Noggle, J. H. (1985) *F-Curve*, LEDS Publishing Co., Research Triangle Park, NC.
Pace, N. (1975) *CRC Crit. Rev. Biochem. 2*, 1-43.
Pinkofsky, H. B., Ginsburg, A., Reardon, I., & Heinrikson, R. L. (1984) *J. Biol. Chem. 259*, 9616-9622.
Privalov, P. L. (1979) *Adv. Protein Chem. 33*, 167-241.
Privalov, P. L. (1982) *Adv. Protein Chem. 35*, 1-104.
Ragone, R., Colonna, G., Balestrieri, C., Servillo, L., & Irace, G. (1984) *Biochemistry 23*, 1871-1875.
Ragone, R., Colonna, G., Bismuto, E., & Irace, G. (1987) *Biochemistry 26*, 2130-2134.
Sãnchez-Ruiz, J. M., Lõpez-Lacomba, J. L., Cortijo, M., & Mateo, P. L. (1988) *Biochemistry 27*, 1648-1652.
Schellman, J. A. (1975) *Biopolymers 14*, 999-1018.
Schellman, J. A. (1976) *Biopolymers 15*, 999-1000.
Schellman, J. A. (1987a) *Annu. Rev. Biophys. Biophys. Chem. 16*, 115-137.
Schellman, J. A. (1987b) *Biopolymers 26*, 549-559.
Shapiro, B. M., & Ginsburg, A. (1968) *Biochemistry 7*, 2153-2167.
Shortle, D. (1987) in *Protein Structure, Folding, and Design*, pp 353-361, Alan R. Liss, New York.
Shrake, A., & Rupley, J. K. (1980) *Biochemistry 19*, 4044-4051.
Shrake, A., & Ross, P. D. (1988) *J. Biol. Chem. 263*, 15392-15399.
Shrake, A., Powers, D. M., & Ginsburg, A. (1977) *Biochemistry 16*, 4372-4381.
Shrake, A., Whitley, E. J., Jr., & Ginsburg, A. (1980) *J. Biol. Chem. 255*, 581-589.
Shrake, A., Ginsburg, A., Wedler, F. C., & Sugiyama, Y. (1982) *J. Biol. Chem. 257*, 8238-8243.
Shrake, A., McFarland, P. J., Fisher, M. T., & Ginsburg, A. (1988) *Biophys. J. 53*, 292a.
Stadtman, E. R., & Ginsburg, A. (1974) *Enzymes (3rd Ed.) 10*, 755-807.
Stadtman, E. R. Smyrniotis, P. Z., Davis, J. N., & Wittenberger, M. E. (1979) *Anal. Biochem. 95*, 275-285.
Sturtevant, J. M. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2236-2240.
Woolfolk, C. A., Shapiro, B. M., & Stadtman, E. R. (1966) *Arch. Biochem. Biophys. 116*, 177-192.

# Phosphonate Analogues of Carboxypeptidase A Substrates Are Potent Transition-State Analogue Inhibitors†

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ABSTRACT: Analogues of tri- and tetrapeptide substrates of carboxypeptidase A in which the scissile peptide linkage is replaced with a phosphonate moiety ($-PO_2^-$–O–) were synthesized and evaluated as inhibitors of the enzyme. The inhibitors terminated with either L-lactate or L-phenyllactate [designated (O)Ala and (O)Phe, respectively] in the $P_1'$ position. Transition-state analogy was shown for a series of 14 tri- and tetrapeptide derivatives containing the structure RCO-Ala$^P$-(O)Ala [RCO-A$^P$(O)A, A$^P$ indicates the phosphonic acid analogue of alanine] by the correlation of the $K_i$ values for the inhibitors and the $K_m/k_{cat}$ values for the corresponding amide substrates. This correlation supports a transition state for the enzymatic reaction that resembles the tetrahedral intermediate formed upon addition of water to the scissile carbonyl group. The inhibitors containing (O)Phe at the $P_1'$ position proved to be the most potent reversible inhibitors of carboxypeptidase A reported to date: the dissociation constants of ZAF$^P$(O)F, ZAA$^P$(O)F, and ZFA$^P$(O)F are 4, 3, and 1 pM, respectively. Because of the high affinity of these inhibitors, their dissociation constants could not be determined by steady-state methods. Instead, the course of the association and dissociation processes was monitored for each inhibitor as its equilibrium with the enzyme was established in both the forward and reverse directions. A phosphonamidate analogue, ZAA$^P$F, in which the peptide linkage is replaced with a $-PO_2^-$–NH– moiety, was prepared and shown to hydrolyze rapidly at neutral pH ($t_{1/2}$ = 20 min at pH 7.5). This inhibitor is bound an order of magnitude *less* tightly than the corresponding phosphonate, ZAA$^P$(O)F, a result that contrasts with the 840-fold higher affinity of phosphonamidates for thermolysin [Bartlett, P. A., & Marlowe, C. K. (1987) *Science 235*, 569-571], a zinc peptidase with a similar arrangement of active-site catalytic residues.

Substrate analogues in which the scissile peptide linkage is replaced with a tetrahedral phosphorus ester or amide moiety are potent inhibitors of zinc peptidases (Komiyama et al., 1975; Weaver et al., 1977; Kam et al., 1979; Nishino & Powers, 1979; Hoffmann & Rottenberg, 1980; Jacobsen & Bartlett, 1981a,b; Thorsett et al., 1982; Bartlett & Marlowe, 1983, 1987a,b; Galardy et al., 1983; Grobelny et al., 1985a; Yamauchi et al., 1985; Mookthiar et al., 1987; Karanewsky et al., 1988). The basis for this inhibition is attributed to the similarity of the tetrahedral phosphorus species to the presumed tetrahedral intermediate arising from addition of a zinc-bound water molecule to the substrate carbonyl group (Tronrud et al., 1970; Nishino & Powers, 1979). There is both thermodynamic as well as structural evidence in support of this concept. For thermolysin (TLN), the $K_i$ values for a series of phosphonamidate tripeptide inhibitors show a strong cor-

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relation with the $K_m/k_{cat}$ values of the corresponding substrates (Bartlett & Marlowe, 1983), as expected if the inhibitors are transition-state analogues (Thompson, 1973; Wolfenden, 1976). Moreover, crystallographic studies of a number of complexes of phosphorus-containing inhibitors with thermolysin (Tronrud et al., 1970, 1987; Holden et al., 1987; Matthews, 1988) and carboxypeptidase A (CPA) (Christianson & Lipscomb, 1988) reveal a relationship between the tetrahedral phosphorus moieties and the functional groups in the active site which is consistent with this view.

Although CPA is one of the most studied of the zinc peptidases, there is still debate as to the details of its mechanism. In one view, cleavage of the peptide linkage involves a single nucleophilic event, with addition of a zinc-bound water molecule to the carbonyl group and, following proton-transfer steps, subsequent collapse of the tetrahedral intermediate (Lipscomb, 1970; Breslow & Wernick, 1977; Auld et al., 1984; Christianson & Lipscomb, 1989). In an alternative view, two nucleophilic steps are involved, namely, formation and hydrolysis of an acyl-enzyme anhydride (Lipscomb, 1970; Makinen et al., 1982; Sander & Witzel, 1985; Suh et al., 1985, 1986). The former sequence is often referred to as the "general base" mechanism, the latter as the "anhydride" mechanism, reflecting the disparate roles that the carboxylate of Glu-270 is envisaged to play. There is significant structural similarity between the active sites of thermolysin and carboxypeptidase A (Kester & Matthews, 1975), especially with respect to the orientation of the catalytic zinc ion and glutamate carboxylate. In contrast to CPA, however, no evidence for covalently bound intermediates has been reported for thermolysin, and the general base mechanism has been persuasively argued (Hangauer et al., 1984; Matthews, 1988).

One of the fundamental differences between these two mechanistic sequences is that the tetrahedral intermediates in the anhydride mechanism are covalently linked to the enzyme, through Glu-270, whereas the corresponding species in the general base mechanism is bound only through hydrogen bonding and coordination to the zinc atom (Figure 1). Only the latter tetrahedral intermediate can be effectively mimicked by anionic phosphorus-containing substrate analogues, since such inhibitors are unable to bond covalently with an active-site nucleophile. For example, anionic phosphonates are not effective inhibitors of the serine peptidases (Lamden & Bartlett, 1983; Bartlett & Lamden, 1986), for which there is unequivocal evidence for a covalent acylation/deacylation mechanism.

A limited number of phosphorus-containing inhibitors have been reported for CPA, with inhibition constants between 10 nM and 10 $\mu$M (Kam et al., 1979; Hoffman & Rottenberg, 1980; Jacobsen & Bartlett, 1981a,b, 1982; Grobelny et al., 1985a; Yamauchi et al., 1985). However, there have been no phosphorus-containing inhibitors reported that are larger than dipeptides, and there has been no investigation in which the $K_i$ values of a series of such inhibitors can be compared with the binding properties of related substrates to probe the "transition-state analogy" of such compounds. It was therefore of interest to determine whether tri- and tetrapeptide phosphonate analogues are bound strongly to CPA, and, most importantly, whether they are transition-state analogues.

Thompson (1973) and Wolfenden (1976), and later Bartlett and Marlowe (1983), have addressed explicitly the question of how transition-state analogy of enzyme inhibitors can be demonstrated. Although such inhibitors are expected to be tightly bound, high affinity is neither a necessary nor a sufficient criterion for the transition-state analogue designation.

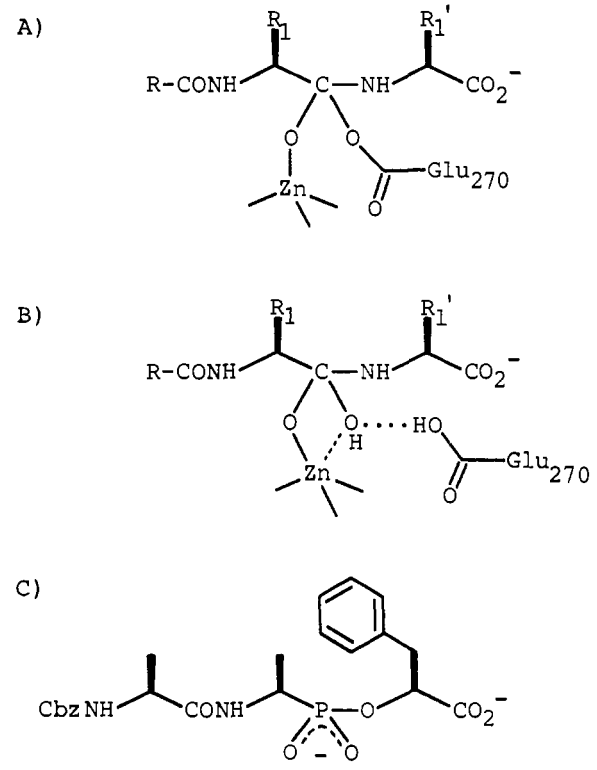


FIGURE 1: Comparison between tetrahedral structures of (A) the intermediates in the anhydride mechanism and (B) the general base mechanism of carboxypeptidase A and (C) a phosphonate tripeptide analogue [Cbz-Ala-Ala$^P$-(O)Phe = ZAA$^P$(O)F].

Enzymes accelerate the reactions they catalyze through a combination of proximity and selective binding effects: proximity through the appropriate positioning of catalytic residues and substrates and selective binding through more favorable interaction with the substrate in its transition-state than in its ground-state configuration (Jencks, 1975; Fersht, 1985; Kraut, 1988). A difference between ground-state and transition-state interactions is revealed as a difference in substituent effects on $K_S$ and on $K_S/k_{cat}$ for a series of related substrates. A criterion for transition-state analogy, therefore, is whether the substituent effects observed for the $K_i$ values of a series of inhibitors are the same as those found for the $K_S/k_{cat}$ values of the related substrates. As a corollary, if the inhibitors are ground-state or multisubstrate analogues, the substituent effects found for $K_i$ are expected to be similar to those for $K_S$.

A number of important assumptions are made in the derivation of these relationships (Bartlett & Marlowe, 1983, 1987b; Rich & Northrup, 1988). It is assumed that the rate constant for the non-enzyme-catalyzed transformation of substrate to product is invariant for the series and that the same chemical step comprises the transition state for each of the substrates used in the correlation. It is also important that association or dissociation steps not play a rate-limiting role for the substrates chosen, because it is the kinetically more accessible Michaelis constants, $K_m$, as opposed to the true substrate dissociation constants, $K_S$, that are available for such a correlation. Examples of the successful application of these criteria, as well as of situations in which the assumptions break down, have been observed in evaluation of phosphonamidate and phosphonate inhibitors of the zinc peptidase thermolysin (Bartlett & Marlowe, 1983, 1987a,b) and *gem*-diol inhibitors of collagenase (van Wart et al., 1988).

In the above-mentioned investigations of phosphorus-containing peptides as inhibitors of thermolysin, we concluded

from the observed correlation between inhibitor $K_i$ values and substrate $K_m/k_{cat}$ values that the inhibitors are indeed transition-state analogues and that phosphonamidates and phosphonates can be regarded as good mimics of the tetrahedral intermediate arising from addition of water to the peptide carbonyl group.[1] It is important to note that the noncovalent complexes between the peptidase and the phosphorus analogues cannot mimic effectively the covalently linked tetrahedral intermediates of the anhydride mechanism. In extending our study of phosphonate inhibitors to CPA, we sought to use the $K_i$ vs $K_m/k_{cat}$ correlation in the opposite logical sense: on the basis of the evidence that the phosphonates are analogues of the tetrahedral intermediate in the general base mechanism, the observation of a transition-state analogy for these inhibitors would be further indication of a similarity in mechanism with thermolysin and additional support for the general base catalyzed process. We now report such a correlation for CPA, using phosphonate tri- and tetrapeptide analogues, as well as the synthesis and evaluation of several exceedingly potent inhibitors of this enzyme.

## Experimental Procedures

*Synthesis of Inhibitors.* The tri- and tetrapeptide inhibitors were synthesized by using methodology previously reported for related compounds (Bartlett & Jacobsen, 1983a; Bartlett & Marlowe, 1983, 1987b). The final products were converted to the dilithium salts and fully characterized, the absence of phosphorus-containing impurities was demonstrated by $^{31}P$ NMR spectroscopy, and the absolute purity of the inhibitors was determined by elemental analysis. The syntheses of $ZAA^P(O)F$ and $ZAA^PF$ are presented below as representative procedures; spectral characterization of the intermediates and full experimental details and characterization of the other inhibitors may be found in the supplementary material.

Dimethyl [(1*R*)-*N*-(Phenylmethoxycarbonyl)-1-aminoethyl]phosphonate ($ZA^P$ Dimethyl Ester). To a stirred slurry of 1.02 g (8.13 mmol) of (*R*)-1-aminoethylphosphonic acid [obtained from Fluka or synthesized and resolved according to the method of Kafarski et al. (1983)] were added 1.37 g (16.3 mmol) of $NaHCO_3$ and 1.73 g (16.4 mmol) of $Na_2CO_3$ in 8.2 mL of 2 N NaOH. After the mixture was cooled in an ice–water bath, 1.2 mL (8.8 mmol) of benzyl chloroformate (CbzCl) was added slowly by syringe. Two additional 1.2-mL portions of CbzCl were added at 1-h intervals. After the mixture was stirred at 21 °C overnight, 2 mL of 2 N NaOH and 20 mL of $H_2O$ were added. This solution was washed twice with ether and then acidified with concentrated aqueous HCl. The milky solution was extracted three times with EtOAc, and the organic layers were combined, washed with brine, dried (molecular sieves), and evaporated under reduced pressure to give 1.8 g of a white foam: $^1H$ NMR; $^{31}P$ NMR.

To 1.65 g of this foam was added 50 mL of trimethyl orthoformate, and the resulting solution was heated at 120 °C for 48 h. The volatile reactants and products were removed by rotary evaporation, and the resulting residue was purified by silica gel chromatography (5% $EtOH/CH_2Cl_2$) to give 853 mg (51% yield) of the dimethyl phosphonate as a colorless oil: $[\alpha]^{24}_D$ −17.5° (*c* 1, $CHCl_3$); IR; $^1H$ NMR; $^{31}P$ NMR.

Methyl Hydrogen [(1*R*)-*N*-(Phenylmethoxycarbonyl)-1-aminoethyl]phosphonate ($ZA^P$ Monomethyl Ester). A solution of 743 mg (2.59 mmol) of the dimethyl ester above in 4 mL of methanol and 3 mL of 2 N NaOH was stirred at 21 °C overnight. After dilution with 20 mL of water, the solution was washed twice with $CHCl_3$, acidified with 2 mL of concentrated HCl, and extracted four times with $CHCl_3$. The combined $CHCl_3$ extracts were dried ($MgSO_4$), and the solvent was evaporated to give 637 mg (90% yield) of the monoester as a white solid: mp 119–122 °C; $[\alpha]^{25}_D$ −22.1° (*c* 1, $CHCl_3$); IR; $^1H$ NMR; $^{31}P$ NMR.

*O*-[[(1*R*)-*N*-(Phenylmethoxycarbonyl)-1-aminoethyl]-methoxyphosphinyl]-L-3-phenyllactic Acid Methyl Ester [$ZA^P(O)F$ Dimethyl Ester]. A solution of 700 mg (4.22 mmol) of L-(−)-3-phenyllactic acid in 20 mL of $Et_2O$ was treated at 0 °C with an ethereal solution of diazomethane until the yellow color persisted. The reaction mixture was then stirred at 21 °C open to the atmosphere until the yellow color disappeared, and the ether was removed by rotary evaporation. The methyl ester was redissolved in $CH_2Cl_2$, dried over $MgSO_4$, and filtered, 700 μL (5.0 mmol) of triethylamine was added, and the solution was stored over $CaSO_4$ and 3-Å molecular sieves while the phosphonic acid was activated.

A solution of 1.07 g (3.91 mmol) of the monomethyl ester, mono acid of Cbz-$Ala^P$ in 10 mL of dry $CH_2Cl_2$ was treated with 360 μL (4.93 mmol) of $SOCl_2$ and stirred for 3 h. The solvent was removed by short-path distillation at 70 °C and then under vacuum to yield the chloridate as a yellow oil. This material was dissolved in 10 mL of dry $CH_2Cl_2$ and combined with the filtered solution of methyl 3-phenyllactate and triethylamine prepared previously. After 5 days at 21 °C, the $CH_2Cl_2$ was removed by rotary evaporation and the residue was dissolved in EtOAc. The EtOAc solution was washed with $H_2O$, saturated $NaHCO_3$, 3 N HCl, and brine and dried ($MgSO_4$), and the solvent was evaporated to give the coupled product as a yellow oil. Column chromatography (silica gel, 85:15 EtOAc/hexanes) afforded 944 mg (55% yield) of the coupled diester as a mixture of diastereomers at phosphorus. Characterization (separate diastereomers): $^1H$ NMR; $^{13}C$ NMR; $^{31}P$ NMR; (mixture) IR; MS. Anal. Calcd for $C_{21}H_{26}NO_7P$: C, 57.93; H, 6.02; N, 3.22; P, 7.11. Found: C, 58.01; H, 6.10; N, 3.10; P, 7.09.

*O*-[[(1*R*)-*N*-[*N*-(Phenylmethoxycarbonyl)-L-alanyl]-1-aminoethyl]methoxyphosphinyl]-L-3-phenyllactic Acid Methyl Ester [$ZAA^P(O)F$ Dimethyl Ester]. A suspension of 98 mg (0.225 mmol) of $ZA^P(O)F$ dimethyl ester and 28 mg of 10% Pd/C in 5 mL of EtOAc was stirred under 1 atm of $H_2$ for 1 h. The solution was filtered through a 0.45-μm filter to remove the catalyst, and the solvent was removed by rotary evaporation. The anhydride from 159 mg (0.714 mmol) of *N*-Cbz-L-alanine was prepared in 10 mL of $CH_2Cl_2$ with 74 mg (0.358 mmol) of dicyclohexylcarbodiimide at 0 °C over a 2-h period, and the solution was filtered onto the aminoalkyl phosphonate. After stirring overnight at 12 °C, the solution was evaporated and the residue was dissolved in EtOAc and washed with water, 2 N NaOH, and brine, dried ($MgSO_4$), and evaporated. The crude product was purified by chromatography (silica gel, 3% EtOH/EtOAc) to give 100 mg (88% yield) of the two phosphonate diastereomers as a colorless oil. Characterization (separate diastereomers): $^1H$ NMR; $^{13}C$ NMR; $^{31}P$ NMR; (mixture) IR; MS. Anal. Calcd for $C_{24}H_{31}N_2O_8P$: C, 56.91; H, 6.17; N, 5.53; P, 6.12. Found: C, 56.67; H, 6.28; N, 5.42; P, 6.21.

[1] A distinction has been made between transition-state analogues and high-energy or reaction intermediate analogues (Schloss & Cleland, 1982). The tetrahedral intermediate of Scheme I is a reaction intermediate as opposed to a transition state; however, the structural difference between the tetrahedral intermediate and the transition state is likely to be smaller than the difference between either of them and the phosphonate analogues. Put another way, our ability to design and synthesize transition-state analogues is of inherently lower accuracy than the difference between transition states and high-energy reaction intermediates; hence, the distinction is more a semantic than a practical one.

*O*-[[(1*R*)-*N*-[*N*-(Phenylmethoxycarbonyl)-L-alanyl]-1-aminoethyl]hydroxyphosphinyl]-L-3-phenyllactic Acid Dilithium Salt [ZAA$^P$(O)F]. The tripeptide phosphonate dimethyl ester (111 mg, 0.22 mmol) was treated with 0.46 mmol of lithium *n*-propyl mercaptide in 1 mL of hexamethylphosphoramide (HMPA) (Bartlett & Johnson, 1970) and stirred at 21 °C for 2.5 h. The reaction mixture was diluted with water and washed four or five times with $CHCl_3$ to remove the HMPA, and the aqueous phase was lyophilized to yield a white powder. The crude product was purified by reverse-phase HPLC [C18 support, 47% MeOH:53% 0.1 N triethylammonium bicarbonate (TBK) buffer, pH 7.4, as eluant], and the triethylammonium salt obtained after lyophilization was converted to the lithium salt by passage through a small column of Dowex 50W-X8 cation-exchange resin in the lithium form. Lyophilization yielded 40 mg of a white powder that was 87% inhibitor by weight, as determined by phosphorus analysis (37% yield): UV ($H_2O$) $\lambda_{max}$ = 257 nm ($\epsilon$ = 540 $M^{-1}$ $cm^{-1}$); $[\alpha]^{24}_D$ −40° (*c* 0.62, $H_2O$); IR (KBr) 3400 (br), 3070, 3040, 2990, 2950, 1710, 1615, 1530, 1455, 1420, 1335, 1210, 1065, 960, 910, 790, 750, 704 $cm^{-1}$; $^1H$ NMR ($D_2O$, $CH_3CN$ = 2.0 ppm) $\delta$ 0.85–1.00 [br m, 0.9 (minor conformer)], 1.14 [dd, 2.1 (major conformer), $J$ = 7.1, 15.1], 1.27 (d, 3, $J$ = 7.0), 3.02 (m, 2), 3.70–3.85 [br m, 0.3 (minor conformer)], 3.89 [dq, 0.7 (major conformer; $J$ = 15.2, 7.3], 4.03 (br q, 1, $J$ = 7.1), 4.65–4.73 (m, 1), 4.95–5.10 (m, 2), 7.2–7.4 (m, 10); $^{13}C$ NMR ($D_2O$, $CH_3CN$ = 1.3 ppm) $\delta$ 16.21, 17.54, 40.46, 43.83 (d, $J$ = 152.5), 51.35, 67.44, 76.65, 127.11, 128.12, 128.83, 129.21, 130.27, 136.77, 137.86, 157.99, 175.04, 178.84; $^{31}P$ NMR (81.7 MHz, $D_2O$) $\delta$ 20.48. HRMS (FAB, $MH^+$) Calcd. for $C_{22}H_{26}N_2O_8PLi_2$: 491.1747. Found: 491.1755.

*N*-[[(1*R*)-*N*-[*N*-(Phenylmethoxycarbonyl)-L-alanyl]-1-aminoethyl]hydroxyphosphinyl]-L-phenylalanine (ZAA$^P$F). The phosphonamide dimethyl ester (synthesis described in the supplementary material) (66 mg, 0.13 mmol) was treated with 910 $\mu$L of a 0.43 M solution of lithium *n*-propyl mercaptide (0.4 mmol) in HMPA. After stirring at 21 °C for 2 h, the mixture was diluted with 10 mL of $H_2O$ and washed five times with $CHCl_3$ to remove HMPA. The aqueous solution was immediately frozen and lyophilized to give 66 mg of a white powder. $^{31}P$ NMR ($D_2O$) $\delta$ 19.33 + 19.45 (total 10%), 21.09 + 21.19 (total 21%), 21.49 (62%), 30.67 (residual HMPA, 7%). Due to the hydrolytic instability of this compound, solutions of the inhibitor were purified as needed by reverse-phase HPLC (Vydac pH-stable C8 reverse-phase column, 9 mm × 25 cm, eluted with 15% $CH_3CN$:85% 0.15 M TBK, pH 9.8). The concentration of the phosphonamidate ZAA$^P$F in the purified solutions was determined from the UV absorbance at $\lambda$ = 257 nm, assuming an extinction coefficient of 540 $M^{-1}$ $cm^{-1}$, as observed for the corresponding phosphonate ZAA$^P$(O)F. The solutions were stored in an ice bath and used within a few hours of preparation; typical concentrations produced with this procedure were 0.1–0.2 mM: MS (FAB$^+$) *m/z* 579 (M + $Et_3NH^+$), 102 (100%, $Et_3NH^+$).

The products from hydrolytic decomposition of ZAA$^P$F were isolated and shown to be phenylalanine and ZAA$^P$ by spectral and chromatographic comparison with authentic samples.

## Enzyme Assays

*General.* All solutions were prepared by using doubly distilled or distilled, deionized water. Stock assay solutions were filtered (0.45-$\mu$m pore size) prior to use. Assays were performed on a Cary 219 UV–vis spectrophotometer equipped with an OLIS Model 3820 data aquisition computer. Temperature regulation (25.0 ± 0.2 °C) was provided by a Lauda Model RM 20 circulating constant-temperature bath connected to a water-jacketed sample cell holder. The concentrations of phosphonate inhibitor stock solutions were determined by careful dilution of a precisely weighed sample of the inhibitor and corrected for the percent phosphorus determined by elemental analysis.

*Carboxypeptidase A Stock Solutions.* Carboxypeptidase A (CPA, EC 3.4.17.1) was obtained from Sigma Chemical Co. (catalog no. C 0386, Allan form, twice crystallized, from bovine pancreas, aqueous suspension). To 500 $\mu$L of distilled water at 1–5 °C was added 500 $\mu$L of the CPA suspension. This solution was centrifuged for 10 min at 10 000 rpm, and the solid was resuspended in 1000 $\mu$L of double-distilled water. After recentrifugation and decantation of the supernatant, the solid residue was dissolved in 500 $\mu$L of 10% aqueous LiCl (1–5 °C). After standing at 5 °C overnight, the solution was centrifuged as above and the supernatant was collected and used as the CPA stock solution. This solution was stable for at least 2 weeks when stored at 5 °C. The concentration of CPA was determined from the absorbance at 278 nm ($\epsilon_{278}$ = 6.42 × $10^4$ $M^{-1}$ $cm^{-1}$) (Simpson et al., 1963) and was typically 0.4–0.5 mM.

Although concentrated CPA stock solutions in 10% LiCl are stable for up to 2 weeks, upon dilution to concentrations below 1 $\mu$M the enzyme loses activity rapidly ($t_{1/2} \approx$ 60 min), even at 0 °C. Addition of 0.1 mg/mL of bovine serum albumin (BSA) resulted in substantial stabilization of the CPA solutions (>80% activity after 6 h) without affecting the kinetic properties of the enzyme. Working stock solutions were therefore prepared by dilution of the initial stock solution into 10% LiCl containing 0.1 mg/mL BSA or a solution of the assay buffer containing 0.1 mg/mL BSA.

For long-term stabilization under dilute conditions, $ZnCl_2$ was also included in the buffer. We found that 50 pM samples of CPA retain greater than 75% of their original activity when incubated at 25 °C for 12 days in assay buffer containing 0.1 mg/mL bovine serum albumin (BSA) and 1 $\mu$M $ZnCl_2$.

*CPA Assays.* Unless otherwise noted, all assays of (O)-Phe-containing inhibitors were performed under the following conditions: 25 °C, pH 7.5, 50 mM Tris-HCl, 0.5 M NaCl. The buffer in the assays of (O)Ala-containing inhibitors was identical except for a Tris concentration of 25 mM. CPA activity was assayed with *N*-[3-(2-furyl)acryloyl]-L-phenylalanyl-L-phenylalanine [FuAFF purchased from Calbiochem-Behring Corp. (catalog no. 345115) or Sigma Chemical Co. (catalog no. F-7133)] as substrate (Riordan & Holmquist, 1984). The substrate solution was typically prepared by addition of 100 mg (222 $\mu$mol) of FuAFF to 900 mL of aqueous solution containing 50 mmol of Tris (basic form) and 0.5 mol of NaCl; the solution was stirred at 50 °C until all the substrate dissolved (1–2 h) and, after cooling to 25 °C, was brought to pH 7.5 with concentrated HCl, followed by dilution to 1000 mL. Solutions prepared as described above had $\lambda_{max}$ = 305.5 nm and $\epsilon_{305.5}$ = 25 000 ± 900 $M^{-1}$ $cm^{-1}$ (average and standard deviation from six preparations).

CPA concentrations of 0.1–0.3 nM were typically used for routine assays. Assays were followed by the absorbance change at 325 or 330 nm [$\Delta\epsilon_{330}$ = 2100 ± 100 $M^{-1}$ $cm^{-1}$ (average and standard deviation from three preparations)] and were linear for 20% of the reaction. The rates of FuAFF hydrolysis at substrate concentrations from 10 to 100 $\mu$M were obtained and fit to a hyperbolic curve by using Clelan's HYPER program to obtain $K_m$ [40.4 ± 2.1 $\mu$M (4 determinations)] and $k_{cat}$ [329 ± 30 $s^{-1}$ (4 determinations)] (Cleland, 1979). For enzyme solutions that were more dilute than 0.1 nM, long assay times

Table I: Kinetic Constants for Substrates and $K_i$ Values for Inhibitors Terminating in Ala-Ala[a]

| | substrate[b] | | | inhibitor[c] |
|---|---|---|---|---|
| | $K_m$ (mM) | $k_{cat}$ ($s^{-1}$) | $K_m/k_{cat}$ ($\mu$M s) | $K_i$ ($\mu$M) |
| Ac-D-AAA | 97 | 12 | 8100 | 90 |
| AcA-D-AAA | 200 | 20 | 10000 | 53 |
| ZA-D-AAA | 47 | 9.7 | 4790 | 43 |
| AcAAA | 72 | 69 | 1000 | 35 |
| AcAAAA | 118 | 186 | 634 | 8.5 |
| ZAAAA | 18 | 37 | 492 | 6.4 |
| PpAAAA | 50 | 38 | 1320 | 5.6 |
| PpAAA | 11 | 37 | 300 | 3.4 |
| ZGAA | 21 | 143 | 145 | 3.0 |
| ZFAAA | 5.6 | 35 | 160 | 2.0 |
| ZLAAA | 11 | 70 | 159 | 1.2 |
| Z-D-AAA | 1.0 | 12 | 87 | 0.93 |
| ZAAA | 0.45 | 86 | 5.2 | 0.076 |
| ZFAA | 0.23 | 50 | 4.6 | 0.056 |

[a] Abbreviations: Ac, acetyl; Z, carbobenzoxy; Pp, 3-phenylpropanoyl. [b] Substrate data taken from Abramowitz et al. (1967) and Abramowitz-Kurn and Schechter (1974). [c] The inhibitors contain a $-PO_2^-$–O– moiety in place of the C-terminal Ala-Ala peptide linkage.

(60 min) and a sensitive spectrophotometer were required; however, we were able to measure CPA at concentrations as low as 1 pM.

### *Inhibition by Tri- and Tetrapeptide Analogues Terminating in (O)Ala*

The inhibition constants for the (O)Ala analogues were determined from simple plots of $v_0/v_i$ versus [I] at a substrate concentration equal to $K_m$ and inhibitor concentrations ranging from 0 to 20 × $K_i$. The results are presented in Table I, along with the kinetic characteristics for the corresponding substrates. A full analysis was carried out for the most tightly bound derivative, ZFA$^P$(O)A, and it was shown to be a simple competitive, reversible inhibitor; the remaining inhibitors were assumed by analogy to be competitive as well.

### *Inhibition by Tripeptide Analogues Terminating in (Y)Phe (Y = O and NH)*

*Computer Simulation of Binding Data.* For kinetic systems not exhibiting simple exponential behavior, a program was developed to simulate the kinetic behavior by numerical integration of the appropriate partial differential equations describing the kinetic scheme. The program allowed modification of initial conditions and rate constants, and the resulting simulations were displayed in graphical form superimposed on the experimental data. Rate constants were adjusted interactively to give the best fit to the data. Integration was performed by using Euler's method; 500 steps were typically sufficient to give accurate integration. The program was written in BASIC and implemented on a North Star HORIZON computer equipped with a Micro-Angelo graphics board (Scion Corp.). While this system was satisfactory for our needs, Barshop, Wrenn, and Frieden (1983) have developed a much more sophisticated program for simulation of kinetic processes.

*Determination of the On-Rate ($k_{on}$) for ZAA$^P$(O)F.* To a solution of FuAFF and ZAA$^P$(O)F in the assay buffer was added the stock solution of CPA in buffer containing 0.1 mg/mL BSA; the final concentration of FuAFF was 109 $\mu$M, and the concentrations of CPA and ZAA$^P$(O)F were varied from 0.20 to 0.40 nM and from 11.5 to 230 nM, respectively. The assays were followed until there was no further change in absorbance (12–180 min), and the resulting curves were fitted to a first-order equation by a nonlinear, least-squares program. A plot of the apparent on-rate ($k_{app}$) versus inhibitor concentration is linear with a slope of 5.82 × $10^4$ $M^{-1}$ $s^{-1}$ (Figure 2), reflecting an on-rate of 2.1 × $10^5$ $M^{-1}$ $s^{-1}$, assuming competitive inhibition (points from two highest substrate concentrations not used).

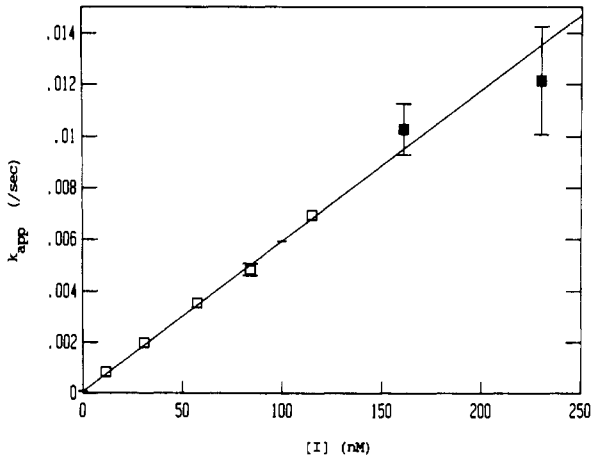


FIGURE 2: Determination of on-rate for binding of ZAA$^P$(O)F: $k_{on}$ = 2.1 × $10^5$ $M^{-1}$ $s^{-1}$. Each point is the average of three determinations; the two points at the highest inhibitor concentrations were not included in the linear fit.

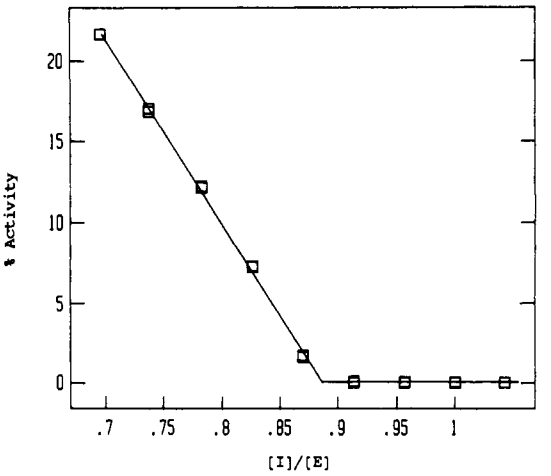


FIGURE 3: Active-site titration of CPA with ZAA$^P$(O)F. The nominal CPA concentration is 0.40 $\mu$M.

The on-rates for the other tight-binding inhibitors were determined in a similar manner; the results are presented in Table II.

*Stoichiometry of CPA and ZAA$^P$(O)F.* To a solution of CPA in buffer containing 0.1 mg/mL BSA were added varying amounts of a solution of ZAA$^P$(O)F such that the final concentration of CPA was 0.40 $\mu$M and the final concentrations of the inhibitor varied from 0.27 to 0.42 $\mu$M. After sitting for at least 20 min, by which time complete binding had occurred, the activity of these solutions was determined and compared to that of solutions that were not treated with inhibitor. A plot of the percent activity of the solutions versus the nominal inhibitor/enzyme ratio was used to determine the stoichiometry of the inhibitor and enzyme solutions; full inhibition was achieved at a nominal ratio [I]/[E] = 0.88 (Figure 3). With the assumption that the true stoichiometry is 1:1, for the experiments involving $K_i$ determination of ZAA$^P$(O)F, the concentrations of inhibitor and enzyme are corrected from their nominal values by factors of 1.07 and 0.94, respectively.

For the other tight-binding inhibitors, the corresponding correction factors for inhibitor concentrations ([I]/[I]$_0$) varied from 0.89 to 0.99; the correction factors for [E]/[E]$_0$ were the inverse.

Table II: Association and Dissociation Rates and Inhibition Constants of Tight-Binding Inhibitors

| inhibitor[a] | concentrations[b] (pM) [E] | [I] | [EI] | % activity at equil[c] | $K_i$[d] (pM) | $k_{off}$[e] ($10^{-7}$ $s^{-1}$) | $k_{on}$[e] ($10^5$ $M^{-1}$ $s^{-1}$) | $k_{on}$[f] ($10^5$ $M^{-1}$ $s^{-1}$) |
|---|---|---|---|---|---|---|---|---|
| $ZAA^P(O)F$ | 45.4 | 49.5 | | 18.1 ± 1.2 | 2.7 ± 0.4 | 7.0 | 2.5 | |
| | | | | | | | | 2.1 |
| | 0.4 | | 45.0 | 23.8 ± 1.4 | 3.3 ± 0.5 | 8.1 | 2.5 | |
| $ZFA^P(O)F$ | 59.0 | 60.0 | | 10.7 ± 2.3 | 0.9 ± 0.4 | 2.2 | 2.2 | |
| | | | | | | | | 2.2 |
| | 1.0 | | 59.0 | 10.7 ± 4.3 | 0.9 ± 0.6 | 2.2 | 2.2 | |
| $ZAF^P(O)F$ | 56.0 | 55.9 | | 23.4 ± 1.4 | 3.8 ± 0.3 | 6.8 | 1.8 | |
| | | | | | | | | 2.0 |
| | 0.1 | | 55.9 | 24.5 ± 1.5 | 4.4 ± 0.8 | 11 | 2.5 | |
| $DsAA^P(O)F$ | 54.7 | 53.6 | | 45.6 ± 1.9 | 20 ± 2 | 30 | 1.5 | |
| | | | | | | | | 1.7 |
| | 1.1 | | 53.6 | 51.0 ± 0.9 | 28 ± 2 | 50 | 1.8 | |
| $ZAA^PF$ | | | | | 32[g] | | | 1.6 |
| $ZAG^P(O)F$ | | | | | 710[h] | | | 6.6 |
| ZA-D-$A^P(O)F$ | | | | | 93 000[h] | | | |

[a] Single-letter codes used for the amino acids; Z, Cbz; Ds, dansyl; (O) represents the phosphonate ester linkage. [b] Concentrations at start of equilibration, with [E] and [I] corrected from nominal values as a result of titration experiments. [c] Average and standard deviation of last five assays in approach to equilibrium. [d] Unless otherwise indicated, $K_i$ values and standard deviation were calculated on the basis of percent activity observed at equilibrium. [e] On- and off-rates derived from computer simulation of equilibration processes. [f] On-rates determined in separate experiments at higher [E] and [I] and in the presence of substrate. [g] $K_i$ value determined as described in text. [h] $K_i$ value determined by steady-state methods.

*Determination of $K_i$ for Inhibition of CPA by $ZAA^P(O)F$.* A solution of the CPA·$ZAA^P(O)F$ complex, prepared and quantitated as described above, was diluted with assay buffer containing 0.1 mg/mL BSA and 1 μM $ZnCl_2$ to give a solution containing 45 pM of the enzyme–inhibitor complex plus 0.4 pM free inhibitor. Another solution was prepared by addition of $ZAA^P(O)F$ to CPA in the same buffer at final concentrations of CPA of 45.4 pM and of $ZFA^P(O)F$ of 49.5 pM. The activity of these solutions was monitored by removing aliquots at various times and mixing with an equal volume of substrate solution (final concentration of FuAFF of 105 μM; typical assay time, 60 min). The percent activity (Figure 4) was calculated relative to control samples at similar CPA concentrations but in the absence of inhibitor; the control samples retained more than 75% activity over the course of the experiment (300 h). All rates were corrected for small residual rates observed upon assay in the absence of CPA (equal to ca. 10% of the enzymatic rate at the lowest activity observed). $K_i$ was determined directly as the equilibrium constant by averaging the residual enzyme activity in the last five assays (Table II). The curves were fit to a simple equilibrium process ($E + I \rightleftarrows EI$) with a simulation program by varying the absolute values of $k_{off}$ and $k_{on}$ while fixing the ratio $k_{off}/k_{on}$ equal to the observed $K_i$. The results of this experiment are given in Table II, along with those from the other tight-binding inhibitors. The experiment with $ZAA^P(O)F$ was also repeated (data not shown) with essentially identical results ($K_i$ = 2.9 ± 0.3 for the on-experiment and $K_i$ = 3.1 ± 0.1 for the off-experiment).

*Inhibition of CPA by $ZAG^P(O)F$ and ZA-D-$A^P(O)F$*

The two phosphonates $ZAG^P(O)F$ and ZA-D-$A^P(O)F$ were evaluated under steady-state conditions in the presence of substrate because of their relatively low affinity, 0.71 and 93 nM, respectively. For $ZAG^P(O)F$, the approach to steady-state inhibition was characterized by subtracting the steady-state rate from the reaction progress curve and fitting the resulting exponential decay to obtain the apparent on-rate ($k_{app}$). A linear dependence on inhibitor concentration was observed for $k_{app}$, leading to a calculated rate constant for association, $k_{on}$, of 6.6 × $10^5$ $M^{-1}$ $s^{-1}$. Time-dependent binding was also observed for ZA-D-$A^P(O)F$; however, at concentrations high enough to obtain significant inhibition the on-rate was too fast to obtain an accurate estimate of $k_{on}$ without stopped-flow methods.

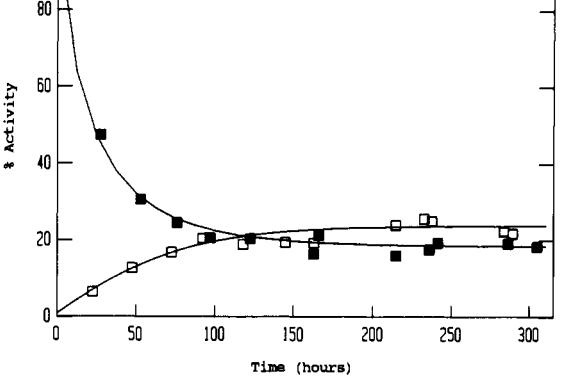


FIGURE 4: Equilibration of CPA with $ZAA^P(O)F$: (□) [EI] = 45.0 pM, [E] = 0.4 pM; (■) [E] = 45.4 pM, [I] = 49.5 pM. The solid lines represent simulations with the following parameters: on-experiment, $k_{on}$ = 2.5 × $10^5$ $M^{-1}$ $s^{-1}$, $k_{off}$ = 7.0 × $10^{-7}$ $s^{-1}$, $K_i$ = 2.8 pM; off-experiment, $k_{on}$ = 2.5 × $10^{-5}$ $M^{-1}$ $s^{-1}$, $k_{off}$ = 8.1 × $10^{-7}$ $s^{-1}$, $K_i$ = 3.3 pM.

*Inhibition of CPA by the Phosphonamidate $ZAA^PF$*

*Hydrolysis of $ZAA^PF$. Determination by UV.* A sample (0.6–1.0 mg) of $ZAA^PF$ was dissolved in 1 mL of the assay buffer, and the absorbance at 230 nm was monitored at 25 °C for 2–3 h. A nonlinear, least-squares analysis of the first-order decay process from three experiments provided a value for $k_{hyd}$ of (5.92 ± 0.17) × $10^{-4}$ $s^{-1}$.

*Determination by Loss of Inhibitory Activity.* To a solution of the assay buffer (3590 μL) was added 10 μL of a freshly purified solution of $ZAA^PF$ (0.104 mM on the basis of absorbance at 257 nm, $\epsilon_{257}$ = 540 $M^{-1}$ $cm^{-1}$) to give a 0.289 μM solution of the phosphonamidate. At various times, 90 μL of this solution was mixed with 10 μL of a 2.15 μM solution of CPA in the assay buffer (also containing 0.1 mg/mL BSA), and after an additional 2 min (at which time all inhibitor was bound), an appropriately diluted sample of the CPA·$ZAA^PF$ solution was assayed for CPA activity. The decrease in inhibition relative to a control as a function of time was analyzed similarly to provide a value for $k_{hyd}$ of (5.2 ± 0.5) × $10^{-4}$ $s^{-1}$ (three experiments).

*Determination of the On-Rate ($k_{on}$) for $ZAA^PF$ Inhibition of CPA.* To a solution of FuAFF in the assay buffer were added CPA (in buffer containing 0.1 mg/mL BSA) and im-

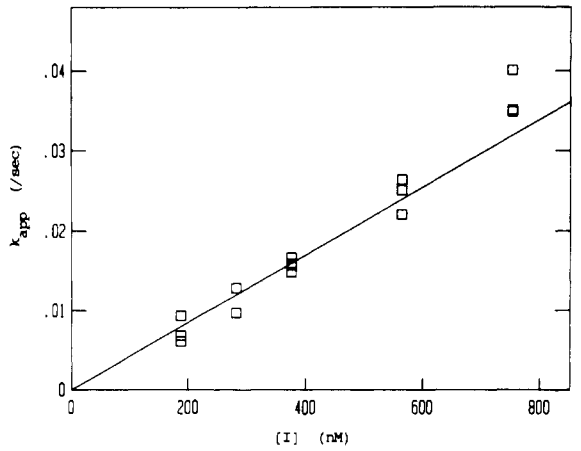


FIGURE 5: Determination of on-rate for binding of ZAA$^P$F: $k_{on}$ = 1.6 × 10$^5$ M$^{-1}$ s$^{-1}$. The data at the highest inhibitor concentration are not included in this calculation, and the line is forced through the origin.

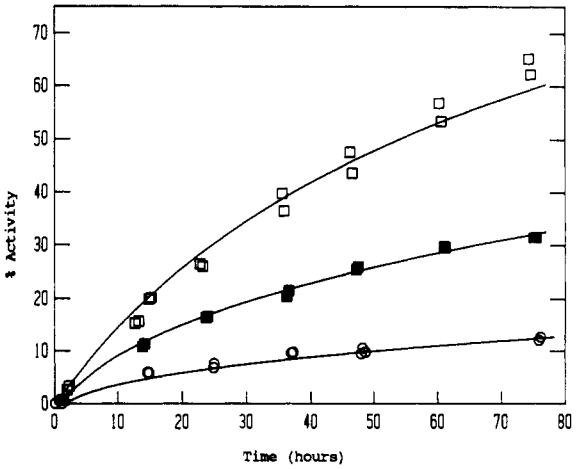


FIGURE 6: Dissociation of complex between CPA and ZAA$^P$F: (□) [EI] = 5.37 nM, [I] = 5.63 nM; (■) [EI] = 53.7 nM, [I] = 56.3 nM; (○) [EI] = 537 nM, [I] = 563 nM. The curves represent the simulation of the mechanism of eq 1 using the following parameters: $k_{hyd}$ = 5.91 × 10$^{-4}$ s$^{-1}$, $k_{on}$ = 1.55 × 10$^5$ M$^{-1}$ s$^{-1}$, $k_{off}$ = 5.0 × 10$^{-6}$ s$^{-1}$.

mediately thereafter freshly purified ZAA$^P$F as a solution in 15% $CH_3CN$:0.15 M TBK, pH 9.8 (as eluted from HPLC). The concentration of FuAFF in the assay mixture was 110 μM, and the concentrations of CPA and ZAA$^P$F were varied from 0.54 to 3.2 nM and from 188 to 752 nM, respectively. The stock solutions of CPA and ZAA$^P$F were such that it was not necessary to add more than 20 μL of either to obtain the desired concentrations in the assay solution (final volume, 1000 μL). In a control experiment it was observed that addition of 10 μL of the 15% $CH_3CN$:0.15 M TBK solution alone did not affect the rate of CPA-catalyzed hydrolysis of FuAFF.

The assays were followed until there was no further change in absorbance (maximum assay time 16 min); the total absorbance change always corresponded to less than 20% hydrolysis of the substrate. Since the apparent on-rate ($k_{app}$) was at least 10 times greater than the rate of inhibitor hydrolysis ($k_{hyd}$), the inhibition curves were nearly first order and an estimate of $k_{app}$ could be obtained by using a nonlinear, least-squares exponential fit program. This initial estimate of $k_{app}$ was then corrected for the presence of inhibitor hydrolysis in the following manner: By use of the simulation program and the initial estimate of $k_{app}$ and the measured value of $k_{hyd}$, an inhibition curve was generated on the basis of the kinetic mechanism of eq 1 ($k_{off}$ = 0). The exponential fit

$$EI \underset{k_{on}}{\overset{k_{off}}{\rightleftharpoons}} E + I \xrightarrow{k_{hyd}} I^* \qquad (1)$$

program was then used to obtain the rate constant ($k$) for the best first-order fit to the simulated curve. The ratio $k_{app}/k$ provided a correction factor that was applied to the initial estimate of $k_{app}$. This approach was justified since errors in the estimates of $k_{app}$ were less than 10% in each case.

A plot of the corrected apparent on-rates ($k_{app}$) versus inhibitor concentrations (Figure 5) was linear with a slope of 4.4 × 10$^4$ M$^{-1}$ s$^{-1}$, determined by constraining the line to pass through the origin. If the line is not so constrained, the calculated line has a negative $y$ intercept, leading to an overestimate of $k_{on}$; the probable cause of this behavior is an anomalous burst in absorbance that is apparent immediately after mixing ($t_{1/2}$ < 1 min, ΔAU ≃ 0.005). (This "burst" is unrelated to the inhibitor, since it was observed on mixing substrate and enzyme as well.) The on-rate ($k_{on}$) was calculated by multiplying the slope of the plot of $k_{app}$ versus [I] by ($1 + S/K_m$), to give a value of 1.6 × 10$^5$ M$^{-1}$ s$^{-1}$.

*Recovery of Activity from a Complex of CPA and ZAA$^P$F: Determination of $k_{off}$ and $K_i$.* To a solution of the assay buffer containing 0.1 mg/mL BSA (3570 μL) was added 400 μL of a 5.37 μM CPA stock followed by addition of 30 μL of a 0.146 mM solution of the inhibitor ZAA$^P$F in 15% $CH_3CN$:85% 0.15 M TBK, pH 9.8. After standing for 10 min, this solution was diluted with assay buffer containing 0.1 mg/mL BSA to give solutions containing 537, 53.7, and 5.37 nM CPA. The activity of these solutions, relative to control solutions without inhibitor, was monitored over 80 h by removing aliquots and assaying with FuAFF (assay times, 4–8 min). Computer simulations of the percent activity versus time according to eq 1 using the previously determined values of $k_{on}$ (1.55 × 10$^5$ M$^{-1}$ s$^{-1}$) and $k_{hyd}$ (5.9 × 10$^{-4}$ s$^{-1}$) while varying $k_{off}$ gave the best fit to all the data when $k_{off}$ was set to 5.0 × 10$^{-6}$ s$^{-1}$ (Figure 6); the best fit to individual dilutions varied only 5% from this average value. The $K_i$ value was calculated from the ratio $k_{off}/k_{on}$ = 32 pM.

*Inhibition of CPA by ZAA$^P$*

The $K_i$ value of the phosphonic acid component from hydrolysis of ZAA$^P$F (ZAA$^P$) was shown to be ca. 0.3 mM; it is ca. 0.2 mM in the presence of equimolar amounts of either phenylalanine or β-phenyllactate.

## Discussion

*Stability of Inhibitors.* The phosphonate ester analogues are stable in aqueous solution over a wide pH range, as expected for anionic monoesters. In contrast, the phosphonamidate ZAA$^P$F hydrolyzes with a half-life of 20 min at pH 7.5, although it is more stable at higher pH. Phosphonamidates are in general more readily hydrolyzed than the corresponding esters; however, the lability of this particular amidate is even higher than that of related analogues that we have studied previously. This instability can be attributed both to the free carboxylate at the $P_1'$ position as well as the peptide linkage between $P_1$ and $P_2$. A similarly placed carboxylate group in a variety of closely related systems has been shown to catalyze substitution at phosphorus via intramolecular nucleophilic attack (Mulliez, 1981; Jacobsen & Bartlett, 1983ab, and references cited therein). Nevertheless, the carboxylate is not entirely responsible for the hydrolytic sensitivity of the tripeptide phosphonamidate, since the dipeptide derivatives ZG$^P$F and Z-β-A$^P$F are stable for more than 2 days at pH 7.5 (Jacobsen & Bartlett, 1981; Yamauchi et al., 1985). This difference in stability between *N*-carbamoyl and *N*-acyl derivatives can also be attributed to intramolecular nucleophilic

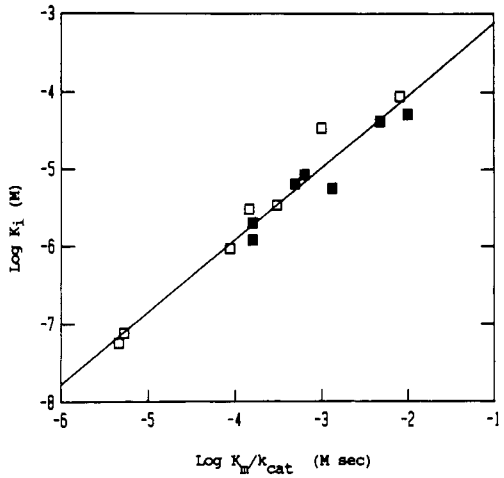


FIGURE 7: Comparison between phosphonate inhibitor $K_i$ and substrate $K_m/k_{cat}$ values: (□) tripeptides; (■) tetrapeptides, slope = 0.93, $r$ = 0.975.

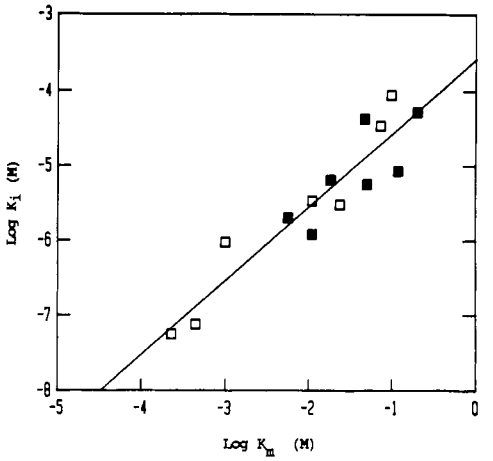


FIGURE 8: Comparison between phosphonate inhibitor $K_i$ and substrate $K_m$ values: (□) tripeptides; (■) tetrapeptides, slope = 0.98, $r$ = 0.928.

attack and has been observed for related phosphinates (Bartlett & Acher, 1986) as well as conventional peptides (Kemp, 1979).

*Transition-State Analogy for Phosphonate Tri- and Tetrapeptide Derivatives.* Schechter et al. (Abramowitz et al., 1967; Abramowitz-Kurn & Schechter, 1974) determined the kinetic constants for an extensive series of oligopeptides in their exploration of the substrate specificity of CPA. For the $K_i$ vs $K_m/k_{cat}$ correlation, two groups of substrates were selected, seven tripeptides and seven tetrapeptides terminating in alanylalanine (Table I). Although aromatic and branched-chain amino acids are preferred at the carboxyl terminus of CPA substrates, such derivatives were not employed in the correlation. The reasons for this exclusion were in part pragmatic, since $K_m$ and $k_{cat}$ data are available for only a limited range of such substrates, and because the corresponding inhibitors bind so tightly that highly accurate $K_i$ values are not readily determined for them. In addition, for substrates that are bound with high affinity and for which turnover is fast, $K_m$ may not reflect the true dissociation constant, and steps other than the carbonyl addition process, the step whose transition state is mimicked by the phosphonate analogues, may be partially or wholly rate-limiting. The structural variation within the 14 substrates arises at the $P_2$ or $P_3$ positions, i.e., sufficiently remote from the site of cleavage that the inherent ease of addition of the scissile carbonyl group is unlikely to be affected. It is reasonable to assume therefore that the rate constant for the noncatalyzed reaction is the same across the series.

For the 14 phosphonate analogues terminating in (O)Ala (Table II), there is a strong correlation between the inhibitor $K_i$ values and the $K_m/k_{cat}$ values of the corresponding substrates (Figure 7), a correlation which is better than that between $K_i$ and $K_m$ alone (Figure 8). Because of the relationship between a free energy difference and the logarithm of an equilibrium constant, Figure 7 represents a direct comparison of substituent effects. The slope for this comparison is close to 1, indicating that the incremental binding energy changes associated with structural variation at the $P_2$ and $P_3$ positions of these inhibitors are nearly the same as those seen in the transition state of the enzymatic reaction. The implication that these inhibitors are transition-state analogues therefore supports a similar transition state for both CPA and TLN, the tetrahedral species arising in the general base mechanism.

For CPA substrates that differ in the $P_2$ and $P_3$ positions, most of the difference in $K_m/k_{cat}$ arises from $K_m$ alone; i.e., there is only modest variation in the $k_{cat}$ values. Thus, the distinction between transition-state analogy (represented by Figure 7) and ground-state analogy (represented by Figure 8) is not as clear-cut with this system as it has been in other cases (Bartlett & Marlowe, 1983; van Wart et al., 1988). Nevertheless, the question to be resolved is not whether the tetrahedral inhibitors mimic the transition state in preference to the ground state form of a CPA substrate, rather, it is whether the transition-state form is a species arising from attack of a water molecule on the carbonyl, which can be mimicked by the phosphonate inhibitors, or a species arising from attack of the basic carboxylate of glutamate-270, which cannot be mimicked by these inhibitors. From this perspective, the correlation of Figure 7, which supports the general base mechanism, is the best that can be expected given the properties of the substrates.

The phosphonates terminating in (O)Phe (Table II) are more tightly bound than expected on the basis of the $K_i$ vs $K_m/k_{cat}$ correlation of the inhibitors terminating in (O)Ala. Thus, for CPA substrates with Phe in the $P_1'$ position, some of the assumptions made in deriving the $K_i$ vs $K_m/k_{cat}$ correlation may not be valid: specifically, the rate-limiting step may not be the chemical transformation, and $K_m$ may not correspond to $K_S$ (Auld et al., 1984). A similar deviation toward higher affinity was observed with inhibitors of TLN that correspond to substrates whose turnover may be diffusion-limited (Bartlett & Marlowe, 1987b). In addition, the larger side chain of Phe in comparison with Ala may affect the nonenzymatic hydrolysis rate.

*Comparison of Phosphonate Ester and Phosphonamidate Analogues: Transition-State Analogy.* Because of the limited stability of the phosphonamidate linkage, it was impractical to undertake the synthesis and evaluation of a series of amide analogues for the purpose of the $K_i$ vs $K_m/k_{cat}$ comparison. On the other hand, insufficient kinetic data are available to attempt a $K_i$ vs $K_m/k_{cat}$ correlation for ester substrates of CPA. The question then arises as to whether the observed correlation is valid for peptide substrates or whether the phosphonates can only mimic the tetrahedral adduct of ester hydrolysis. For the related peptidase TLN, a parallel series of phosphonamidates and phosphonate esters have been synthesized and evaluated (Bartlett & Marlowe, 1983, 1987a). In that case, the amidates proved to be relatively stable because of the absence of a $P_1'$-carboxylate anion. Identical correlations between inhibitor $K_i$ and peptide substrate $K_m/k_{cat}$ values were found for both the amidate and ester inhibitors of TLN, and they were shown by crystallography to adopt identical binding orientations in

the active site (Tronrud et al., 1987). Thus, even when there is a significant difference in absolute binding affinity, phosphonate esters can demonstrate the same transition-state analogy as phosphonamidates.[2]

*Potent Inhibition by Analogues with Phe and (O)Phe at the C-Terminus. Phosphonamidate ZAA$^P$F.* The tripeptide phosphonamidate ZAA$^P$F proved to be so tightly bound that its inhibition constant could not be determined under steady-state conditions. Moreover, the hydrolytic instability of this inhibitor meant that the binding affinity could not be determined under equilibrium conditions either, since at inhibitor concentrations that allow residual enzyme activity the rate at which the inhibitor associates with the enzyme is slower than the rate at which it hydrolyzes. The $K_i$ value was therefore derived from the values determined for $k_{on}$ and $k_{off}$ (Figures 4 and 5; Table II).

A source of error in determining $K_i$ for ZAA$^P$F arises from the method used to determine the inhibitor concentration. Since the material decomposes during the course of isolation and lyophilization, it was not possible to quantitate its purity in the dry state or determine its extinction coefficient accurately. We made the assumption that the extinction coefficient for ZAA$^P$F is similar to that of the phosphonate ZAA$^P$(O)F. While the error in inhibitor concentration is directly reflected in that of the on-rate measurement, the facile hydrolysis of the phosphonamidate means that this error has little effect in the off-experiment. In the latter case it is the concentration of enzyme that is important. When the estimate of $k_{off}$ was varied and its effect on the simulated curves in Figure 6 evaluated, it was clear that changes of 15% cause significantly poorer fits. With these sources of error in $k_{on}$ and $k_{off}$, as well as those in determining $K_m$ and the substrate concentrations, a conservative estimate of $K_i$ for ZAA$^P$F and its uncertainty would be 32 ± 10 pM.

*Phosphonates.* The phosphonate esters terminating in a Phe unit are remarkably potent inhibitors of CPA. ZAA$^P$(O)F, ZAF$^P$(O)F, and ZFA$^P$(O)F, with $K_i$ values between 1 and 4 pM, are more than 3 orders of magnitude more tightly bound than any other inhibitors of this peptidase and to our knowledge show the highest affinity of any noncovalently bound, synthetic peptidase inhibitors that have been described. Unlike the phosphonamidate ZAA$^P$F, the phosphonates are indefinitely stable under the assay conditions. Thus, determination of their on-rates was straightforward, and there was no need to correct for inhibitor hydrolysis [data for ZAA$^P$(O)F shown in Figure 2]. It is interesting to note that the apparent on-rates ($k_{app}$) for the tripeptide phosphonate esters all fall in the range 1.7–2.5 × $10^5$ $M^{-1}$ $s^{-1}$, except for ZAG$^P$(O)F, which binds with a faster on-rate ($k_{on}$ = 6.6 × $10^5$ $M^{-1}$ $s^{-1}$). This exception is reminiscent of the behavior of Gly$^P$-containing inhibitors of TLN, which bind faster than analogues with α-substitution at the corresponding position (Bartlett & Marlowe, 1987b).

For observation of the equilibrium between CPA and each of the inhibitors, i.e., to measure the rate of dissociation of the E–I complex ($k_{off}$) or to see residual activity in the presence of the inhibitor, it was necessary to work at such low concentrations, and therefore with such long equilibration times, that it was impractical to carry out the experiments in the presence of substrate. The enzyme and inhibitor were incubated in the absence of substrate, and aliquots were removed for determination of the remaining activity. In view of the slow dissociation rates for these inhibitors (see below), the addition of a competitive substrate did not perturb the position of the equilibrium between EI and E + I over the time course of an assay, even when the assays approached 60 min in duration. However, it was important that the enzyme be stable for the length of time necessary to reach equilibrium with the inhibitor; this was achieved in buffers containing 0.1 mg/mL of bovine serum albumin and 1 μM $ZnCl_2$.

The equilibrium between CPA and each of the inhibitors was approached from both directions, by combining enzyme and inhibitor at high dilution (ca. 50 pM) and monitoring the progressive loss of activity and by forming the enzyme–inhibitor complex with nearly stoichiometric amounts of the components at high concentration and watching the return of activity on dilution to low concentration (also ca. 50 pM). In each of these experiments, the position of equilibrium provides a separate determination of $K_i$ via

$$K_i = \frac{E_F I_F}{EI} = \frac{(E_T v_i/v_0)(I_T - (E_T - (E_T v_i/v_0)))}{E_T - (E_T v_i/v_0)} \quad (2)$$

where $E_F$ and $I_F$ are free enzyme and inhibitor, respectively, EI is the enzyme–inhibitor complex, $E_I$ and $I_T$ are total enzyme and inhibitor, respectively, and $v_0$ and $v_i$ are the velocity in the absence or presence of inhibitor, respectively, and, through the use of a computer simulation program, of the individual rate constants $k_{off}$ and $k_{on}$. In the simulations, $k_{off}$ and $k_{on}$ were varied while their ratio was held equal to $K_i$ until a best fit to the experimental points was observed (Table II). The self-consistency of the rate constants obtained in the forward and reverse directions indicated that the equilibrium was a simple one and that the approach was valid.

Enzyme activity in the presence of inhibitor and as a function of time was calculated by comparison with controls incubated under identical conditions but in the absence of inhibitor. In all cases these control rates were >75% of their initial values over the period of the experiment. Nevertheless, the inhibited protease may lose activity at a slower rate, particularly if autolysis is a significant factor in the decomposition of free enzyme. The $K_i$ values determined in the dissociative experiments were consistently above those calculated from the associative experiments, as expected if the inhibitors protect as well as inhibit CPA. The fact that for ZFA$^P$(O)F the same values were calculated in the on- and off-experiments is perhaps fortuitous, given the overall accuracy of the determinations (see below).

The largest source of error in these experiments comes from inaccuracies in estimating the *relative* concentrations of the inhibitor and CPA. Because of the extremely tight binding of these inhibitors, this inaccuracy can be minimized by using the inhibitor as an active-site titrant: at high concentrations (e.g., 1 μM) the binding of the (O)Phe tripeptides to CPA is essentially stoichiometric; a typical titration is depicted in Figure 3. With the assumption that inhibition results from a 1:1 complex, the nominal ratio of inhibitor to enzyme as percent activity extrapolated to zero reveals the inaccuracy in this ratio. The difference between nominal and observed relative concentrations was less than 15% in all cases, except for ZFA$^P$(O)F for which 25% more inhibitor than expected was necessary to inhibit CPA fully. Since we had no objective

[2] Interestingly, it is for the hydrolysis of esters, particularly those which resemble poor or unusual peptide substrates, that much of the evidence in favor of the anhydride mechanism has been adduced (Makinen et al., 1976; Suh et al., 1985, 1986; Sander & Witzel, 1985). Moreover, it has been demonstrated that product release is the rate-limiting step in ester hydrolysis by CPA (Galdes et al., 1983; Geoghegan et al., 1986; Auld et al., 1986), i.e., that the difference between the ground and transition states of the transformation is not a question of the difference between trigonal or tetrahedral geometry of the reacting species.

criteria for determining where to assign the inaccuracy, the same correction was applied to both concentrations.

In view of the errors in the assays at the low levels of activity measured in the equilibration experiments (as reflected in the standard deviations of the percent activity at equilibrium, Table II), the potential differences in stability of the free and inhibited enzyme, and the inaccuracy in determination of the absolute concentrations of the enzyme and inhibitor solutions, the total error in the $K_i$ values may be as large as a factor of 2 for the most tightly bound inhibitors. However, the consistency of the values for $k_{on}$ and $k_{off}$ as determined by independent methods suggests that the $K_i$ values are correct within this factor.

The on-rate constants ($k_{on}$) of approximately $2 \times 10^5$ $M^{-1}$ $s^{-1}$ determined for the potent phosphonate and phosphonamidate inhibitors (Table II) are much slower than the usually accepted rate of $10^7$–$10^8$ $M^{-1}$ $s^{-1}$ for diffusion-controlled association of a small molecule with a protein (Gutfreund, 1974; Brouwer & Kirsch, 1982; Hardy & Kirsch, 1984). It is known that chloride ion is an inhibitor of CPA with a $K_i$ of 45 mM (Williams & Auld, 1986). Since the concentration of chloride in the assay mixture is 0.54 M, apparent slow binding may result from only a small fraction of the enzyme being in a form that can bind inhibitor.[3] Whether other processes such as conformational changes or water displacement (Holden et al., 1987; Bartlett & Marlowe, 1987b) play a role in the slow binding process cannot be addressed at this point. However, it is clear that there is no correlation between slow binding behavior per se and inhibitor affinity.

*Comparison of Phosphonate Ester and Phosphonamidate Analogues: Absolute Affinity.* Among the phosphorus-containing CPA inhibitors previously reported, only a small difference in binding affinity was observed between phenylalanine and phenyllactate derivatives, i.e., between amide and ester analogues (Jacobsen & Bartlett, 1982; Hoffman & Rottenberg, 1980). With the higher affinity phosphorus analogues of tripeptide ZAAF, the phosphonate ester is bound an order of magnitude more *tightly* than the phosphonamidate. This contrasts dramatically with observations of analogous inhibitors for TLN, in which the esters are bound 840-fold more *weakly* than the amidates (Bartlett & Marlowe, 1987a). In the latter case, the more favorable binding of the amidates was attributed to a specific hydrogen-bonding interaction between the amidate NH group and a backbone carbonyl oxygen on the enzyme (Tronrud et al., 1987; Bash et al., 1987). Although no structure of a complex between one of the tri- or tetrapeptide phosphonate analogues and CPA is yet available, it appears that an analogous interaction is not possible within the CPA binding site. This conclusion arises from computer modeling studies[4] and analogy to the structure of the complex between CPA and a related dipeptide phosphonamidate Cbz-Gly$^P$-Phe ($ZG^PF$) (Christianson & Lipscomb, 1988).

*Comments on the Transition-State Analogy of Gly$^P$-Containing Zinc Peptidase Inhibitors.* Christianson and Lipscomb (1988), in connection with their crystallographic studies of the complex between CPA and $ZG^PF$, note that the carbobenzoxy moiety occupies the side-chain pocket at the $S_1$ site. Interestingly, although the active-site cavities are quite different for CPA and TLN, Tronrud et al. (1987) found a similar anomaly in the complexes of the latter enzyme with inhibitors containing Cbz-Gly$^P$ at the $P_1$ position: the carbobenzoxy group does not bind where the $P_2$ residue is normally found. For both enzymes, such a binding conformation is not possible for substrates or inhibitors with residues other than glycine at the $P_1$ position, since side chains with the L configuration would encounter severe steric interaction either with the protein or with the Cbz group. For TLN, inhibitors with the phosphorus analogue of phenylalanine at the $P_1$ position have been shown to bind in the expected fashion (Holden et al., 1987; B. W. Matthews, personal communication); unfortunately, similar experimental information for the binding orientation of phosphonate CPA inhibitors with a $P_1$ substituent (such as those reported here) is not yet available, although it seems likely that they too bind in the "normal" fashion.[3]

The question then arises, how "anomalous" is the binding orientation of the Cbz-Gly$^P$-containing inhibitors in these zinc peptidases? This characterization, and the suggestion that such inhibitors therefore cannot be transition-state analogues, rest on the assumption that the corresponding substrates containing Cbz-Gly bind in the same manner as normal substrates. However, there is evidence to the contrary: in the case of CPA, it is precisely for substrates such as Cbz-Gly-Phe that substrate activation and other kinetic anomalies are observed at high substrate concentrations (Auld & Vallee, 1970, and references cited therein), behavior that is indicative of multiple binding modes and the possible formation of ternary complexes. Moreover, in the case of TLN, the series of inhibitors for which transition-state analogy was explicitly demonstrated were the Cbz-Gly$^P$ derivatives (Marlowe & Bartlett, 1983) and included $ZG^PLL$ and $ZG^P(O)LL$, whose anomalous binding was demonstrated crystallographically (Tronrud et al., 1987). To our knowledge the closest non-phosphorus-containing substrate analogue for which a structure of the enzyme complex is available is the ketone analogue of benzoyl-Gly-Phe ($COCH_2$ in place of the peptide linkage; Grobelny et al., 1985b). This compound binds to CPA as the tetrahedral hydrate, although the benzamido moiety is apparently disordered (Christianson et al., 1987). It is clear that enzyme complexes of Cbz-Gly$^P$ derivatives are not good models for the way in which substrates with residues other than glycine at the $P_1$ position bind, either in the ground or the transition state. However, it is possible that CPA and TLN substrates containing Cbz-Gly as the $P_1$ residue bind differently than $P_1$-substituted or longer substrates and that the inhibitors containing Cbz-Gly$^P$ are effective transition-state analogues for them.

## Supplementary Material Available

Spectral characterization of the compounds described above and full experimental details for the synthesis and characterization of the other inhibitors reported in this paper; experimental procedures and results for the enzyme binding studies not described above (35 pages). Ordering information is given on any current masthead page.

## References

Abramowitz, N., Schechter, I., & Berger, A. (1967) *Biochem. Biophys. Res. Commun. 29*, 862–867.

Abramowitz-Kurn, N., & Schechter, I. (1974) *Isr. J. Chem. 12*, 543–555.

[3] In preliminary experiments, we observed a 3–4-fold increase in the on-rate for $ZFA^P(O)F$ when the chloride ion concentration in the assay buffer was lowered to 66 mM ($K_m$ for the substrate FuAFF decreased by approximately 35% under these conditions).

[4] The binding interaction between CPA and $ZFA^P(O)F$ was modeled by starting with the structure of the CPA-potato inhibitor complex (Rees & Lipscomb, 1982). After overlap of the backbone atoms of $ZFA^P(O)F$ with those of the potato inhibitor in the $P_1$–$P_3$ positions, it is possible both to orient the phenyllactate residue in the $P_1'$ binding pocket as observed for other derivatives [inter alia, Christianson et al. (1985) and Christianson and Lipscomb (1986)] and to position the phosphonate moiety relative to the zinc atom and the catalytic glutamate carboxylate as seen in analogous TLN complexes (Holden et al., 1987).

Auld, D. S., Galdes, A., Geoghegan, K. F., Holmquist, B., Martinelli, R. A., & Vallee, B. L. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 5041–5045.

Auld, D. S., Geoghegan, K., Galdes, A., & Vallee, B. L. (1986) *Biochemistry 25*, 5156–5159.

Barshop, D. A., Wrenn, R. F., & Frieden, C. (1983) *Anal. Biochem. 130*, 134–145.

Bartlett, P. A., & Johnson, W. S. (1970) *Tetrahedron Lett.*, 4459–4462.

Bartlett, P. A., & Marlowe, C. K. (1983) *Biochemistry 22*, 4618–4624.

Bartlett, P. A., & Lamden, L. A. (1986) *Bioorg. Chem. 14*, 356–377.

Bartlett, P. A., & Acher, F. (1986) *Bull. Soc. Chim. Fr.*, 771–775.

Bartlett, P. A., & Marlowe, C. K. (1987a) *Science 235*, 569–571.

Bartlett, P. A., & Marlowe, C. K. (1987b) *Biochemistry 26*, 8553–8561.

Bash, P. A., Singh, U. C., Brown, F. K., Langridge, R., & Kollman, P. A. (1987) *Science 235*, 574–576.

Breslow, R., & Wernick, D. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 1303–1307.

Brouwer, A., & Kirsch, J. F. (1984) *Biochemistry 23*, 1275–1282.

Christianson, D. W., & Lipscomb, W. N. (1986) *J. Am. Chem. Soc. 108*, 4998–5003.

Christianson, D. W., & Lipscomb, W. N. (1988) *J. Am. Chem. Soc. 110*, 5560–5565.

Christianson, D. W., & Lipscomb, W. N. (1989) *Acc. Chem. Res. 22*, 62–69.

Christianson, D. W., Kuo, L. C., & Lipscomb, W. N. (1985) *J. Am. Chem. Soc. 107*, 8281–8283.

Christianson, D. W., David, P. R., & Lipscomb, W. N. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 1512–1515.

Cleland, W. W. (1979) *Methods Enzymol. 63*, 103–138.

Fersht, A. (1985) *Enzyme Structure and Mechanism*, 2nd ed., Freeman, San Francisco, CA.

Galardy, R. E., Kontoyiannidou-Ostrem, V., & Kortylewicz, Z. P. (1983) *Biochemistry 22*, 1990–1995.

Galdes, A., Auld, D. S., & Vallee, B. L. (1986) *Biochemistry 25*, 646–651.

Geoghegan, K. F., Galdes, A., Hanson, G., Holmquist, D., Auld, D. S., & Vallee, B. L. (1986) *Biochemistry 25*, 4469–4674.

Grobelny, D., Goli, U. B., & Galardy, R. E. (1985a) *Biochem. J. 232*, 15–19.

Grobelny, D., Goli, U. B., & Galardy, R. E. (1985b) *Biochemistry 24*, 7612–7617.

Gutfreund, H. (1974) *Prog. Biophys. Mol. Biol. 29*, 161–195.

Hangauer, D. G., Monzingo, A. F., & Matthews, B. W. (1984) *Biochemistry 23*, 5730–5741.

Hardy, L. W., & Kirsch, J. F. (1984) *Biochemistry 23*, 1275–1282.

Hoffmann, W., & Rottenberg, M. (1980) in *Enzyme Inhibitors* (Brodbeck, U., Ed.) pp 19–26, Verlag Chemie, Basel.

Holden, H. M., Tronrud, D. E., Monzingo, A. F., Weaver, L. H., & Matthews, B. W. (1987) *Biochemistry 26*, 8542–8553.

Jacobsen, N. E., & Bartlett, P. A. (1981a) *J. Am. Chem. Soc. 103*, 654–657.

Jacobsen, N. E., & Bartlett, P. A. (1981b) in *Phosphorus Chemistry* (Quin, L. D., & Verkade, J., Eds.) ACS Symposium Series 171, pp 221–224, American Chemical Society, Washington, DC.

Jacobsen, N. E., & Bartlett, P. A. (1983a) *J. Am. Chem. Soc. 105*, 1613–1619.

Jacobsen, N. E., & Bartlett, P. A. (1983b) *J. Am. Chem. Soc. 105*, 1619–1626.

Jencks, W. P. (1975) *Adv. Enzymol. Relat. Areas Mol. Biol. 43*, 219–410.

Kafarski, P., Lejczak, F., & Szewczyk, J. (1983) *Can. J. Chem. 61*, 2425–2430.

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

Karanewsky, D. S., Badia, M. C., Cushman, D. W., DeForrest, J. M., Dejneka, T., Loots, M. J., Perri, M. G., Petrillo, E. W., Jr., & Powell, J. R. (1988) *J. Med. Chem. 31*, 204–212.

Kemp, D. S. (1979) in *The Peptides* (Gross, E., & Meienhofer, J., Eds.) Vol. 1, pp 315–383, Academic Press, New York.

Kester, W. R., & Matthews, B. W. (1977) *J. Biol. Chem. 252*, 7704–7710.

Komiyama, T., Suda, H., Aoyagi, T., Takeuchi, T., Umezawa, H., Fujimoto, K., & Umezawa, S. (1975) *Arch. Biochem. Biophys. 171*, 727–731.

Kraut, J. (1988) *Science 242*, 533–540.

Lamden, L. A., & Bartlett, P. A. (1983) *Biochem. Biophys. Res. Commun. 112*, 1085–1090.

Lipscomb, W. N. (1970) *Acc. Chem. Res. 3*, 81–89.

Makinen, M. W., Yamamura, K., & Kaiser, E. T. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3882–3886.

Makinen, M. W., Fukuyama, J. M., & Kuo, L. C. (1982) *J. Am. Chem. Soc. 104*, 2667–2669.

Matthews, B. W. (1988) *Acc. Chem. Res. 21*, 333–340.

Mookthiar, K. A., Marlowe, C. K., Bartlett, P. A., & Van Wart, H. E. (1987) *Biochemistry 26*, 1962–1965.

Morrison, J. F., & Walsh, C. (1988) *Adv. Enzymol. Relat. Areas Mol. Biol.* (in press).

Mulliez, M. (1981) *Tetrahedron 37*, 2027–2041.

Nishino, N., & Powers, J. C. (1979) *Biochemistry 18*, 4340–4347.

Rees, D. C., & Lipscomb, W. N. (1982) *J. Mol. Biol. 160*, 475–498.

Rich, D. H., & Northrup, D. B. (1988) in *Computer Aided Drug Design* (Perun, T. J., & Propst, C. L., Eds.) Marcel Dekker, New York (in press).

Riordan, J. F., & Holmquist, B. (1984) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., Ed.) 3rd ed., Vol. V, pp 44–55, Verlag Chemie, Deerfield Beach, FL.

Sander, M. E., & Witzel, H. (1985) *Biochem. Biophys. Res. Commun. 132*, 681–687.

Schloss, J. V., & Cleland, W. W. (1982) *Biochemistry 21*, 4420–4427.

Simpson, R. T., Riordan, J., & Vallee, B. L. (1963) *Biochemistry 2*, 612–622.

Suh, J., Cho, W., & Chung, S. (1985) *J. Am. Chem. Soc. 107*, 4530–4535.

Suh, J., Hong, S.-B., & Chung, S. (1986) *J. Biol. Chem. 261*, 7112–7114.

Thompson, R. C. (1973) *Biochemistry 12*, 47–51.

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.

Tronrud, D. E., Monzingo, A. F., & Matthews, B. W. (1970) *Eur. J. Biochem. 157*, 261–268.

Tronrud, D. E., Holden, H. M., & Matthews, B. W. (1987) *Science 235*, 571–574.
Weaver, L. H., Kester, W. R., & Matthews, B. W. (1977) *J. Mol. Biol. 114*, 119–132.
Williams, A. C., & Auld, D. S. (1986) *Biochemistry 25*, 94–100.
Williams, J. W., & Morrison, J. F. (1979) *Methods Enzymol. 63*, 437–467.
Wolfenden, R. (1976) *Annu. Rev. Biochem. Bioeng. 5*, 271–306.
Yamauchi, K., Ohtsuki, S., & Kinoshita, M. (1985) *Biochim. Biophys. Acta 827*, 275–282.

# Active-Site Modification of Mammalian DNA Polymerase β with Pyridoxal 5′-Phosphate: Mechanism of Inhibition and Identification of Lysine 71 in the Deoxynucleoside Triphosphate Binding Pocket†

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ABSTRACT: Pyridoxal 5′-phosphate is a potent inhibitor of the DNA polymerase activity of recombinant rat DNA polymerase β. Kinetic studies indicate that the mechanism of PLP inhibition is complex. In a lower range of PLP concentration, inhibition is competitive with respect to substrate dNTP, whereas at higher levels of PLP several forms of enzyme combine with PLP and are involved in the overall inhibition, and a possible model for these interactions during the catalytic process is suggested. Reduction of the PLP-treated enzyme with sodium [$^3$H]borohydride results in covalent incorporation of about 4 mol of PLP/mol of enzyme, and the modified enzyme is not capable of DNA polymerase activity. The presence of dNTP during the modification reaction blocks incorporation of 1 mol of PLP/mol of enzyme, and the enzyme so modified is almost fully active. This protective effect is not observed in the absence of template-primer. Tryptic peptide mapping of the PLP-modified enzyme reveals four major sites of modification. Of these four sites, only one is protected by dNTP from pyridoxylation. Sequence analysis of the tryptic peptide corresponding to the protected site reveals that it spans residues 68–80 in the amino acid sequence of the enzyme, with Lys 71 as the site of pyridoxylation. These results indicate that Lys 71 is at or near the binding pocket for the dNTP substrate.

All known DNA polymerases have a common catalytic mechanism and exhibit an absolute dependence on the template (with the exception of terminal deoxynucleotidyltransferase) for DNA synthesis (Kornberg, 1980). While template-dependent substrate selection is a crucial step in error-free DNA replication or repair processes, the mechanism of base selection and the structural components of the enzyme protein involved in this process have not been clarified. To gain insight into structural components involved in the recognition and binding of individual reaction components of the polymerase reaction, we have used site-specific reagents that are capable of producing covalent linkage at the site of their reaction on the enzyme protein. Definition of that site, in turn, reveals the active-site domain, as well as an important amino acid residue(s) in that domain which is essential for catalysis. Thus, we found that pyridoxal 5′-phosphate (PLP), under appropriate conditions, is a reagent with specificity for the substrate deoxynucleoside triphosphate (dNTP) binding site in many DNA polymerases (Modak, 1976; Modak & Dumaswala, 1981). For example, lysine residues invoved in the substrate binding function of *Escherichia coli* DNA polymerase I (Basu & Modak, 1987), murine leukemia virus reverse transcriptase (Basu et al., 1988), and human immunodeficiency virus reverse transcriptase (Basu et al., 1989) have been identified via their covalent modification with PLP followed by peptide mapping and amino acid sequencing. We have now extended this analysis to mammalian DNA polymerase β as a representative of mammalian DNA polymerases, since the primary amino acid sequence of this enzyme has been deduced from the nucleotide sequence of a cDNA (Zmudzka et al., 1986). The successful subcloning of the coding sequence in an expression vector has been accomplished (Abbotts et al., 1988a), and this has made available sufficient quantities of enzyme protein for detailed structural studies. Furthermore, the relatively small size ($M_r$ = 40 000) and simple structure in the form of a single polypeptide chain have made this enzyme most attractive for structure function studies. In this paper, we describe the mechanism of PLP-mediated inactivation of β-polymerase and report the identification of a PLP-reactive lysine residue that appears to be in the substrate binding pocket of the enzyme.

## Materials and Methods

[$^3$H]dTTP was from New England Nuclear. Tritiated $NaBH_4$ was from ICN. To prepare the template-primer complex, poly(dA) and $d(T)_{14}$ were mixed in a weight ratio of 2:1 in 10 mM KCl. Poly(rA)·$(dT)_{12-18}$ was obtained from P-L Biochemicals. The template-primers were heated in boiling water for 3 min and were then allowed to cool to room

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