° under anhydrous conditions. The resin was washed with dimethylformamide, dimethylformamide–10% H_2O (v/v), and ethanol, and then dried to constant weight to yield a product containing 0.199 mmol of tyrosine/g of resin (28.6%). The nonapeptidyl-polymer was synthesized by using conditions described previously after which 1.67 g of material was treated with HF–5% anisole at 0° for 1.5 hr to yield, after work-up, 174 μmol (67%) of crude **12.** In a parallel experiment, 1.66 g of peptidyl-resin was treated with an equal volume of HF–50% anisole for 1.5 hr at 0° to yield, after evaporation at high vacuum, extraction, and lyophilization, 221 μmol (85%) of crude product. In general, yields from cleavages conducted in HF–50% anisole are greater than from those conducted in HF–5% anisole under otherwise identical conditions.

Crude **12** was dissolved in 1 *M* AcOH and chromatographed on Sephadex G-15 (2.5 × 190 cm) in the same solvent to yield a fraction eluting between 464 and 547 ml which, when lyophilized, yielded 43.8 μmol of peptide (46%). Partly purified peptide (62.4 μmol) was dissolved in 13 ml of 0.01 *N* HCl and chromatographed on Bio-Gel P-2 (2.5 × 194 cm) in the same solvent to yield a fraction eluting between 946 and 1048 ml, which was lyophilized and relyophilized from 1 *M* AcOH to yield 44.9 μmol of homogeneous **12** (72%): Pro, 1.93; His, 2.00; Phe, 1.02; Leu, 0.93;[1] Tyr, 2.05; Val, 1.02; R_f(T3) 0.33; R_f(T4) 0.07; R_f(T8) 0.68; R_{His}(E1) 0.61; $[M]^{18}_{578\ nm}$ −1141° (*c*, 5.5 m*M*, 1.0 *M* AcOH); $\epsilon_{276\ nm}$, 3281 (*c*, 224 μ*M*, 1 *M* AcOH).

Prolylprolylprolylhistidylprolylphenylalanylhistidylleucylphenylalanylvalyltyrosine ((*Pro*)₃-[*Phe⁶*]*octapeptide*) (**13**). *N-tert*-Butoxycarbonyl-*O*-benzyltyrosine cesium salt was prepared by the method reported by Gisin (1973) and used to esterify Bio-Beads SX-1 (200–400 mesh, 0.69 mmol of Cl^-/g of resin). The product contained 0.132 mmol of tyrosine/g of product (20%). Completion of synthetic operations yielded 2.74 g of peptidyl-polymer

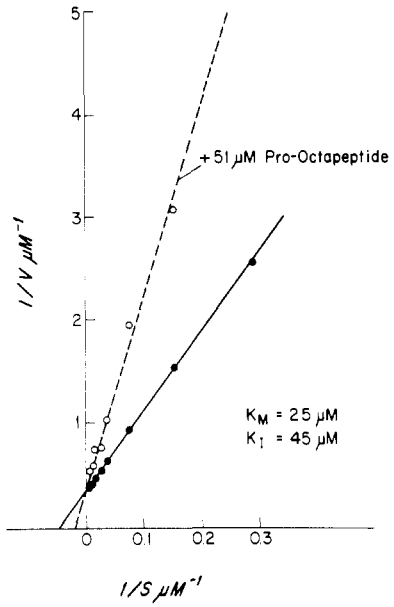

FIGURE 6: Competitive inhibition of renin by Pro-octapeptide. Lineweaver-Burk plots of the reaction between human renin (0.006 GU/ml) and tetradecapeptide substrate with (○) and without (●) the presence of 51 μM of Pro-octapeptide. K_m and K_i values were 25 and 45 μM, respectively. Incubation time was 2 hr at 37° at pH 7.5.

(74%) which was split into two equal batches. One lot was treated with liquified HF–5% anisole (v/v) for 90 min at 0° and the other lot was cleaved in a parallel operation under similar conditions with HF–50% anisole (v/v). After evaporation the residues were independently extracted with 1 M AcOH, and the solutions were then filtered and lyophilized to yield 121 μmol (81%) of crude **13** from the cleavage conducted in the presence of 5% anisole, and 124 μmol (83%) of crude peptide from the parallel cleavage conducted in the presence of 50% anisole. Crude **13** (370 mg) from the HF–50% anisole cleavage was dissolved in 40 ml of 1 M AcOH with gentle warming and a small amount of insoluble material removed by centrifugation. Supernatant (20.8 g) was chromatographed on Sephadex G-15 (2.5 × 194 cm) at a flow rate of 32 ml hr^{-1}. The fraction eluting between 448 and 544 ml was lyophilized to yield 21.5 μmol of peptide (36%). A fraction eluting between 400 and 432 ml contained 5.8 μmol (10%) of the truncated sequence phenylalanylvalyltyrosine. This fraction was not observed when crude peptide from cleavage in the presence of 5% anisole was chromatographed under identical conditions. Partly purified peptide was dissolved in 0.01 N HCl, clarified by centrifugation, and chromatographed on Bio-Gel P-2 (2.5 × 194 cm) in 0.01 N HCl. The desired peptide eluted between 775 and 892 ml, and was lyophilized and relyophilized from 1 M AcOH to yield 39.0 μmol of $(Pro)_3$-[Phe^6]octapeptide (88%). A truncated sequence, valyltyrosine (2.0 μmol, 5.5%), elutes between 935 and 957 ml and is present in extracts from both cleavages: Pro, 4.28; His, 1.92; Phe, 1.98; Leu, 0.90; Val, 0.91; Tyr, 0.95;[1] R_f(T3) 0.65; R_f(T6) 0.34; R_f(T8) 0.58 (streak); R_{His}(E1) 0.61; $[M]^{20}_{578}$ −2339° (c, 2.74 mM, 1.00 M AcOH); $\epsilon_{276\,nM}$ 1773 (c, 524 μM, 1.00 M AcOH).

Determination of Inhibitor Constant (K_i). Human renin (68/356) was obtained from the Division of Biological Standards, London. The source of the tetradecapeptide and human plasma used as substrate was as previously described (Poulsen et al., 1973).

The peptides were dissolved and diluted in 0.01 N HCl until soluble when pH was adjusted to pH 7.5. The peptide solution was diluted further to appropriate concentrations with 0.1 M Tris-HCl containing 0.5% albumin.

Renin (0.006 Goldblatt unit (GU)/ml) was incubated for 2 hr at 37° with a 7 μM concentration of tetradecapeptide and varying concentrations of the inhibitory peptide. The diluent was 0.1 M Tris-HCl containing 0.5% albumin. The release of angiotensin I was determined by radioimmunoassay (Haber et al., 1969; Poulsen, 1969). A peptide concentration giving approximately 50% inhibition was selected for the determination of K_i as described below. To determine K_m, renin (0.006 Goldblatt unit (GU)/ml) was incubated for 2 hr at 37° with incremental concentrations of tetradecapeptide varying between 3 and 108 μM in a volume of 160 μl at pH 7.5 in 0.1 M Tris-HCl containing 0.5% albumin. To determine the inhibitor constant (K_i), a parallel series of experiments were performed exactly as described above but in the presence of a single concentration (3–190 μM) of the inhibitory peptide, the concentration being selected as described above. The cross-reaction of the tetradecapeptide with antibody was 1%; it was less than 0.5% with the peptides both before and after exposure to renin. Controls for cross-reactivity were performed with each experiment and appropriate corrections were made when significant (Poulsen et al., 1973).

The inhibitor constant was obtained as previously described (Poulsen et al., 1973) from a weighted least-squares fit of a Lineweaver-Burk plot (Figure 6). Each point was given a weight proportional to the initial velocity (Dowd and Riggs, 1965).

To determine K_m, 20-μl aliquots of serial dilutions of the plasma described above were incubated with 5 μl of human renin (4×10^{-3} GU/ml); 5 μl of a solution containing 3 M Tris-HCl (pH 7.3), 200 mM EDTA, 12 mM 2,3-dimercaptopropanol, and 25 mM 8-hydroxyquinoline; and 10 μl of 0.1 M Tris-HCl (pH 7.5) containing 0.5% albumin. The mixture was incubated for 45 min at 37° and placed in ice-water. Complementary amounts of plasma were then added as 20 μl of a plasma dilution in 0.1 M Tris-HCl (pH 7.5) containing 0.5% albumin to give the same amount of plasma protein in all tubes. Tracer and antibody (1000 μl) were then added following the radioimmunoassay procedure described by Poulsen and Jorgensen (1974). The standard curve was constructed in the same medium but was kept at 4°. To determine K_i, parallel series of experiments were performed in which the 10-μl Tris buffer was replaced with 10 μl of a solution containing 40 and 20 μM Pro-[Phe^6]octapeptide, respectively. Correction for cross-reactivity and calculation of result were performed as described above.

Results

Modification of Solubility. The octapeptide segment of the tetradecapeptide substrate is moderately soluble at pH 7.5 (Table I, peptide 1). Addition of either one or three prolyl residues to the amino terminus increases solubility nearly threefold (peptides 2 and 3), but there is little difference in the effects on solubility between the mono- and the triproline substitution. An additional fourfold increase in solubility is achieved with the pentaprolyl derivative (peptide 4). The [D-Leu^6]octapeptide (peptide 6) has about the same solubility at pH 7.4 as the parent octapeptide. Addition of proline to the amino terminus of this derivative should produce an excellent inhibitor, but decreased solubility is observed (peptide 7). The triprolyl derivative of the

Table I: Inhibitors of Renin.[a]

Peptide		K_i (μM)	Solubility μM, 20°, pH 7.5
1	His-Pro-Phe-His-Leu-Leu-Val-Tyr	*b*	161
2	Pro—	42	324
3	Pro-Pro-Pro—	157	374
4	Pro-Pro-Pro-Pro-Pro—	140	1328
5	Ile—	29	72
6	—D-Leu—	*b*	137
7	Pro—D-Leu—	*b*	41
8	Pro-Pro-Pro—D-Leu—	*b*	365
9	—Thr—	*b*	300
10	—serinol	*b*	81
11	Pro—Phe—	4	412
12	Pro—Tyr—	12	303
13	Pro-Pro-Pro—Phe—	3	224

[a] Inhibition studies were performed at pH 7.4 and solubility studies at pH 7.5. [b] Absence of inhibition with one-third saturated solution.

[D-Leu^6]octapeptide does show an increase in solubility (peptide 8), and it is approximately as soluble as the $(Pro)_3$-octapeptide (peptide 3). The solubility of Pro-[Phe^6]octapeptide (peptide 11) is approximately equal to the Pro-octapeptide (peptide 2). Two additional prolines at the amino terminus do not increase solubility (peptide 13) in agreement with the comparison between peptides 2 and 3.

Replacement of levoenantiamorphs of hydrophobic residues may be made in position 6 without affecting solubility. Substitution of the hydrophobic residue, valine, in position 7 by an isosteric hydrophilic threonine markedly increased solubility (peptide 9). The addition of serinol at the carboxyl terminus, however, decreased solubility (peptide 10), as did extension of the peptide by addition of isoleucine to the amino terminus (peptide 5).

Inhibition Studies. Although moderately soluble, neither the octapeptide nor the [D-Leu^6]octapeptide showed any inhibition of proteolysis of the tetradecapeptide substrate by renin at pH 7.4 (Table I). The Pro- and Ile-octapeptides were moderately potent competitive inhibitors. The $(Pro)_3$- and $(Pro)_5$-octapeptides, although more soluble, had inhibitory constants approximately fourfold greater than the Pro- and Ile-octapeptides. The addition of three prolines to the amino terminus of the [D-Leu^6]octapeptide did not yield an inhibitor, although they increased its solubility. The [Thr^7]octapeptide was also inactive, although it was soluble. Substitution of the prolyloctapeptide with a phenylalanyl or tyrosyl residue at position 6 gave soluble, effective competitive inhibitors. The $(Pro)_3$-[Phe^6]octapeptide had indistinguishable inhibitory properties from the Pro-[Phe^6]octapeptide. The Pro-[Tyr^6]octapeptide was significantly less potent as an inhibitor than either of the peptides containing a phenylalanine substitution at position 6.

Further insight into the lack of activity of the [D-Leu^6]octapeptide at pH 7.5 was obtained when it and the more soluble $(Pro)_3$-[D-Leu^6]octapeptide were examined at pH 5.5. As found previously, the [D-Leu^6]octapeptide had K_i of 3 μM; the $(Pro)_3$ derivative was, however, inactive.

Using the three best inhibitors, Pro-[Phe^6]octapeptide, Pro_3-[Phe^6]octapeptide, and Pro-[Tyr^6]octapeptide, a complete inhibition of the reaction could be obtained with the use of 140–250 μM of peptide and 7 μM tetradecapeptide.

Competitive inhibition could also be demonstrated with respect to the natural protein renin-substrate of renin in human plasma. A concentration of 110 μM Pro-[Phe^6]octapeptide completely inhibits the reaction between human renin and normal human plasma. K_i in human plasma (Figure 7) for Pro-[Phe^6]octapeptide was determined to be 9 μM. This is in agreement with K_i of 4 μM when the peptide competes with tetradecapeptide (Table I).

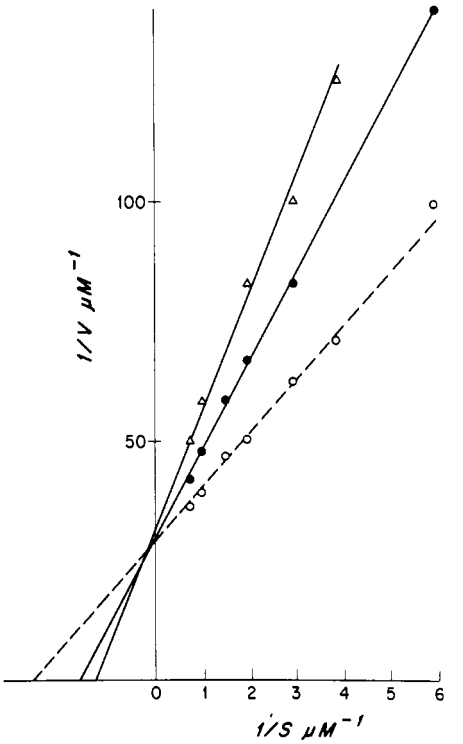

FIGURE 7: Competitive inhibition of the reaction between human renin and protein renin-substrate in plasma by Pro-[Phe^6]octapeptide. The reaction was performed in the presence of 10 μM (△), 5 μM (●), and without (○) Pro-[Phe^6]octapeptide. Renin concentration was 8×10^{-4} GU/ml, and incubation time was 45 min at 37° and pH 7.5. K_m and K_i values were 0.4 and 9 μM, respectively.

Discussion

Three approaches were used to increase solubility of the small peptide renin inhibitors. Addition of prolyl residues to the amino terminus and replacement of the valine at position 7 with threonine were successful. Only the former, however, was effective in yielding active inhibitors. The third approach, patterned on the work of Kokubu et al.

(1968), was ineffective in increasing solubility; the octapeptidylserinol was only ⅓ as soluble as the octapeptide. Extending the peptide chain at the amino terminus with isoleucine, which is the next residue in renin substrate, decreased solubility. Extension of the chain by proline rather than isoleucine increased solubility as well as the activity of the octapeptide. Additional proline residues continued to increase solubility, but inhibitory activity was somewhat decreased. Solubility of the [D-Leu^6]octapeptide was decreased, however, by the addition of a single proline residue to the amino terminus. When three prolines were added, solubility increased, but no activity could be demonstrated, which indicates that the [D-Leu^6]octapeptide could not interact with the active site of renin at pH 7.4 even when it is soluble. This may relate to (1) alterations in the conformation of renin with pH, (2) changes in peptide conformation with pH, or (3) interactions between the amino terminal prolines and the rest of the peptide. The last interpretation is likely to be correct since the (Pro)$_3$-[D-Leu^6]octapeptide is also inactive at pH 5.5, while [D-Leu^6]octapeptide is a potent inhibitor (Poulsen et al., 1973).

In the most effective inhibitor, phenylalanine is substituted for leucine at position 6 and solubility is increased by adding proline to the amino terminus. The increased effectiveness of this compound in relation to the Pro-octapeptide may relate either to increased hydrophobicity of the region around the cleavage site, which leads to a higher affinity for renin, or to the decreased rate of cleavage of the peptide. It should be noted that if the peptide is cleaved by renin, the K_i value is modified by the rate of cleavage (Dixon and Webb, 1964). An increase in hydrophilic properties near the carboxyl end of this peptide leads to decreased activity as shown by the observations that substitution of threonine for valine at position 7 or tyrosine for phenylalanine at position 6 decreases activity of the inhibitor.

In crude extracts of human kidneys, Skeggs et al. (1969) have described an enzyme called pseudorenin, which cleaves tetradecapeptide at the leucylleucine bond yielding angiotensin I. This enzyme has its optimum at pH 4.5, and is remarkable in that it does not cleave the protein renin-substrate. Although purified 600-fold (Haas et al., 1965), the international standard for human renin could hypothetically contain pseudorenin as an impurity. It is, however, very unlikely that the activity of such an impurity would influence the present results for the following reasons. (1) The pseudorenin is 1360-fold less active at pH 7.5 used in this research than at its optimum pH 4.5 (Skeggs et al., 1969). (2) Having determined K_m and V_{max} for the reaction at pH 5.5 (Poulsen et al., 1973) and pH 7.5 (this paper), using a tetradecapeptide concentration of 0.5 μM and a renin concentration of 1×10^{-3} GU ml^{-1}, the reaction rate was calculated to be 190 nM hr^{-1} at pH 5.5 and 7 nM hr^{-1} at pH 7.5, a 28-fold difference. For pure human pseudorenin and the same tetradecapeptide concentration, the difference should be 550-fold (as read from Figure 1; Skeggs et al., 1969). Thus, if all renin activity at pH 5.5 was due to pseudorenin (which is unlikely), only 5% of the activity at pH 7.5 could be caused by this impurity. (3) Almost the same K_i value is found for Pro-[Phe^6]octapeptide when using tetradecapeptide and plasma as substrate, although pseudorenin does not attack plasma substrate. (4) Finally, Levine et al. (1972) demonstrated that a 50% acetone treatment of crude hog renin destroyed 90% of the pseudorenin. Such an acetone treatment is step 5 in the 8-step procedure used by Haas et al. (1965) to prepare the human renin for the international standard used in the present study.

Demonstration of the effective inhibition of the proteolysis of protein substrate in plasma by renin at pH 7.4 suggests that Pro-[Phe^6]octapeptide will provide a model for construction of renin inhibitors effective in vivo.

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