Inhibition of Angiotensin Converting Enzyme by Phosphonic Amides and Phosphonic Acids†

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ABSTRACT: N^{α}-Phosphoryl-L-alanyl-L-proline is a reversible, competitive inhibitor of the zinc protease angiotensin converting enzyme with a K_i of 1.4 nM. Alanine and proline presumably occupy the S_1' and S_2' subsites on the enzyme, and the phosphate is positioned at the active site zinc to mimic the tetrahedral transition state for amide hydrolysis. (Phenethylphosphonyl)-L-alanyl-L-proline extends this inhibitor toward the S_1 subsite from the P–N bond that is at the position of the scissile amide bond in the natural substrate angiotensin I. This inhibitor has a K_i of 0.5 nM, 3-fold lower than that of the parent phosphorylated dipeptide and 200 000-fold lower than that of the parent carboxylated amide (3-phenylpropanoyl)-L-alanyl-L-proline. This decrease in K_i is presumably due to occupation of the S_1 subsite by the phenethyl side chain, which duplicates the side chain of the phenylalanine at the P_1 position in the natural substrate. (Phenethyl-*O*-ethylphosphonyl)alanylproline preserves the tetrahedral phosphorus but is 2000-fold less potent than the unesterified phosphonic amide, demonstrating that, in addition to tetrahedral phosphorus, an ionizable basic oxygen atom is required for tight binding. [[(Benzoylamino)methyl]phosphonyl]alanylproline, which extends phosphorylalanylproline to mimic the synthetic substrate benzoylglycylalanylproline, has 7000-fold reduced potency (K_i = 10 μM) compared to that of phosphorylalanylproline and is equipotent to its carboxylic amide analogue benzoylglycylalanylproline. Therefore, substitution of phosphonic amide for carboxylic amide in inhibitors at the position that interacts with the cationic zinc site of converting enzyme can but does not necessarily increase binding to the enzyme. The discrepancy in the K_i's of the two inhibitors is not explained by the difference in affinity of their phosphonic acid moieties for converting enzyme. Phenethylphosphonic acid and [(benzoylamino)methyl]phosphonic acid have K_i's of 3.2 mM and 1.8 mM, respectively. Substitution of phosphonate for carboxylate in the inhibitor at its carboxy terminus, which interacts with the second cationic site, presumably arginine, does not affect enzyme–inhibitor binding. For example, substitution of a phosphonic acid function for the carboxylic acid group at the carboxy terminus of N^{α}-phosphorylglycylglycine gives [[(N^{α}-phosphorylglycyl)-amino]methyl]phosphonic acid, the K_i of which (35 μM) is experimentally indistinguishable from that of the parent carboxylic acid (K_i = 25 μM).

Angiotensin converting enzyme is a dipeptidyl carboxypeptidase (EC 3.4.15.1) that catalyzes the hydrolysis of the carboxy-terminal dipeptide histidylleucine from the decapeptide angiotensin I to produce the pressor octapeptide angiotensin II. Several potent inhibitors of the enzyme have been shown to be orally active antihypertensive agents in animals and man (Cushman & Ondetti, 1980; Sweet et al., 1981). N^{α}-Phosphoryl-L-alanyl-L-proline, an analogue of the transition state for amide hydrolysis, was shown to be a competitive inhibitor of converting enzyme with a K_i = 1.4 nM (Galardy, 1980). The tetrahedral phosphorus atom in these compounds is thought to be coordinated to the active site zinc of the enzyme, mimicking the transition state for amide hydrolysis in analogy with the thermolysin inhibitor phosphoramidon (Weaver et al., 1977). In addition to the active site zinc, converting enzyme possesses a second cationic site that binds the C-terminal carboxylate of substrates and inhibitors (see Figure 1). This second cationic site is probably an arginine, shown to be essential by chemical modification with butanedione (Bunning et al., 1978).

Three series of phosphonic acids and amides have been tested against converting enzyme for inhibitory activity compared to that of their parent carboxylic acids and amides. [[(Benzoylamino)methyl]phosphonyl]alanylproline and (phenethylphosphonyl)alanylproline [also reported by Thorsett et al. (1982)] substitute the phosphonic amide function for the scissile carboxylic amide of hippurylalanylproline and (3-phenylpropanoyl)alanylproline, respectively. The proposed mode of binding of these compounds to converting enzyme is shown in Figure 1. All these inhibitors are expected to occupy the S_1, S_1', and S_2' subsites on converting enzyme with the scissile carboxylic amide or the phosphonic amide positioned at the cationic active site zinc atom. [(Benzoylamino)-methyl]phosphonic acid and phenethylphosphonic acid mimic the parent carboxylic acids hippuric acid and 3-phenylpropanoic acid, respectively, and presumably occupy only the S_1 site.

[(Glycylamino)methyl]phosphonic acid and [[(N^{α}-phosphorylglycyl)amino]methyl]phosphonic acid substitute phosphonate for C-terminal carboxylate in glycylglycine and N^{α}-phosphorylglycylglycine, respectively, inhibitors that occupy only the S_1' and S_2' sites, as shown in Figure 2. These phosphonic acids substitute phosphonate for carboxylate at the position that interacts with the second cationic site on the enzyme, which is proposed to be arginine. This substitution was expected to decrease the K_i (increase binding to the second cationic site) in analogy with the decrease in K_i observed upon substitution of phosphonate for carboxylate in several other enzyme–inhibitor systems (Brand & Lowenstein, 1978). The phosphorus-containing inhibitors reported here have K_i's that range from equipotent to 200 000-fold more potent than those of their carboxylic analogues.

Experimental Procedures

Hippurylhistidylleucine and (aminomethyl)phosphonic acid were from Sigma. Chloroform was washed with concentrated sulfuric acid and water and then distilled from phosphorus

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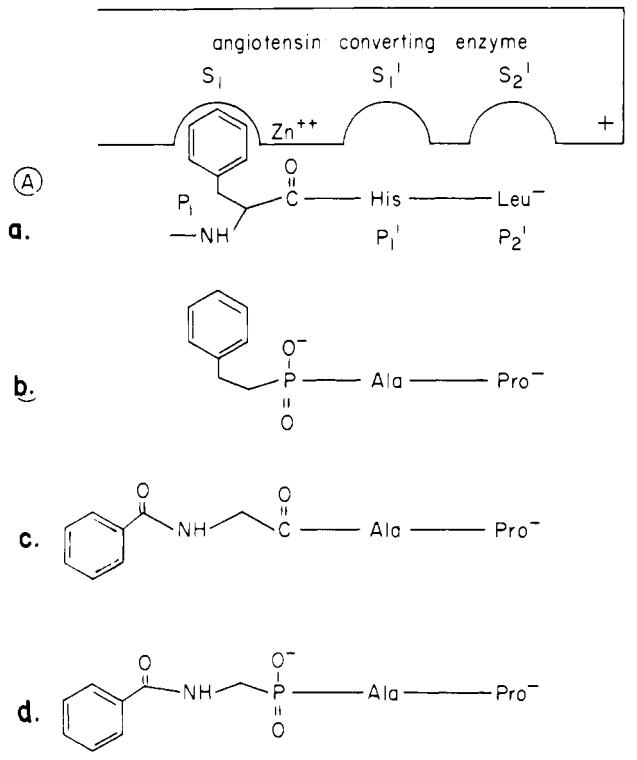

FIGURE 1: A model for the active site of converting enzyme adapted from Cushman & Ondetti (1980): (a) C-terminal tripeptide of the natural substrate angiotensin I; (b) (phenethylphosphonyl)alanylproline (VIII); (c) benzoylglycylalanylproline; (d) [[(benzoylamino)-methyl]phosphonyl]alanylproline (III).

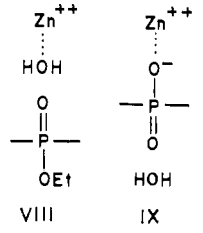

FIGURE 2: Proposed mode of binding of inhibitors VIII and IX at the active site zinc of converting enzyme, adapted from Breslow (1978).

pentoxide. Triethylamine and *N*-ethylmorpholine were distilled from phthalic anhydride. Thionyl chloride was distilled from boiled linseed oil. Protected intermediates were purified by column chromatography on silica gel 60 F-254 (EM Reagents) in chloroform/methanol or chloroform/ethanol.

Melting points were taken on a hot stage and are corrected. Proton nuclear magnetic resonance (NMR) spectra were recorded on a Varian EM-390 or XL-200. Proton chemical shifts are in ppm downfield from tetramethylsilane in organic solvents and from sodium 4,4-dimethyl-4-silapentane-1-sulfonate in deuterium oxide. Thin-layer chromatography (TLC) was on silica gel 60 F-254 (EM Reagents). Compounds were visualized by the following methods: ninhydrin (0.4 g in 100 mL of acetone) for deprotected amines, exposure to hydrochloric acid fumes followed by ninhydrin for phosphorylated amines, phosphomolybdate spray for phosphorylated amines, ultraviolet light, and iodine vapor. Analytical thin-layer solvent systems were, by volume, (A) chloroform/methanol/acetic acid (17:2:1), (B) chloroform/acetic acid (19:1), (C) chloroform/methanol/acetic acid (7:8:11), (D) 1-propanol/concentrated ammonium hydroxide (84:37), (E) acetonitrile/concentrated ammonium hydroxide (3:1), (F) acetonitrile/concentrated ammonium hydroxide (2:1), (G) 1-butanol/acetic acid/water (4:1:1), (H) 1-butanol/pyridine/acetic acid/water (15:10:3:12), (I) acetonitrile/water (3:1), (J) 1-propanol/methanol/3% ammonium hydroxide (13:4:3), and (K) EtOAc/pyridine/HOAc/water (15:15:1:3). Preparative TLC in system J was on plates prepared with a 1-mm layer of silica gel 60 H F-254 (EM Reagents) without binder and without heat activation. High-pressure liquid chromatography (HPLC) was with a Varian 5000 liquid chromatograph on a Unimetrics RP-18 column, 4.6 mm × 25 cm. Elution at 1 mL min^{-1} was with a gradient of 0–70% acetonitrile over 15 min followed by 70% acetonitrile for 5 min. The aqueous phase was 10 mM H_3PO_4 adjusted to pH 7 with LiOH. Retention times after the solvent front are reported as R_t. Paper electrophoresis was at approximately 40 V/cm for about 1 h on Whatman No. 3 MM paper in 1.7% *N*-ethylmorpholine adjusted to pH 7.9 with acetic acid. Relative migration is given as $R_{f,elec}$ compared to a standard.

[*(Benzoylamino)methyl*]*phosphonic Acid* (*I*). Benzoyl chloride (2.6 g, 19 mmol) was added in three portions over 2 h at 25 °C to a solution of (aminomethyl)phosphonic acid (1.78 g, 17 mmol) in 8 mL of water containing 5 g of $NaHCO_3$. After an overnight standing, the reaction was diluted to 100 mL, the pH was adjusted to 4.0 with HCl, and benzoic acid was extracted into ether and discarded. The solution was chromatographed on 105 mL of AG 50-X2 (H^+ form), eluting with water to give 3.0 g (92%) of I: mp 172–174 °C; TLC $R_{f,D}$ 0.35, $R_{f,G}$ 0.35, $R_{f,H}$ 0.66; HPLC R_t 7 min; NMR [$(CD_3)_2SO$] δ 3.45 (2 d, 2 H, CH_2P, J_{CH_2-NH} 6 Hz, J_{CH_2-P} 12 Hz), 7.45 (m, 5 H, Ph), 8.35 (t, 1 H, NH, J_{CH_2-NH} 6 Hz), 8.92 [br s, 2 H, $P(OH)_2$].

[[*(Benzoylamino)methyl*]*phosphonyl*]-L-*alanyl*-L-*proline Benzyl Ester Potassium Salt* (*II*). To 1.3 g of I (6.8 mmol) was added 2 mL of thionyl chloride. After 20 min at room temperature, the thionyl chloride was evaporated and the residue evaporated twice from $CHCl_3$. To this residue was added L-alanyl-L-proline benzyl ester hydrochloride (Galardy, 1982) (2.2 g, 6.8 mmol) and *N*-ethylmorpholine (2.34 g, 20.4 mmol) in 15 mL of $CHCl_3$ at 0 °C. After an overnight standing at 0 °C, the solution was evaporated to an oil that was triturated with water. The oil was then dissolved in 2 mL of MeOH and diluted with 1.5 equiv of KOH in 20 mL of water. The resulting oil was triturated 3 times with water and dried to give 1.03 g (35%) of II: TLC $R_{f,A}$ 0.75, $R_{f,D}$ 0.82, $R_{f,F}$ 0.62, $R_{f,I}$ 0.75; HPLC R_t 17 min; NMR ($CDCl_3$) δ 1.20 (d, 3 H, Ala CH_3), 1.80–2.10 (m, 4 H, Pro CH_2 β,δ), 3.25–4.25 (4 m, 6 H, PCH_2, Pro CH_2 δ, Pro CH α, Ala CH α), 4.87 (m, 2 H, $PhCH_2$), 7.00 (s, 10 H, Ph), 7.60 (m, PNH), 8.00 (t, 1 H, PhCONH).

[[*(Benzoylamino)methyl*]*phosphonyl*]-L-*alanyl*-L-*proline Diammonium Salt* (*III*). To II (311 mg, 0.61 mmol) in 5 mL of MeOH and 2 mL of water at −18 °C was added 1 equiv of 1 N HCl and 300 mg of 5% palladium on carbon. Hydrogen was bubbled through this solution for 6 h at 1 atm at −18 °C, 2 equiv of 1 N NaOH was added at −18 °C (to pH 7–8), and the mixture was evaporated to dryness at room temperature after removal of the catalyst to give 150 mg of a mixture containing alanylproline, I, III, and two other products containing phosphorus. Preparative TLC yielded 35 mg (13%) of III: TLC $R_{f,D}$ 0.50, $R_{f,F}$ 0.63, $R_{f,H}$ 0.58, $R_{f,J}$ 0.56; HPLC R_t 9 min. NMR of III in D_2O showed the unexchangeable protons of II but without the benzyl protons of II. III was also purified by HPLC from the reaction mixture in 5% yield, determined by its extinction coefficients: ϵ_{226} 10 300; ϵ_{270} 950.

Phenethylphosphonic Acid Diethyl Ester (*IV*). IV was prepared by a general method (Kosolapoff, 1944) from

(bromoethyl)benzene (16.4 mL, 0.12 mol) and triethyl phosphite (20.6 mL, 0.12 mol) in 46% yield: bp_{760} 335–340 °C; TLC $R_{f,A}$ 0.57, $R_{f,B}$ 0.44; NMR [$(CD_3)_2SO$] δ 1.20 (t, 6 H, CH_3), 1.98 (m, 2 H, PCH_2), 2.75 (m, 2 H, $PhCH_2$), 3.94 (m, 4 H, OCH_2), 7.05 (s, 5 H, Ph).

Phenethylphosphonic Acid Monoethyl Ester (V). IV (1.48 g, 6.1 mmol) was refluxed for 5 h in 12 mL of dioxane, 4 mL of water, and 6.1 mL of 1.0 N KOH and evaporated. The residue was dissolved in water, extracted 3 times with $CHCl_3$, acidified to pH 1 with HCl, and extracted 6 times with $CHCl_3$. The acidic $CHCl_3$ extracts were combined and evaporated to yield 1 g (76%) of V: TLC $R_{f,C}$ 0.74, $R_{f,D}$ 0.62, $R_{f,E}$ 0.45, $R_{f,F}$ 0.73; NMR ($CDCl_2$) δ 1.23 (t, 3 H, CH_3), 2.05 (m, 2 H, CH_2P), 2.90 (m, 2 H, $PhCH_2$), 4.04 (m, 2 H, OCH_2), 7.10 (s, 5 H, Ph), 12.5 (s, 1 H, OH). V slowly decomposed unless converted to its sodium salt.

Phenethylphosphonic Acid (VI). Phenethylphosphonic acid was prepared from IV according to Jensen & Noller (1949): mp 136–137 °C (lit. mp 136–138 °C).

(Phenethyl-O-ethylphosphonyl)-L-alanyl-L-proline Benzyl Ester (VII). V (0.42 g, 2 mmol) was refluxed in 10 mL of $SOCl_2$ for 40 min and evaporated, and the residue was evaporated twice from $CHCl_3$. To this residue were added L-alanyl-L-proline benzyl ester hydrochloride (0.61 g, 2 mmol) and Et_3N (0.55 mL, 3.9 mmol) in 5 mL of $CHCl_3$ at 20 °C. After an overnight standing, VII (800 mg, 86%) was isolated by partitioning into EtOAc and washing with 5% $NaHCO_3$, 5% citric acid, and water: TLC $R_{f,A}$ 0.13, $R_{f,D}$ 0.70, $R_{f,G}$ 0.62; NMR ($CDCl_3$) δ 1.23 (m, 6 H Ala CH_3, OCH_2CH_3), 2.05 (m, 6 H, Pro CH_2 β,γ, PCH_2), 2.90 (m, 2 H, $PhCH_2$), 3.65 (m, 2 H, Pro CH_2 δ), 4.05 (m, 4 H, OCH_2, Ala CH α, Pro CH α), 4.60 (m, 1 H, Ala NH), 5.15 (d, 2 H, $PhCH_2$), 7.15 (s, 5 H, Ph), 7.28 (s, 5 H, Ph).

(Phenethyl-O-ethylphosphonyl)-L-alanyl-L-proline (VIII). VII (230 mg, 0.49 mmol) was dissolved in 4 mL of MeOH, 1 mL of water, and 1 mL of HOAc containing 50 mg of 5% palladium on carbon. Hydrogen was bubbled through this solution for 6 h at 1 atm at 20 °C. Removal of the catalyst followed by evaporation yielded 190 mg (100%) of VIII: TLC $R_{f,D}$ 0.58, $R_{f,F}$ 0.58, $R_{f,G}$ 0.60; NMR ($CDCl_3$) δ 1.30 (m, 6 H, Ala CH_3, OCH_2CH_3), 2.05 (m, 6 H, Pro CH_2 β,γ, PCH_2), 2.90 (m, 2 H, $PhCH_2$), 3.62 (m, 2 H, Pro CH_2 δ), 4.2 (m, 5 H, OCH_2, Ala CH α, Pro CH α, Ala NH), 7.15 (s, 5 H, Ph), 11.1 (s, 1 H, Pro COOH).

(Phenethylphosphonyl)-L-alanyl-L-proline Dilithium Salt (IX). VIII (160 mg, 0.41 mmol) was dissolved in 1.0 N LiOH (2.05 mL, 2.05 mmol). After 3 days at 37 °C, the solvent was evaporated, and the residue was precipitated from MeOH with acetone to give 145 mg of IX (95%): TLC $R_{f,D}$ 0.43, $R_{f,E}$ 0.17, $R_{f,F}$ 0.29, $R_{f,G}$ 0.27; NMR (D_2O) 1.20 (2 d in ratio 3:1, 3 H, Ala CH_3 s-trans and s-cis), 1.82 (m, 6 H, Pro CH_2 β,γ, $PhCH_2$), 2.62 (m, 2 H, PCH_2), 3.80 (br m, 4 H, Pro CH δ, Pro CH_2 α, Ala CH α), 7.15 (s, 5 H, Ph).

(Phenethylphosphonyl)-L-alanyl-L-proline Benzyl Ester Monopotassium Salt (X). X was prepared from VI and alanylproline benzyl ester hydrochloride analogously to II in 18% yield: TLC $R_{f,A}$ 0.71, $R_{f,B}$ 0.42, $R_{f,I}$ 0.75; NMR ($CDCl_3$) δ 1.23 (d, 3 H, Ala CH_3), 1.90 (m, 6 H, Pro CH_2 β,γ, PCH_2), 2.85 (m, 2 H, $PhCH_2$), 3.50 (m, 2 H, Pro CH_2 α), 4.2 (m, 2 H, Ala CH α, Pro CH α), 5.06 (d, 2 H $PhCH_2O$), 7.18 (m, 10 H, Ph).

(3-Phenylpropanoyl)-L-alanyl-L-proline Benzyl Ester (XI). To 301 mg of 3-phenylpropanoic acid (2 mmol) and 628 mg of alanylproline benzyl ester hydrochloride (2 mmol) in 10 mL of $CHCl_3$ were added 0.27 mL of *N*-ethylmorpholine (2 mmol) and 412 mg of dicyclohexylcarbodiimide (2 mmol). After an overnight standing, XI was isolated as an oil (0.74 g, 88% yield) by partition into EtOAc as described for II: $R_{f,A}$ 0.62, $R_{f,B}$ 0.53, $R_{f,I}$ 0.82.

(3-Phenylpropanoyl)-L-alanyl-L-proline (XII). Hydrogenolysis of 0.59 g of XI in MeOH with palladium on charcoal gave 0.33 g (72%) of XII: $R_{f,A}$ 0.44, $R_{f,B}$ 0.03; NMR ($CDCl_3$), δ 1.30 (d, 3 H, Ala CH_3), 2.00 (m, 4 H, Pro CH_2 β,γ), 2.50 (m, 2 H, $PhCH_2$), 2.95 (m, 2 H, CH_2CO), 3.68 (m, 2 H, Pro CH_2 δ), 4.54, 4.83 (2 m, 2 H, Ala CH α, Pro CH α), 6.99 (d, 1 H, Ala NH), 7.22 (s, 5 H, Ph), 9.81 (br s, 1 H, COOH).

(Dibenzylphosphoryl)glycine Methyl Ester (XIII). To 2.0 g (16 mmol) of glycine methyl ester hydrochloride in 100 mL of $CHCl_3$ were added 4.46 mL (32 mmol) of Et_3N and 16 mmol of dibenzyl chlorophosphite (Atherton et al., 1948). After 12 h at 25 °C, the mixture was diluted into EtOAc and washed with water, 5% sodium bicarbonate, 5% citric acid, and brine, and dried to give 3.5 g (63%) of XIII: mp 90–92 °C; TLC $R_{f,A}$ 0.53, $R_{f,B}$ 0.79, $R_{f,C}$ 0.83; NMR ($CDCl_3$) δ 3.58 (s, 3 H, OCH_3; m, 3 H, $PNHCH_2$), 5.03 (d, 4 H, $PhCH_2$), 7.30 (s, 10 H, Ph).

(Dibenzylphosphoryl)glycine (XIV). To 2.10 g (6 mmol) of XIII in 80 mL of MeOH was added 6.0 mL of 1.0 N KOH. After 3 h at 25 °C, the MeOH was evaporated, and the resulting solution was extracted with ether and then acidified to pH 3 with HCl and extracted with EtOAc. The EtOAc extract was evaporated to give 1.82 g of XIV (100% yield): TLC $R_{f,A}$ 0.68, $R_{f,B}$ 0.34, $R_{f,C}$ 0.78; NMR ($CDCl_3$) δ 3.55 (d, 2 H, Gly CH_2), 4.00 (br m, 1 H, NH), 4.95 (d, 4 H, $PhCH_2$), 7.20 (s, 10 H, Ph), 12.1 (s, 1 H, COOH).

(Dibenzylphosphoryl)glycine N-Hydroxysuccinimide Ester (XV). To 0.435 g (1.3 mmol) of XIV and 0.157 g (1.37 mmol) of *N*-hydroxysuccinimide in 3 mL of dimethoxyethane at 0 °C was added 0.268 g (1.3 mmol) of dicyclohexylcarbodiimide. After removal of dicyclohexylurea, the product was crystallized from 2-propanol in 78% yield: mp 110–111 °C; TLC $R_{f,A}$ 0.75, $R_{f,B}$ 0.62, $R_{f,I}$ 0.87; NMR ($CDCl_3$) δ 2.76 (m, 4 H, CH_2CH_2), 3.60 (m, 1 H, PNH), 3.94 (m, 4 H, Gly CH_2), 5.04 (d, 4 H, $PhCH_2$), 7.30 (s, 10 H, Ph).

[[[(Dibenzylphosphoryl)glycyl]amino]methyl]phosphonic Acid (XVI). XV (423 mg, 1 mmol) was reacted with 111 mg (1 mmol) of (aminomethyl)phosphonic acid (Sigma) in 2 mL of dimethoxyethane and 2.2 mL of water containing 2 mmol of $NaHCO_3$. After 12 h at 25 °C, the reaction mixture was acidified with 2 mmol of 1.0 N HCl at 0 °C and extracted 7 times with Et_2O. The aqueous layer was then extracted with EtOAc until no product remained. Concentration of the EtOAc gave XVI in 51% yield: mp 115 °C; TLC $R_{f,D}$ 0.47, $R_{f,E}$ 0.62, $R_{f,K}$ 0.68; NMR [$(CD_3)_2SO$] δ 3.46 (m, 4 H, Gly CH_2, CH_2P), 5.0 (d, 4 H, $PhCH_2$), 5.35 (m, 1 H, PNH), 7.30 (s, 10 H, Ph), 7.85 [s, 2 H, $P(OH)_2$; m, 1 H, CONH].

[[(Phosphorylglycyl)amino]methyl]phosphonic Acid Tetrapotassium Salt (XVII). Hydrogen at 1 atm was bubbled through a solution of 43 mg (0.1 mmol) of XVI and 0.4 mL of 1.0 N KOH in MeOH/H_2O at 0 °C containing 8 mg of 55% palladium on carbon for 1 h. After removal of the catalyst, evaporation gave 30 mg (75%) of XVII: $R_{f,B}$ 0.06, $R_{f,E}$ 0.20, $R_{f,elec}$ 2.0 ($R_{f,elec}$ Gly-Gly 1.0). Proton-decoupled ^{31}P NMR spectrum at pD 10 in D_2O showed two resonances of equal intensity at 2.6 and 5.8 ppm downfield from the calculated position of an external standard of H_3PO_4.

[(Glycylamino)methyl]phosphonic Acid (XVIII). XVII (7 mg, 17 μmol) in 200 μL of acetic acid and 50 μL of water was heated to 110 °C for 10 min, dried, and precipitated from dry MeOH with acetone to give XVIII: TLC $R_{f,E}$ 0.49, $R_{f,F}$ 0.05,

Table I: Phosphonic Amide and Acid Inhibitors of Angiotensin Converting Enzyme

inhibitor	K_i (μM), mode
$PhCONHCH_2PO_2Ala$-Pro-OBzl (II)	400, mixed
$PhCONHCH_2PO_2Ala$-Pro (III)	10, mixed
$PhCH_2CH_2P(O)OEtAla$-Pro (VIII)	2, competitive
$PhCH_2CH_2PO_2Ala$-Pro-OBzl (X)	68, competitive
$PhCH_2CH_2PO_2Ala$-Pro[a] (IX)	0.0005, competitive
OPO_3Ala-Pro[b]	0.0014, competitive
$PhCONHCH_2PO_3$ (I)	1800, mixed
$PhCH_2CH_2PO_3$ (VI)	3200, mixed
$PO_3NHCH_2CONHCH_2PO_3$ (XVII)	35, competitive
$NH_2CH_2CONHCH_2PO_3$ (XVIII)	2000, not determined

[a] Lit. IC_{50} 0.007 μM (Thorsett et al., 1982). [b] Galardy, 1982.

$R_{f,K}$ 0.14, $R_{f,elec}$ 1.5 ($R_{f,elec}$ Gly-Gly 1.0).

Angiotensin Converting Enzyme. Converting enzyme was partially purified (Galardy, 1982) from frozen rabbit lungs, omitting the last step of lectin affinity chromatography (Das & Soffer, 1975). Yields were lower than those achieved by Das and Soffer. The specific activity of converting enzyme averaged 19 units/mg of protein. One unit of converting enzyme hydrolyzes 1 μmol of hippurylhistidylleucine/min at 37 °C in 100 mM potassium phosphate buffer, pH 8.3, 300 mM in sodium chloride.

Kinetic Studies. All inhibitors were assayed with hippurylhistidylleucine as substrate in 50 mM Tris-HCl[1] adjusted to pH 7.5 with sodium hydroxide, 300 mM in sodium chloride (Cheung & Cushman, 1973; Galardy, 1980), by the fluorometric assay and a single 30-min time point. K_m was 0.5 mM. The reaction was initiated by the addition of enzyme to a final concentration of 0.05 nM. At this enzyme concentration, depletion of inhibitor by enzyme is insignificant (Webb, 1961). Hydrolysis was linear with time to well beyond 30 min with less than 5% total substrate hydrolyzed for all substrate concentrations. K_i's were determined by averaging the K_i's found from a Lineweaver–Burk plot and a Dixon plot. Every K_i was determined at least twice. The standard deviations of the mean-reported K_i's average about 30% of the value. When these K_i's are compared to K_i's and IC_{50}'s from the literature determined under different conditions, the buffer composition and pH are given for the literature references. K_i's determined under different conditions are not strictly comparable.

Results

The K_i's and modes of inhibition of converting enzyme by the phosphonic acids and amides are given in Table I. The K_i of [[(benzoylamino)methyl]phosphonyl]alanylproline III (10 μM) is indistinguishable from that of its analogous carboxylic amide, hippurylalanylproline, K_i = 7 μM in 100 mM phosphate buffer at pH 8.3, 300 mM in sodium chloride (Cheung et al., 1980). In contrast, the K_i of (phenethylphosphonyl)alanylproline (IX) (0.5 nM) is 200 000-fold lower than that of its analogue, (3-phenylpropanoyl)alanylproline, K_i = 100 μM. Esterification of either the carboxy terminus or one of the phosphonic oxygens in IX gives a dramatic increase in K_i. The K_i's of [(benzoylamino)methyl]phosphonic acid (I) and phenethylphosphonic acid (VI), whose phosphonic acid function should be positioned at the active site zinc, are similar to each other while the K_i's of their respective carboxylate analogues are not similar (hippuric acid K_i = 16 mM and 3-phenylpropanoic acid K_i = 1.2 mM). The K_i's of [[(phosphorylglycyl)amino]methyl]phosphonic acid (XVII) and [(glycylamino)methyl]phosphonic acid (XVIII) (see Table I) where the phosphonic function is positioned at the cationic arginine subsite of the enzyme are indistinguishable from the K_i's of their respective carboxylate analogues, phosphorylglycylglycine (K_i = 25 μM; Galardy, 1982) and glycylglycine (K_i estimated to be about 2 mM from an IC_{50} of 7.2 mM in 100 mM phosphate buffer at pH 8.3, 300 mM in sodium chloride; Cheung et al., 1980).

Discussion

The inhibitors of Table I that occupy the S_1, S_1', and S_2' subsites on converting enzyme (III and IX) are designed to be transition-state analogues of substrate hydrolysis. Extension of phosphorylalanylproline (Galardy, 1982) to [[(benzoylamino)methyl]phosphonyl]alanylproline (III) gives an inhibitor that mimics the synthetic substrate hippurylalanylproline. The substitution of a phosphonic amide for the phosphoric amide in the parent compound phosphorylalanylproline was not expected to affect interaction with the active site zinc since alkylation of one phosphate oxygen atom in the phosphoric amide did not change the K_i (Galardy, 1982). However, [[(benzoylamino)methyl]phosphonyl]alanylproline (III), K_i = 10 μM, is a poor inhibitor of converting enzyme compared to phosphorylalanylproline with K_i = 1.4 nM. This is surprising since benzoylglycylalanylproline binds 4 times more strongly to the enzyme than alanylproline (Cheung et al., 1980). In fact, [[(benzoylamino)methyl]phosphonyl]alanylproline has a K_i indistinguishable from that of benzoylglycylalanylproline, (10 μM and 7 μM in pH 8.3 phosphate buffer, 300 mM in sodium chloride, respectively; Cheung et al., 1980). The tetrahedral phosphorus of III, therefore, is not correctly positioned to mimic the tetrahedral transition state for hydrolysis of the peptide substrate.

In contrast, extension of phosphorylalanylproline to mimic the P_1 position side chain only of the natural substrate angiotensin I gives (phenethylphosphonyl)alanylproline (IX) with K_i = 0.5 nM, 3-fold lower than that of the parent compound. The K_i of IX is 20 000-fold lower than the K_i of angiotensin I (K_i = 10 μM in pH 8.3 phosphate buffer, 300 mM in sodium chloride; Cheung et al., 1980), 4000-fold lower than that of phenylalanylalanylproline (K_i = 2 μM), and 200 000-fold lower than that of its carboxylate analogue (3-phenylpropanoyl)-alanylproline (K_i = 100 μM). This result suggests that the phenethyl group and the phosphonic amide are both correctly positioned in the P_1 subsite and at the active site zinc, respectively, and that IX is a transition-state analogue of natural substrate hydrolysis. Inhibition of converting enzyme by IX is therefore analogous to inhibition of thermolysin by the transition-state analogue phosphoramidon (Weaver et al., 1977). The large difference in the K_i's of III and IX is not explainable by a difference in affinity of the moieties occupying the S_1 site since [(benzoylamino)methyl]phosphonic acid and phenethylphosphonic acid have similar K_i's (Table I). However, it is surprising that the relative K_i's of (phenethylphosphonyl)alanylproline and [[(benzoylamino)methyl]-phosphonyl]alanylproline are reversed compared to their respective carboxylate analogues (see Results). The relative K_i's of III and IX are consistent with the relative IC_{50}'s of [(benzoyl-1-amino-2-phenylethyl)phosphonyl]alanylproline and IX, 33 μM and 7 nM, respectively, in 50 mM borate/phosphate buffer at pH 8.0, 170 mM in sodium chloride reported by Thorsett et al. (1982). The K_i's of II and X, the carboxylate benzyl esters of III and IX, are greatly increased compared to those of the free acids as expected for an enzyme that

[1] Abbreviations: IC_{50}, concentration of inhibitor producing 50% inhibition at a given enzyme and substrate concentration; Tris, tris(hydroxymethyl)aminomethane.

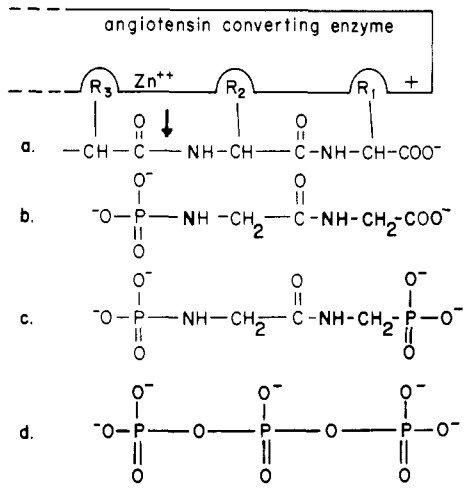

FIGURE 3: Proposed mode of binding of phosphorylated inhibitors to the active site of angiotensin converting enzyme: (a) substrate, the arrow shows the bond cleaved by the enzyme; (b) phosphorylglycylglycine; (c) [[(phosphorylglycyl)amino]methyl]phosphonic acid; (d) tripolyphosphate.

requires a free acid in the P_2' position (Cushman & Ondetti, 1980). The benzyl ester X has a K_i 136 000-fold greater than its parent compound, IX.

The 4000-fold decrease in potency observed in (phenethyl-*O*-ethylphosphonyl)alanylproline (VIII) compared to IX demonstrates that tetrahedral phosphorus is not sufficient for strong inhibition, one ionizable phosphate oxygen is also required. This observation is consistent with the hypothesis of Breslow (1978), concerning the mechanism of the zinc protease carboxypeptidase A. In this hypothesis, ester substrates can use an anhydride mechanism because an ester carbonyl oxygen is not basic enough to displace a water molecule bound to the active site zinc. The amide carbonyl of a peptide substrate is basic enough to displace zinc-bound water, and peptide substrates are directly hydrolyzed by water rather than using the anhydride mechanism. In converting enzyme, the nonionizable phosphonate oxygen of VIII is not basic enough to displace zinc-bound water, and therefore, VIII does not bind properly to the active site zinc. The ionizable phosphonate oxygen in IX is basic enough to displace zinc-bound water and allows tetrahedral phosphate to occupy the position of the scissile amide carbonyl of a peptide substrate. The proposed modes of binding of VIII and IX at the active site zinc of converting enzyme are shown in Figure 2, adapted from Breslow (1978).

Three factors suggested that substitution of terminal phosphate for terminal carboxylate would increase the binding (decrease the K_i) of converting enzyme inhibitors by increasing the affinity to the second cationic site on the enzyme, presumably an arginine. First, tripolyphosphate, a competitive inhibitor that does not remove zinc from the enzyme, was found to be equipotent to phosphorylglycylglycine. Tripolyphosphate (K_i = 18 μM) is a better inhibitor of converting enzyme than N^{α}-phosphorylglycylglycine (K_i = 25 μM; Galardy, 1982). This relatively high potency is surprising since tripolyphosphate lacks side chains, a hydrogen-bond donor in the position of the phosphoramidate nitrogen, and the amide carbonyl of the dipeptide, all of which are important for maximum interaction with the enzyme (Galardy, 1982; Condon et al., 1982; Thorsett et al., 1982; Cushman et al., 1977). Second, phosphonate is more basic than carboxylate and its doubly hydrogen-bonded complex with guanidine may be stronger than a carboxylate–guanidine complex (Cotton et al., 1974). Finally, several phosphonate analogues have K_i's much lower than the K_i's of their respective carboxylate substrates. For example, substitution of 3-phosphonoalanine for the aspartate moiety in the substrate adenylosuccinate produced an inhibitor of adenylosuccinase with K_i 500-fold lower than K_m (Brand & Lowenstein, 1978), where K_m and K_i for the substrate were similar. This was interpreted to indicate 500-fold stronger binding of the phosphonate due to the interaction of phosphonate with a "double cationic site" on the enzyme. The proposed mode of binding of tripolyphosphate, [[(phosphorylglycyl)amino]methyl]phosphonic acid, and its carboxylate analogue phosphorylglycylglycine to converting enzyme is shown in Figure 3.

The finding that substitution of terminal phosphonate for carboxylate does not decrease K_i suggests that inhibitors based on glycylglycine may not be properly aligned in the active site to maximize interaction with the second cationic site of converting enzyme. For example, phosphorylalanylproline binds more than 10 000-fold more tightly than phosphorylglycylglycine (Galardy, 1982) and, therefore, is more likely to have all inhibitor–enzyme interactions optimized. However, Petrillo & Ondetti (1982) have recently reported that *N*-(3-mercaptopropanoyl)pyrrolidine-2-phosphonic acid has an IC_{50} of 1.7 μM, nearly 10-fold higher than its rather potent carboxylate analogue *N*-(3-mercaptopropanoyl)proline, IC_{50} of 0.20 μM. Therefore, phosphonate in general must not interact more strongly than carboxylate at a carboxylate binding site. Table IV in Brand & Lowenstein (1978) also lists several examples of carboxylic substrates whose phosphonate analogues bind more weakly.

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Registry No. I, 6881-56-7; II, 84712-31-2; III, 84712-32-3; IV, 54553-21-8; V, 14295-50-2; VI, 4672-30-4; VII, 84712-33-4; VIII, 84712-34-5; IX, 84712-35-6; X, 84712-36-7; XI, 84712-37-8; XII, 84712-38-9; XIII, 84712-39-0; XIV, 84712-40-3; XV, 84712-41-4; XVI, 84712-42-5; XVII, 84712-43-6; XVIII, 30211-73-5; angiotensin converting enzyme, 9015-82-1; benzoyl chloride, 98-88-4; (aminomethyl)phosphonic acid, 1066-51-9; L-alanyl-L-proline benzyl ester hydrochloride, 41591-35-9; (bromoethyl)benzene, 103-63-9; triethyl phosphite, 122-52-1; 3-phenylpropanoic acid, 501-52-0; glycine methyl ester hydrochloride, 5680-79-5; dibenzyl chlorophosphite, 41662-50-4.

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Binding of Hormones and Neuropeptides by Calmodulin†

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ABSTRACT: Calmodulin exhibits high-affinity, calcium-dependent binding of 1 mol/mol of the vasoactive intestinal peptide (VIP), secretin, and either the 42- or 43-residue gastric inhibitory peptide (GIP) with dissociation constants of 0.05–0.14 μM. The affinity of VIP for calmodulin approaches its affinity for the cell-surface VIP receptors. These peptides compete with both smooth muscle myosin light chain kinase and glucagon in calmodulin binding. Calculation of amino acid frequencies for eight calmodulin binding peptides (VIP, GIP, secretin, ACTH, β-endorphin, substance P, glucagon, and dynorphin [Malencik, D. A., & Anderson, S. R. (1982) *Biochemistry 21*, 3480]) shows a below-average incidence of glutamyl residues, above-average incidence of glutaminyl residues, and average incidence of both aspartyl and asparaginyl residues. Predictions of structure from sequence suggest that the bound peptides contain strongly basic turns and coils in close association with regions having above-average β-sheet potential. The temperature dependence of glucagon binding by calmodulin shows that the association is enthalpy driven.

As the major intracellular receptor for calcium, calmodulin is involved in the regulation of diverse cellular functions. The binding of calcium stabilizes one or more specific conformations of the calmodulin molecule recognized by calmodulin-dependent enzymes such as cyclic nucleotide phosphodiesterase, adenylate cyclase, and myosin light chain kinase [cf. reviews by Means (1981) and Cheung (1980, 1982)]. The complementary protein binding sites of both calmodulin and the affected enzymes are yet to be characterized in terms of primary and higher levels of structure. In fact, no calmodulin-dependent enzyme has been completely sequenced up to the present time. In order to obtain information on the protein binding specificity of calmodulin, we have previously studied the calcium-dependent binding of 19 different model peptides ranging in length from 7 to 39 amino acid residues (Malencik & Anderson, 1982; Malencik et al., 1982b). Our survey revealed four peptides that compete for common binding sites on calmodulin with dissociation constants in the micromolar range: adrenocorticotropic hormone (ACTH),[1] β-endorphin, substance P, and glucagon. The report by Weiss et al. (1980), that ACTH and β-endorphin inhibit the purified cyclic nucleotide phosphodiesterase, had suggested to us that calmodulin binds other peptide hormones and neurotransmitters. Regardless of the physiological relevance of these interactions, they may provide clues regarding a recognition sequence for calmodulin. We found that the peptides that calmodulin binds well contain common structural features, notably a strongly basic tripeptide sequence three positions away from a pair of bulky hydrophobic residues. Since this pattern is similar to the recognition sequence for the cAMP-dependent protein kinase, we suggested that calmodulin and protein kinase act on common sequences in proteins subject to dual control by calcium and cAMP. This hypothesis was supported by experiments showing that phosphorylation by the protein kinase affects calmodulin binding by smooth muscle myosin light chain kinase (Conti & Adelstein, 1981; Malencik et al., 1982a), skeletal muscle troponin I, histone H2A, myelin basic protein (Malencik et al., 1982a), and synthetic protein kinase substrates (Malencik et al., 1982b).

This report extends our previous observations with emphasis on other peptides of the glucagon family—secretin, the vasoactive intestinal peptide (VIP), and the gastric inhibitory peptide (GIP) (Table IV). The affinities of these peptides for calmodulin are 10–70 times greater than those of the peptides previously studied and are within an order of magnitude of that for the cell-surface neurohormone receptor in the case of VIP. The close relationships among the peptides permit observations on the effects of amino acid substitutions.

Materials and Methods

Porcine brain calmodulin was prepared according to Schreiber et al. (1981) and subjected to a final purification step by affinity chromatography on a fluphenazine–Sepharose matrix (Charbonneau & Cormier, 1979). This additional

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[1] Abbreviations: Mops, 3-(*N*-morpholino)propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; 9AC, 9-anthroylcholine; cAMP, adenosine cyclic 3′,5′-phosphate; CaM, calmodulin; MLCK, myosin light chain kinase; TnI, troponin I; ACTH, adrenocorticotropic hormone; VIP, vasoactive intestinal peptide; GIP, gastric inhibitory peptide; dansyl, 5-(dimethylamino)-1-naphthalenesulfonyl; *K*, dissociation constant; *F*, observed fluorescence; F_0, fluorescence of unbound ligand; F_∞, fluorescence of totally bound ligand.

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