Inhibition of Angiotensin Converting Enzyme by Phosphoramidates and Polyphosphates†

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ABSTRACT: N^{α}-Phosphoryl-L-alanyl-L-proline is a reversible competitive inhibitor of angiotensin converting enzyme with a K_i of 1.4 nM. Alkylation of one phosphate oxygen with methyl, ethyl, or benzyl does not change the K_i. The high activity of the O-alkylated inhibitors demonstrates that the two phosphate oxygen anions do not constitute a bidentate ligand of the active site zinc ion. Substitution of valyltryptophan, glycylglycine, or δ-aminovaleric acid for alanylproline in the phosphoramidate raises the K_i to 12 nM, 25 μM, and 178 μM, respectively. Methylation of the alanine nitrogen in phosphorylalanylproline raises the K_i to 29 μM. Polyphosphates inhibit converting enzyme with the following K_i's: phosphate, ~300 mM; pyrophosphate, 2 mM; tripolyphosphate, 18 μM; tetrapolyphosphate, 150 μM. The inhibition by tripolyphosphate appears to be competitive and is unaffected by the addition of excess zinc ion. Since the K_i of tripolyphosphate is nearly 10-fold lower than that of *N*-phosphoryl-δ-aminovaleric acid and is near that of N^{α}-phosphorylglycylglycine, its terminal phosphates may bind the zinc site and the cationic site on the enzyme, thus spanning the S_1' and S_2' sites.

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). These inhibitors resulted from extensive structure–activity studies that showed preference for the dipeptide alanylproline with a free terminal carboxyl group in the S_1' and S_2' sites on the enzyme and a carboxyl or mercaptan ligand for the active site zinc ion (Cushman et al., 1977). The above studies culminated in D-(3-mercapto-2-methylpropanoyl)-L-proline (captopril), an orally active inhibitor with K_i = 1.7 nM for converting enzyme (Cushman et al., 1977). On the basis of the inhibition of other zinc proteases by phosphoramidates (Kam et al., 1979) and the inhibition of converting enzyme by monophenyl esters of phosphoramidates (Holmquist & Vallee, 1979), N^{α}-phosphorylalanylproline was shown to be a competitive inhibitor of converting enzyme with K_i = 1.4 nM (Galardy, 1980). Patchett et al. (1980) employed carboxylate as a zinc ligand in the transition-state analogue (1-carboxy-3-phenylpropyl)alanylproline, which has a K_i of 0.2 nM (Herb Bull, personal communication, Merck Sharp & Dohme, Inc., Rahway, NJ). Thorsett et al. (1982) have recently shown that a number of phosphoric and phosphonic amides of alanylproline have I_{50}'s[1] in the 10 nM range. These compounds were proposed to be transition-state analogues of normal substrate hydrolysis in analogy with phosphoramidon, a phosphoramidate inhibitor of the zinc protease thermolysin (Weaver et al., 1977).

This paper describes structure–activity relations for reversible inhibition of converting enzyme by *N*-phosphoryl dipeptides and by polyphosphates. The effect of substitutions in the P_1' and P_2' positions agrees, with one exception, with those established for dipeptide, carboxyl, and mercaptan inhibitors (Cushman et al., 1977; Cheung et al., 1980). The fact that inhibition of converting enzyme by tripolyphosphate is superior to that of phosphorylglycylglycine suggests that it spans the S_1' and S_2' sites, one terminal phosphate a ligand for the active site zinc and the other interacting with the cationic site on the enzyme that normally binds carboxylate anion. Figure 1 shows the active site model for converting enzyme of Cushman et al. (1977), one natural substrate, and the proposed mode of binding of phosphorylalanylproline.

Experimental Procedures

Hippurylhistidylleucine, pyrophosphate, tripolyphosphate, and tetrapolyphosphate were from Sigma. Tripolyphosphate was purified on the sodium form of Bio-Rad AG50-X2 by elution with water to remove an impurity that was fluorescent in the fluorometric assay of converting enzyme. Chloroform and carbon tetrachloride were washed with concentrated sulfuric acid and water and then distilled from phosphorus pentoxide. Triethylamine, *N*-methylmorpholine, and *N*-ethylmorpholine were distilled from phthalic anhydride. 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 spectra were recorded on a Varian EM-390 or XL-200. Chemical shifts are in ppm downfield from tetramethylsilane in organic solvents and from sodium 4,4-dimethyl-4-silapentane-1-sulfonate in deuterium oxide. Paper electrophoresis was at approximately 40 V/cm for about 1 h on Whatman either No. 1 or 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. Thin-layer chromatography was on silica gel 60 F-254 or cellulose F-254 (EM Reagents). Compounds were visualized by the following methods: ninhydrin (0.4 g in 100 mL of acetone) for deprotected and *tert*-butyloxycarbonyl-protected amines; exposure to hydrochloric acid fumes followed by ninhydrin for phosphorylated amines; phosphomolybdate spray for phosphorylated amines (Bandurski & Axelrod, 1951); ultraviolet light; iodine vapor. Thin-layer solvent systems were,

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

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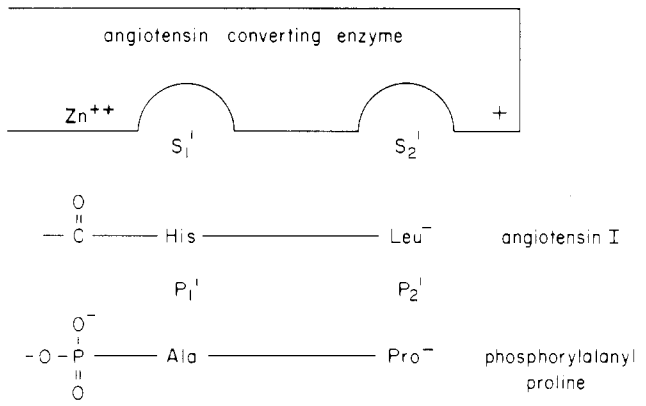

FIGURE 1: Model for active site of converting enzyme adapted from Cushman et al. (1977).

by volume, (A) butanol/acetic acid/water (4:1:1) on silica, (B) chloroform/acetic acid (19:1) on silica, (C) chloroform/methanol/acetic acid (17:2:1) on silica, (D) acetonitrile/water (3:1) on silica, (E) acetonitrile/water (3:1) on cellulose, (F) butanol/pyridine/acetic acid/water (15:10:3:12) on silica, (G) butanol/pyridine/acetic acid/water (15:10:3:12) on cellulose, (H) ethanol/pyridine/acetic acid/water (5:5:1:3) on silica, (I) ethanol/pyridine/acetic acid/water (5:5:1:3) on cellulose, (J) 2-propanol/concentrated ammonium hydroxide/water (4:2:4) on silica, (K) 2-propanol/concentrated ammonium hydroxide/water (4:2:4) on cellulose, (L) 1-propanol/concentrated ammonium hydroxide (8.4:3.7) on silica, (M) 1-propanol/concentrated ammonium hydroxide (8.4:3.7) on cellulose, (N) 0.1 N sodium chloride on cellulose, (O) 0.4 N sodium chloride on cellulose, (P) 1.2 N sodium chloride on cellulose, (Q) ethyl acetate/pyridine/acetic acid/water (15:15:1:3) on silica, and (R) acetonitrile/concentrated ammonium hydroxide (3:1) on silica.

N^{α}-Phosphoryl-L-alanyl-L-proline Tripotassium Salt (IV). To 12 g (50 mmol) of the hydrochloride salt of L-proline benzyl ester (Sigma) and 9.5 g (50 mmol) of *N*-(*tert*-butyloxycarbonyl)-L-alanine in 50 mL of dimethylformamide at 0 °C were added 5.55 mL (50 mmol) of *N*-methylmorpholine and 10.3 g (50 mmol) of dicyclohexylcarbodiimide (Aldrich). After the mixture was allowed to stand overnight at 4 °C, the dicyclohexylurea was collected by filtration, and the filtrate was diluted to 1 L with ethyl acetate, which was extracted with 5% sodium bicarbonate, water, 5% citric acid, water, and brine, and dried over magnesium sulfate. Evaporation yielded 19.1 g (98% yield) of I, *N*-(*tert*-butyloxycarbonyl)-L-alanyl-L-proline benzyl ester: R_{f_B} 0.55, R_{f_C} 0.80, R_{f_D} 0.87.

A 19.1-g sample (49 mmol) of I was deprotected by stirring in 100 mL of 1.6 N HCl in acetic acid at room temperature for 10 min and then poured into 1.5 L of ether with stirring. The precipitate was collected, washed with ether, and dried to give 11.8 g (78% yield) of II, the hydrochloride salt of L-alanyl-L-proline benzyl ester: mp 169–170 °C; R_{f_C} 0.22, R_{f_K} 0.57.

II was acylated with dibenzyl chlorophosphite prepared from dibenzyl phosphite by the method of Atherton et al. (1948). To 3.14 g (10 mmol) of II and 2.7 mL (20 mmol) of triethylamine dissolved in 50 mL of chloroform at 0 °C was added a solution of 10 mmol of dibenzyl chlorophosphite in 25 mL of chloroform. After 18 h at 4 °C, the reaction mixture was diluted with a large volume of ethyl acetate, extracted as described for I, and dried under high vacuum to yield 4 g (75% yield) of III, N^{α}-(dibenzylphosphoryl)-L-alanyl-L-proline benzyl ester: R_{f_B} 0.51, R_{f_C} 0.65, R_{f_D} 0.89; NMR ($CDCl_3$) δ 1.28 (d, 3 H, Ala CH_3), 1.95 (m, 4 H, Pro CH_2 β,γ), 3.43 (m, 2 H, Pro CH_2 δ), 3.86 (m, 1 H, P–NH), 4.01 (m, 1 H, Ala CH α), 4.52 (m, 1 H, Pro CH α), 5.10 (d, 2 H, Ph CH_2), 5.12 (d, 2 H, Ph CH_2), 5.21 (d, 2 H, Ph CH_2), 7.46 (m, 15 H, Ph). Anal. Calcd for $C_{29}H_{33}N_2O_6P$: C, 64.92; H, 6.20; N, 5.22; P, 5.77. Found: C, 65.24; H, 6.20; N, 4.98; P, 5.78.

III was converted to IV by hydrogenolysis in the presence of 3 equiv of potassium hydroxide. To 3.35 g of III (6.25 mmol) in 100 mL of methanol and 50 mL of water were added 18.75 mL of 1.0 N potassium hydroxide and 0.5 g of 5% palladium on carbon. Hydrogen at atmospheric pressure was passed through the solution for 1 h. After removal of the catalyst by filtration, the solution was evaporated to give 2.2 g (93%) of IV. This material was precipitated from the minimum amount of methanol with acetone to give a white solid: R_{f_K} 0.86, positive to ninhydrin spray containing 1% acetic acid, positive to phosphomolybdate spray for phosphate. Sodium dihydrogen phosphate has an R_{f_K} of 0.45 and alanylproline has an R_{f_K} of 0.86. IV is ninhydrin and phosphomolybdate positive upon high-voltage paper electrophoresis at pH 7.9, $R_{f_{elec}}$ 0.75 ($R_{f_{elec}}$ for alanylproline 0.12). Warming IV in acetic acid for a few minutes quantitatively yields alanylproline and free phosphate determined in thin-layer system K and by paper electrophoresis.

N^{α}-Phosphoryl-L-valyl-L-tryptophan Tripotassium Salt (V). V was prepared identically with IV, starting with (*tert*-butyloxycarbonyl)-L-valine and L-tryptophan benzyl ester hydrochloride. Hydrogenolysis of the tribenzyl ester of V gave V in 80% yield: R_{f_K} 0.86, $R_{f_{elec}}$ 0.4 ($R_{f_{elec}}$ for valyltryptophan 0.1).

N^{α}-Phosphoryl-L-alanyl-L-prolinamide Dipotassium Salt (VI). VI was prepared identically with IV, starting with prolinamide (Sigma). Hydrogenolysis of the dibenzyl ester of VI gave VI in 90% yield. VI was always contaminated by a few percent of IV, presumably due to basic hydrolysis of the primary amide. Thin-layer chromatography of VI resulted in the following: R_{f_K} 0.89, R_{f_G} 0.28, R_{f_I} 0.20, R_{f_N} 0.29, R_{f_O} 0.76, R_{f_P} 0.87, $R_{f_{elec}}$ 0.54 ($R_{f_{elec}}$ for IV 0.75, $R_{f_{elec}}$ for alanylproline 0.12).

N^{α}-Phosphorylglycylglycine Tripotassium Salt (VII). VII was prepared identically with IV in 93% yield from its tribenzyl ester: R_{f_K} 0.62, R_{f_J} 0.65, $R_{f_{elec}}$ 0.6 ($R_{f_{elec}}$ for glycylglycine 0.1).

N^{α}-Phosphoryl-δ-aminovaleric Acid Tripotassium Salt (IX). The benzyl ester of δ-aminovaleric acid *p*-toluenesulfonate salt (VIII) was prepared according to the general method of Greenstein & Winitz (1961a) in 75% yield: mp 75–76 °C; R_{f_A} 0.44, R_{f_F} 0.72, R_{f_J} 0.32. IX was prepared from its tribenzyl ester in 86% yield: R_{f_E} 0.43, R_{f_G} 0.80, R_{f_I} 0.87, R_{f_K} 0.87, R_{f_M} 0.22.

N^{α}-(O-Methylphosphoryl)-L-alanyl-L-proline Dipotassium Salt (X). X was prepared from II and the product of the reaction of phenyl phosphoryldichloridate with methanol (Hamer & Tack, 1974) identically with IV. X was isolated in 20% yield from hydrogenolysis of its triester after multiple precipitations from methanol with acetone: R_{f_L} 0.12, R_{f_A} 0.05, R_{f_M} 0.49; NMR (D_2O) δ 1.14 (d, 3 H, Ala CH_3), 1.82 (m, 4 H, Pro CH_2 β,γ), 3.30 (d, 3 H, P–OCH_3, J = 10 Hz), 3.56 (m, 2 H, Pro CH_2 δ), 3.90–4.12 (m, 2 H, Pro CH α, Ala CH α).

N^{α}-(O-Ethylphosphoryl)-L-alanyl-L-proline Dipotassium Salt (XI). XI was prepared identically with X in 20% yield from its triester: R_{f_L} 0.12, R_{f_A} 0.05.

N^{α}-(O-Benzylphosphoryl)-L-alanyl-L-proline Dipotassium Salt (XII). XII was isolated in 68% yield from the triester III by partial hydrogenation in the presence of 3 equiv of potassium hydroxide: R_{f_A} 0.14, R_{f_L} 0.23; NMR (D_2O) δ 1.12 (d, 3 H, Ala CH_3), 1.70 (m, 4 H, Pro CH β,γ), 3.34 (m, 2

H, Pro CH_2 δ), 3.84 (m, 2 H, Ala CH α, Pro CH α), 7.28 (m, 5 H, Ph).

N^{α}-Phosphoryl-N^{α}-methyl-L-proline Tripotassium Salt (XIV). *N*-(*tert*-Butyloxycarbonyl)-*N*-methyl-L-alanine was prepared from *N*-methyl-L-alanine (Chemical Dynamics Corp., South Plainfield, NJ) of specific rotation 5.4° in water (lit. $[\alpha]_D$ = +5.6° in water; Greenstein & Winitz, 1961b) and 2-[[(*tert*-butyloxycarbonyl)oxy]imino]-2-phenylacetonitrile (Itoh et al., 1975) in 87% yield: mp 90–98 °C; R_{f_A} 0.79, R_{f_C} 0.46. XIV was prepared in a manner identical with IV in 96% yield from its tribenzyl ester: R_{f_Q} 0.41, R_{f_R} 0.05, $R_{f_{elec}}$ 0.6 ($R_{f_{elec}}$ for glycylglycine 0.1).

Angiotensin Converting Enzyme. Converting enzyme was partially purified from frozen rabbit lungs according to Das & Soffer (1975), omitting the last step of lectin affinity chromatography. Yields were lower than those achieved by Das & Soffer (1975). The specific activity of converting enzyme averaged 19 units/mg of protein. One unit of converting enzyme hydrolyzes 1 mmol of hippurylhistidylleucine/min in 100 mM potassium phosphate buffer, pH 8.3, 300 mM in sodium chloride (Cheung & Cushman, 1973). Protein was assayed by the binding of Coomassie Brilliant Blue (Bradford, 1976) and with fluorescamine (Bohlen et al., 1973) with bovine serum albumin as a standard.

Kinetic Studies. All inhibitors were assayed with hippurylhistidylleucine as substrate in 50 mM Tris-HCl adjusted to pH 7.5 with sodium hydroxide, 300 mM in sodium chloride (Galardy, 1980), by the fluorometric assay described by Cheung & Cushman (1973) and a single 30-min time point. The 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. The reaction was also initiated by addition of substrate to a mixture of enzyme and inhibitor preincubated for 20 min. 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.

Phosphoramidates not alkylated on phosphate oxygen were dissolved at high concentration (≥1 mg/mL) in distilled water at 0 °C to give a stock solution of pH ~10. This solution was diluted into buffer just before assay. Other phosphoramidates were dissolved in pH 7.5 buffer at 0 °C.

Inhibition of converting enzyme by phosphorylalanylproline was shown to be reversible by dilution of enzyme and inhibitor at a high degree of inhibition to a low inhibitor concentration. The inhibitor was shown to be at equilibrium with enzyme in less than 10 min (i.e., inhibition was complete) by assay of aliquots of an enzyme–inhibitor mixture as a function of time over a 90-min period with a 5-min time point assay.

Results

Lineweaver–Burk and Dixon plots for the inhibition of converting enzyme by N^{α}-(*O*-methylphosphoryl)alanylproline, N^{α}-phosphoryl-N^{α}-methylalanylproline, and tripolyphosphate are shown in Figure 2.

The K_i's and modes of inhibition of converting enzyme by phosphoramidates and polyphosphates are given in Table I. Identical K_i's were found when the reaction was initiated by adding substrate to a mixture of enzyme and inhibitor preincubated for 20 min. Inhibition by phosphorylalanylproline was reversible and did not progress with increasing time of contact between enzyme and inhibitor. The inhibition of converting enzyme by tripolyphosphate at 10 and 40 μM was not affected by the addition of 20 and 80 μM zinc chloride, respectively.

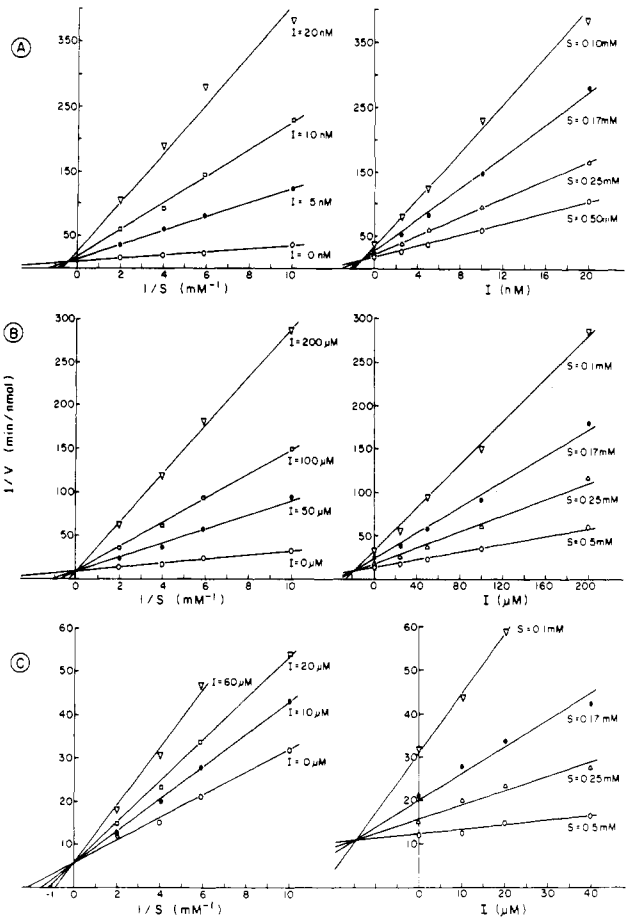

FIGURE 2: Lineweaver–Burk (left side) and Dixon (right side) plots for inhibition of converting enzyme by (A) N^{α}-(*O*-benzylphosphoryl)alanylproline, (B) N^{α}-phosphoryl-N^{α}-methylalanylproline, and (C) tripolyphosphate.

Table I: Inhibitors of Angiotensin Converting Enzyme

inhibitor	K_i (μM)	mode
PO_3AlaPro[a] (IV)	0.001 4	competitive
MeOPO$_2$AlaPro (X)	0.002 6	competitive
EtOPO$_2$AlaPro (XI)	0.001 2	competitive
BzlOPO$_2$AlaPro[a] (XII)	0.001 2	mixed
PO_3AlaProNH$_2$ (VI)	0.033	competitive or mixed
PO_3-δ-aminovaleric acid (IX)	80	competitive
PO_3GlyGly (VII)	25	mixed
PO_3ValTrp (V)	0.012	noncompetitive
PO_3NMeAlaPro (XIV)	29	competitive or mixed
phosphate	~300 000	
pyrophosphate	1 800	mixed
tripolyphosphate	18	competitive
tetrapolyphosphate	150	competitive or mixed
MK-422[b]	0.000 23	competitive

[a] Thorsett et al. (1982) report I_{50}'s of 0.7 μM and 0.04 μM for phosphorylalanylproline and N^{α}-(*O*-benzylphosphoryl)alanylproline, respectively. [b] MK-422 is *N*-(1-carboxy-3-phenylpropyl)-L-proline and is included as a reference compound. Its K_i is reported to be 0.0002 μM (Herb Bull, personal communication, Merck Sharp & Dohme, Inc., Rahway, NJ).

Discussion

Phosphorylalanylproline and Its O-Alkyl Analogues. Phosphorylalanylproline and its *O*-alkyl derivatives exhibit

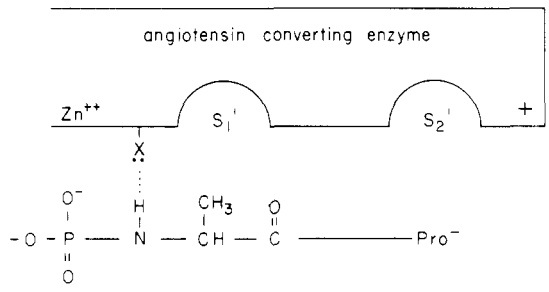

FIGURE 3: Binding of phosphorylalanylproline to converting enzyme showing proton donation by the phosphoramidate nitrogen.

equally low reversible K_i's for converting enzyme of about 1 nM. In contrast, Thorsett et al. (1982) found that phosphorus substituents larger or smaller than $PhCH_2O$– led to decreased inhibition. This remarkably tight binding is equivalent to that found for D-(3-mercapto-2-methylpropanoyl)proline (1.7 nM; Cushman et al., 1977). The K_i of 1.4 nM for phosphorylalanylproline is not consistent with the I_{50} of 0.7 μM reported by Thorsett et al. (1982). The high I_{50} reported by Thorsett et al. probably represents decomposition of the inhibitor, which occurs rapidly below pH 10. On the basis of the extraordinarily tight binding of phosphorylalanylproline to converting enzyme and the fact that the *O*-alkyl inhibitors bind as well as the parent compound, these phosphoramidates are likely to be transition-state analogues of the natural substrate as found by Thorsett et al. (1982) for similar phosphoramidates.

Effect of Substitutions at Positions P_1' and P_2'. The requirement for an unprotected carboxyl terminus in both substrates and inhibitors of converting enzyme is well established (Cushman & Ondetti, 1980). The unexpectedly high activity of phosphorylalanylprolinamide is due to contamination by phosphorylalanylproline, produced by partial hydrolysis of the primary amide in the basic conditions necessary for preservation of the phosphorus–nitrogen bond. Substitution of glycylglycine or δ-aminovaleric acid at the P_1' and P_2' sites increases the K_i as found for the analogous mercaptan inhibitors (Cushman et al., 1977). Substitution of valyltryptophan increases the K_i's compared to alanylproline, contrary to the relative K_i's of the free dipeptides (Cheung et al., 1980), and yields a noncompetitive inhibitor. Therefore, the structure–activity relations already established for the P_1' and P_2' positions cannot be extended to all phosphoramidates.

Phosphoryl-N-methylalanylproline. Substitution of a methyl group for the phosphoramidate amide proton increases the K_i by a factor of 20000. If the nonzwitterionic form of the phosphoramidate is the species that binds to the enzyme, then the *N*-methyl inhibitor can no longer act as a proton donor in the enzyme–inhibitor complex, as demonatrated by Thorsett et al. (1982). Loss of this hydrogen bond could be consistent with the observed large decrease in potency. However, if the zwitterion binds to the enzyme, then N-methylation could be either changing the concentration of this species or sterically interfering with its binding. The log of the ratio of the concentration of zwitterion to nonzwitterion is equal to a pK_{a2} of −7.2, where K_{a2} is the second dissociation constant of the phosphoramidate (Benkovic & Sampson, 1971). pK_{a2} is about 1 pK_a unit below the pK_a of the free amine in the phosphoramidate (Benkovic & Sampson, 1971). If one uses pK_a' = 8.3 for alanylproline and pK_a' = 8.8 for *N*-methylalanylproline (Fasman, 1976), the pK_{a2}'s of their phosphoramidates would be approximately 7.3 and 7.8, respectively. Therefore, both of these phosphoramidates have a significant proportion of nonzwitterionic species present in solution with the phosphoryl-*N*-methylalanylproline having about 3-fold more zitterion than phosphorylalanylproline. It is not known which species binds to the enzyme, and both zwitterion and nonzwitterion are present at significant concentrations. If the zwitterion binds, then the *N*-methyl compound should be a better inhibitor since its zwitterion concentration is higher. The observed 20000-fold decrease in potency must therefore be due to a steric effect or to lack of proton donation by the nonzwitterion species in the enzyme–inhibitor complex.

The crystallographic structure of the complex of phosphoramidon with the zinc protease thermolysin shows that one phosphate oxygen is directed toward the active site zinc and that the phosphoric amide proton is donated in a hydrogen bond to an enzyme carbonyl oxygen (Weaver et al., 1977). The mode of binding of phosphorylalanylproline to converting enzyme is proposed to be similar and is shown in Figure 3. Phosphoramidates are therefore transition-state analogues of normal substrate hydrolysis except that the phosphoramidate nitrogen is a proton donor and the substrate peptide nitrogen normally in this position is a proton acceptor, as for carboxypeptidase A (Quiocho & Lipscomb, 1971). Not shown in this figure (nor in Figure 1) is the hydrogen bond donated to the alanine carbonyl oxygen by an enzyme-bound donor (Cushman et al., 1977; Condon et al., 1982).

Polyphosphates. Tripolyphosphate is about 10-fold more potent an inhibitor than ATP (62% inhibition at 380 μM; Oshima & Nagasawa, 1976) and is a better inhibitor than phosphoryl-δ-aminovaleric acid, phosphorylglycylglycine, and phosphoryl-*N*-methylalanylproline (see Table I). The high inhibitory activity of tripolyphosphate compared to those of tetrapolyphosphate and pyrophosphate (see Table I) indicates that the trimer spans the S_1' and S_2' sites but that the dimer and tetramer are too short and too long, respectively. The K_i of 18 μM for tripolyphosphate is unaffected by the addition of excess zinc and is competitive.

Such strong inhibition is surprising considering that tripolyphosphate lacks the proton-donating phosphoramidate nitrogen, lacks any aliphatic side chains, and appears to be two atoms too short to span the zinc and cationic sites on the enzyme. However, the internal phosphate could conceivably act as a hydrogen-bond acceptor as the amide carbonyl does in mercaptoacyl amino acids (Condon et al., 1982). Tripolyphosphate may bind in an extended conformation without interacting with the S_1' and S_2' side chain sites while peptide substrates and inhibitors bind in a folded conformation with side chains fitting these sites.

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References

Atherton, F. R., Howard, H. T., & Todd, A. R. (1948) *J. Chem. Soc.*, 1106–1110.

Bandurski, R. S., & Axelrod, B. (1951) *J. Biol. Chem. 193*, 405–410.

Benkovic, S. J., & Sampson, E. J. (1971) *J. Am. Chem. Soc. 93*, 4009–4016.

Bohlen, P., Stein, S., Dairman, W., & Udenfried, S. (1973) *Arch. Biochem. Biophys. 155*, 213–220.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Cheung, H. S., & Cushman, D. W. (1973) *Biochim. Biophys. Acta 293*, 451–463.

Cheung, H. S., Wang, F. L., Ondetti, M. A., Sabo, E. F., & Cushman, D. W. (1980) *J. Biol. Chem. 255*, 401–407.

Condon, M. D., Petrillo, E. W., Jr., Ryono, D. E., Reid, J. A., Neubeck, R., Puar, M., Heikes, J. E., Sabo, E. F., Losee, K. A., Cushman, D. W., & Ondetti, M. A. (1982) *J. Med. Chem. 25*, 250–258.

Cushman, D. W., & Ondetti, M. A. (1980) *Prog. Med. Chem. 17*, 41–104.

Cushman, D. W., Cheung, H. S., Sabo, E. F., & Ondetti, M. A. (1977) *Biochemistry 16*, 5484–5491.

Das, M., & Soffer, R. L. (1975) *J. Biol. Chem. 250*, 6762–6768.

Fasman, G. D. (1976) *Handbook of Biochemistry and Molecular Biology, Physical and Chemical Data*, Vol. 1, pp 318, 321, CRC Press, Cleveland, OH.

Galardy, R. E. (1980) *Biochem. Biophys. Res. Commun. 97*, 94–99.

Greenstein, J. P., & Winitz, M. (1961a) *Chemistry of the Amino Acids*, Vol. 2, p 942, Wiley, New York.

Greenstein, J. P., & Winitz, M. (1961b) *Chemistry of the Amino Acids*, Vol. 3, p 2760, Wiley, New York.

Hamer, N. K., & Tack, R. D. (1974) *J. Chem. Soc., Perkin Trans. 2*, 1184–1187.

Holmquist, B., & Vallee, B. L. (1979) *Proc. Natl Acad. Sci. U.S.A. 76*, 6216–6220.

Itoh, M., Hagiwara, D., & Kamiya, T. (1975) *Tetrahedron Lett. 49*, 4393–4394.

Kam, C. M., Nishino, N., & Powers, J. C. (1979) *Biochemistry 18*, 3032–3038.

Oshima, G., & Nagasawa, K. (1976) *J. Biochem. (Tokyo) 31*, 57–63.

Patchett, A. A., Harris, E., Tristram, E. W., Wyvratt, M. J., Wu, M. T., Taub, D., Peterson, E. R., Ikeler, T. J., ten Broeke, J., Payne, L. G., Ondeyka, D. L., Thorsett, E. D., Greenlee, W. J., Lohr, N. S., Hoffsommer, R. D., Joshua, H., Ruyle, W. V., Rothrock, J. W., Aster, S. D., Maycock, A. L., Robinson, F. M., Hirschmann, R., Sweet, C. S., Ulm, E. H., Gross, D. M., Vassil, T. C., & Stone, C. A. (1980) *Nature (London) 288*, 280–283.

Quiocho, R. A., & Lipscomb, W. N. (1971) *Adv. Protein Chem. 25*, 1–78.

Sweet, C. S., Gross, D. M., Arbegast, P. T., Gaul, S. L., Britt, P. M., Ludden, C. T., Weitz, D., & Stone, C. A. (1981) *J. Pharmacol. Exp. Ther. 216*, 558–566.

Thorsett, E. D., Harris, E. E., Peterson, E. R., Greenlee, W. J., Patchett, A. A., Ulm, E. H., & Vassil, T. C. (1982) *Proc. Natl Acad. Sci. U.S.A. 79*, 2176–2180.

Weaver, L. H., Kester, W. R., & Matthews, B. W. (1977) *J. Mol. Biol. 114*, 119–132.

Webb, J. L. (1961) *Enzyme and Metabolic Inhibitors*, Vol. I, pp 66–79, Academic Press, New York.

Equilibrium Binding of ^{125}I-Labeled Adenosinetriphosphatase Inhibitor Protein to Complex V of the Mitochondrial Oxidative Phosphorylation System†

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ABSTRACT: The ATPase inhibitor protein (IF_1) purified from bovine heart mitochondria was labeled with ^{125}I at its single tyrosyl residue under conditions that inhibitor potency was fully preserved. Equilibrium binding studies of [^{125}I]IF_1 to complex V (purified ATP synthetase complex) under conditions favoring inhibition revealed the presence in complex V of saturable high-affinity as well as low-affinity binding sites for [^{125}I]IF_1. The double-reciprocal plot of data concerned with [^{125}I]IF_1 binding to the complex V high-affinity site as a function of added [^{125}I]IF_1 indicated a saturation point of 0.94 mol of [^{125}I]IF_1 bound per mol of complex V and a dissociation constant of 0.75 μM. The amount of [^{125}I]IF_1 bound to complex V or soluble F_1-ATPase at maximal activity inhibition was estimated from titration curves to be 0.75–0.8 mol of [^{125}I]IF_1 per mole of complex V or F_1-ATPase. The conditions required for IF_1 to exert inhibition are incubation of IF_1 with an active ATPase at pH <7.0 in the presence of MgATP (or another hydrolyzable substrate). It was found that under otherwise optimal conditions, the alteration of any one of these factors (i.e., absence of MgATP, pH 8.0, or addition of rutamycin to inhibit ATPase activity) drastically diminished the binding of [^{125}I]IF_1 to the high-affinity site of complex V. It was shown by Galante et al. [Galante, Y. M., Wong, S.-Y., & Hatefi, Y. (1981) *Biochemistry 20*, 2671–2678] that the inhibition of complex V by IF_1 can be reversed by incubating the inhibited enzyme at pH > 7.0 in the absence of MgATP. The present studies have shown that reversal of inhibition is associated with the release of IF_1. In phosphate buffer at pH 8.0, the release of the radiolabeled inhibitor from [^{125}I]IF_1-treated complex V was found to precede the reappearance of ATPase activity, while in bicarbonate buffer of the same ionic strength and pH the reappearance of activity coincided with the release of [^{125}I]IF_1. An increase in ionic strength and the presence of other anions such as sulfite and nitrate, uncouplers, or Ca^{2+} did not duplicate the bicarbonate effect.

The bovine heart mitochondrial ATPase inhibitor protein (IF_1)[1] is a water-soluble, heat-stable polypeptide (Pullman & Monroy, 1963; Brooks & Senior, 1971). IF_1 consists of 84 amino acid residues with a molecular weight of 9578, and its sequence has been recently determined (Frangione et al., 1981). It inhibits ATP hydrolysis when incubated with F_1-ATPase, complex V (purified ATP synthetase complex), or submitochondrial particles at pH <7.0 in the presence of Mg^{2+}

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[1] Abbreviations: IF_1, ATPase inhibitor protein from bovine heart mitochondria; [^{125}I]IF_1, ^{125}I-labeled IF_1; F_1, soluble F_1-ATPase; F_0, membrane sector of the ATPase complex or complex V; NTP, nucleoside triphosphate; Cl_3CCOOH, trichloroacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; OSCP, oligomycin sensitivity conferring protein; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tes, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

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